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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.

LX2 cells treated with succinate for 24 h showed increased protein expression of GPR91, α-SMA, and TIMP-1(Fig. 1A) but not α-SMA mRNA levels (data was not shown). LX2 cells treated with succinate for 8 h demonstrated increased mRNA expression of α-SMA through GPR91 activation (Fig. 1B). LX2 cells treated with succinate for 24 h showed increased expression of collagen type 1 and TGF-β1 measured using RT-PCR (Fig. 1B). LX2 cells transfected with GPR91 siRNA and incubated for 36 h showed decreased GPR91 and α-SMA expression in HSCs (Fig. 1C). These results suggest that succinate lead to HSC activation, through direct activation of GPR91.

3.2. Effects of MCD media, palmitate and malonate on succinate and GPR91 in HSCs

NAFLD is a hepatic complication of obesity and a major cause of persistent liver abnormalities in humans. Feeding mice an MCD diet is a widely used technique to create an animal model of nonalcoholic steatohepatitis (NASH). To directly investigate the influence of MCD media on the expression of GPR91 in HSCs, we cultured LX2 cells in MCD media for 24 h and monitored GPR91 expression, SDH activity, and succinate levels in the LX2 cells after the treatment. The expression of both GPR91 and α-SMA increased (Fig. 2A). LX2 cells cultured in MCD media showed decreased SDH activity and increased succinate concentration in cell lysates compared to controls (Fig. 2B, C) and resulted in increased expression of GPR91 and α-SMA. LX2 cells incubated with palmitate for 30 min resulted in increased protein expression of GPR91 and α-SMA (Fig. 2D), decreased activity of SDH, and elevated concentrations of succinate in cell lysates (Fig. 2E, F). Succinate is converted into fumarate by SDH and malonate is a well-known SDH inhibitor. LX2 cells treated with malonate for 24 h increased the expression of GPR91 and α-SMA (Fig. 2G). Malonate treatment led to decreased SDH activity (Fig. 2H), and the concentration of succinate in cell lysates was 1.5-fold higher in the malonate-treated group compared to controls (Fig. 2I). These results suggest that MCD media, palmitate or malonate can induce activation of HSCs through SDH-succinate-GPR91 signaling.

3.3. Expression of GPR91 and succinate in isolated HSCs of MCD diet fed mice model of NAFLD

To investigate the direct influence of succinate and GPR91 on NAFLD, we isolated mouse primary HSCs and monitored GPR91 expression and succinate concentration. MCD diet fed mice showed increased hepatosteatosis compared to controls (Fig. 3A). The expression of the GPR91 and α-SMA proteins was increased in HSCs isolated from MCD diet fed mice compared to controls (Fig. 3B). Markedly elevated succinate concentrations in HSC lysates and decreased SDH activities (Fig. 3C, D) were observed in MCD diet fed mice compared to controls. Interestingly, succinate levels in the plasma of MCD diet fed mice increased significantly compared to controls (Fig. 3E). These findings implicate that succinate-GPR91 signaling are important for HSC activation and fibrogenesis in vivo.

3.4. Succinate concentration in mice hepatocytes and isolated hepatocytes from MCD diet fed mice model of NAFLD

When alpha mouse liver 12 (AML12) cells were cultured in MCD media, the hepatocytes showed increased succinate concentrations in cell lysates compared to controls (Fig. 4A, B). The isolated hepatocytes of MCD diet fed mice also showed increased succinate level compared to controls (Fig. 4C, D).

4. Discussion

Although succinate has been studied extensively for several decades in terms of energy metabolism, recent studies have demonstrated that it is a cellular signaling molecule in many metabolic diseases [7]. The present study provides novel information regarding the key role of succinate and GPR91 in HSC activation as signaling regulators of hepatic fibrosis.

In the present study, we showed that GPR91 is expressed during HSC activation and succinate-GPR91 signaling stimulates HSC activation *in vitro* experiment, suggesting a profibrogenic function of GPR91. A previous study showed that GPR91 expression was increased in inactivated primary HSCs and the expression decreased with HSC activation [16]. These findings are different from our results showing stimulated GPR91 expression in activated HSCs.

In a cellular level, we demonstrated that palmitate, malonate and MCD media decreased SDH activity and increased cellular succinate concentration and protein expression of GPR91 thereby promoting HSC activation in LX2 cells (Fig. 2). We next assess whether succinate and GPR91 is linked functionally to fibrogenic expression of HSC in animal model of NASH/NAFLD. In the MCD diet fed mice model of NAFLD, there was increased hepatosteatosis and increased plasma succiante concentration compared to control diet fed mice. In the isolated hepatic stellate cells of MCD diet fed mice showed decreased SDH activity and increased succinate level and GPR91 protein expression (Fig. 3). In the previous study [16], Correa P.R. et al., suggested the novel role of GPR91 in hepatic fibrosis and

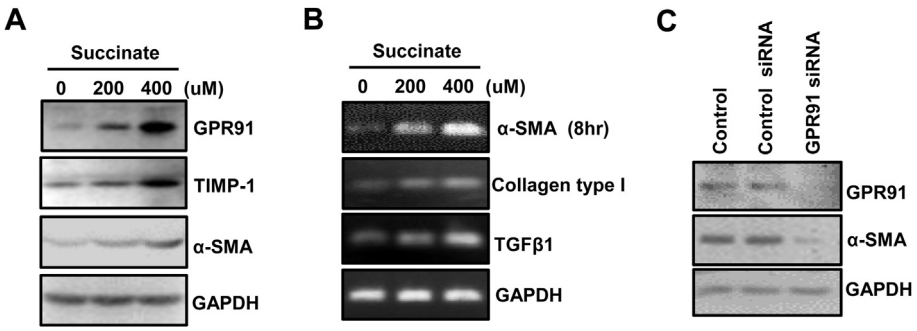

Fig. 1. Stimulation of α-SMA, TIMP-1, TGF-β1, and collagen type I production by succinate in LX2 cells. (A) Western blot analysis of HSC activation target proteins. Cells were treated with 0, 200, and 400 uM succinate for 24 h. Subsequently, GPR91, α-SMA and TIMP-1 were detected using Western blotting. (B) RT–PCR analysis of α-SMA, TGF-β1, and collagen type-I mRNA expression. The total RNA was isolated from LX2 cells and RT-PCR was conducted. (C) Effects of GPR91 siRNA in LX2 cells. LX2 cells were transfected with either control siRNA or siRNA targeting GPR91 for 36 h and α-SMA was determined using Western blotting.

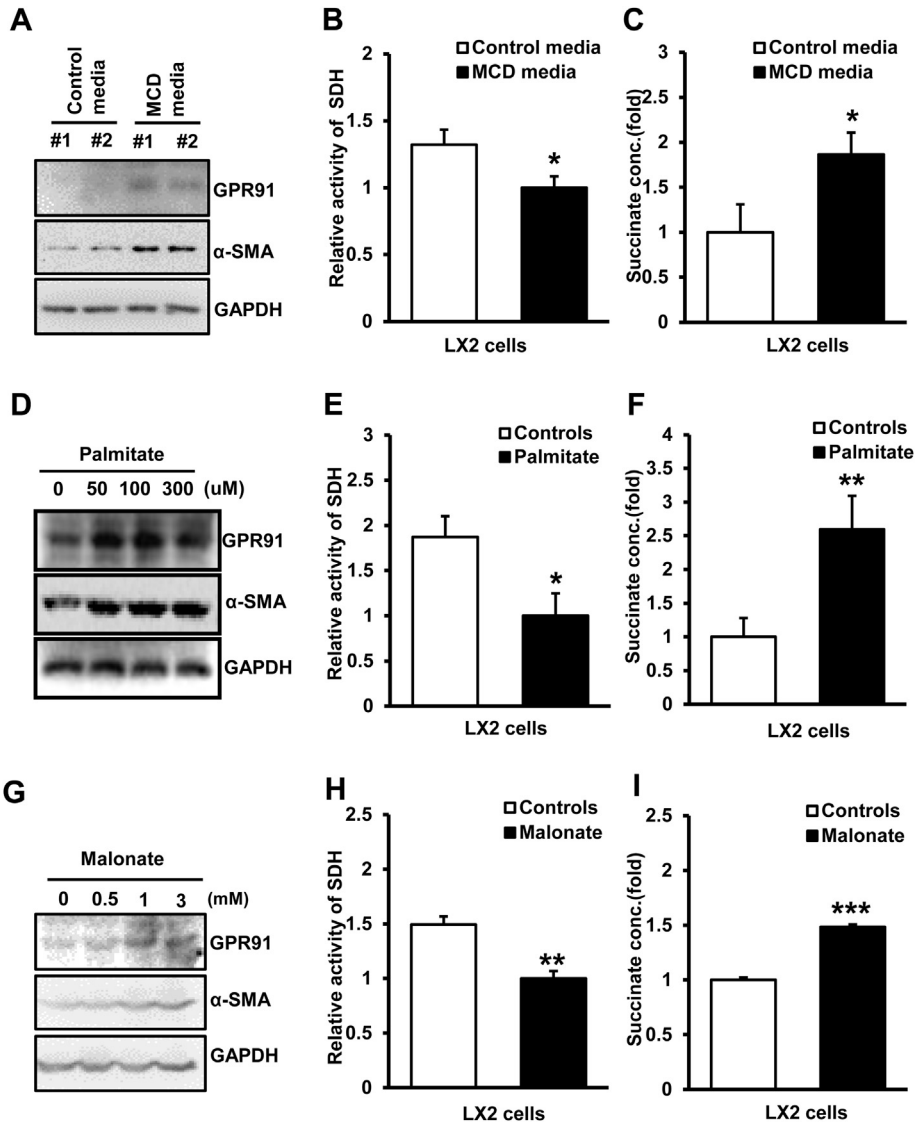

Fig. 2. Effects of MCD media, palmitate and malonate on HSC activation. (A) GPR91 and α-SMA are upregulated in response to treatment with MCD media. LX2 cells were cultured in control or MCD media for 24 h. Cell lysates were prepared and the GPR91 and α-SMA protein levels were analyzed using Western blotting. (B) Effects of MCD media on SDH activity. LX2 cells were cultured in control or MCD media for 24 h and SDH activity was measured in whole cell lysates (n = 3). (C) Succinate is induced by treatment with MCD media. LX2 cells were cultured in control or MCD media for 24 h and succinate concentration was measured in whole cell lysates (n = 6). (D) GPR91 and α-SMA are upregulated in response to palmitate treatment. Cells were cultured in the presence or absence of various concentrations of palmitate for 36 h. Cell lysates were subjected to immunoblotting with specific antibodies. (E) Effects of palmitate on SDH activity. LX2 cells were cultured in the presence or absence of palmitate (300 uM) for 36 h and succinate concentration was measured in whole cell Lysates (n = 3). (F) Succinate is induced by treatment with palmitate. LX2 cells were cultured in the presence or absence of palmitate (300 uM) for 36 h and succinate concentration was measured in whole cell lysates (n = 4). (G) GPR91 and α-SMA are upregulated in response to treatment with malonate. After LX2 cells were cultured for 24 h in malonate at the indicated doses, cells were lysed and subjected to Western blotting. (H) Effects of malonate on SDH activity. LX2 cells were left untreated (control) or treated with malonate (3 mM) for 24 h and SDH activity was measured in whole cell lysates (n = 3). (I) Succinate is induced by treatment with malonate. LX2 cells were left untreated (control) or treated with malonate (3 mM) for 24 h and succinate concentration was measured in whole cell lysates(n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls.

we demonstrated that palmitate and MCD media activate hepatic stellate cells through succinate and its receptor GPR91 signaling in this study suggesting the GPR91 as an anti-fibrogenic target of hepatic fibrogenesis.

The citric acid cycle intermediate succinate is normally present within mitochondria and release into outside cells and blood circulation if the energy demand of local tissues is out of balance and oxygen supply is low [7,18]. Substantial evidence indicates that succinate and metabolic diseases such as diabetes, hypertension and NAFLD are positively associated [7,19,20]. Rats fed high-fat diets show increased succinate concentrations in their serum over time [19]. Intravenous succinate administration increases blood pressure by activating the renin-angiotensin system, and this effect is abolished in GPR91-deficient mice [7]. Baboons with hepatic fibrosis after chronic ethanol consumption show decreased enzyme activity in the mitochondrial respiratory chain including SDH [20], suggesting that the succinate accumulates due to decreased SDH activity. Rats administered ethanol show mitochondrial dysfunction with decreased SDH activity [21]. However, another study using immunohistochemical analysis reported no defects in complex II (SDH) and complex IV activities in aging and cirrhotic livers compared to normal livers [22].

Until now, it is not elucidated well that whether succinate activates GPR91 locally or systemically or whether the excessive succinate accumulation observed in activated HSCs is a cause or a consequence.

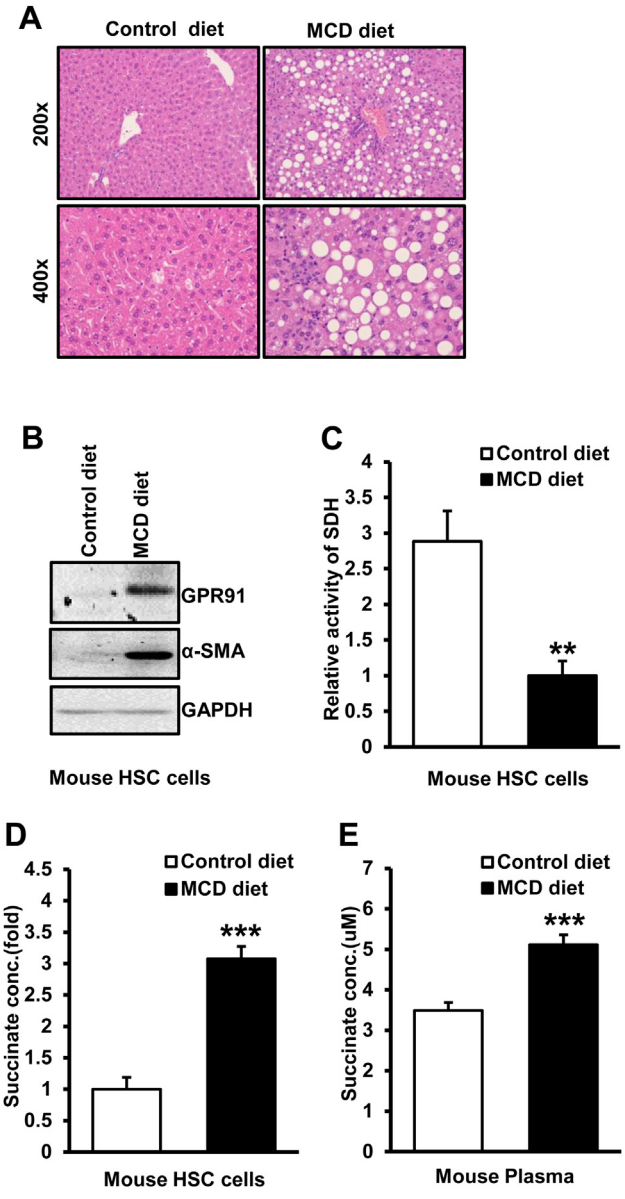

Fig. 3. Expression of GPR91, SDH activity and succinate concentration in isolated HSCs of MCD diet fed mice model of NAFLD. (A) Hepatic steatosis evaluated by H&E staining. H&E staining of liver sections from mice fed the respective diet for 4 weeks (original magnification × 200 and × 400 for inserts). The MCD diet led to increased steatosis compared to the control diet. (B) The MCD diet led to increased HSC activation. Primary HSC cells were isolated from mice fed the MCD or control diet for 4 weeks. The GPR91 and α-SMA protein levels were analyzed using Western blotting. (C) Effects of the MCD diet on SDH activity in isolated primary HSC lysates (n = 3). (D) Succinate is induced by MCD diet treatment. Succinate concentration was measured in isolated primary HSC lysates (n = 3). (E) Plasma succinate measured in mice fed the MCD or control diet for 4 weeks (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls.

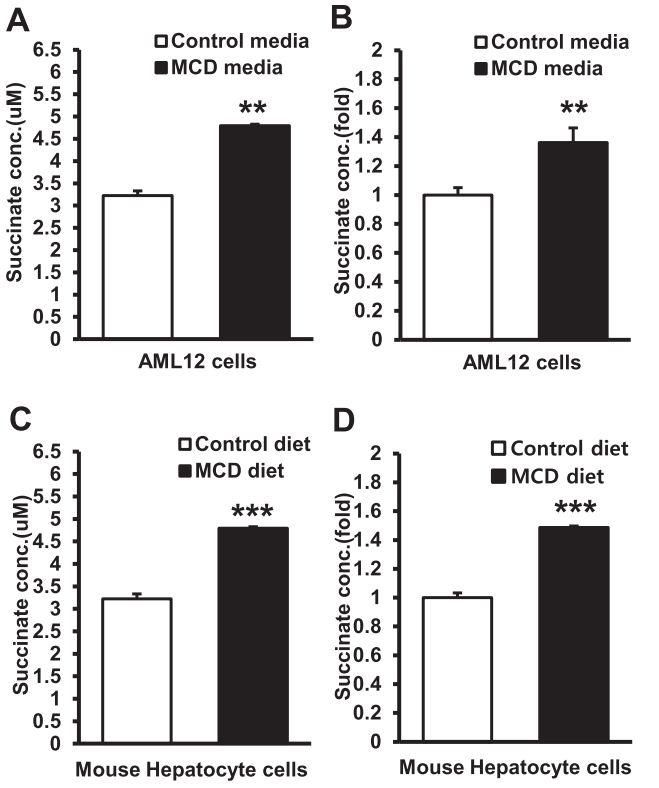

Fig. 4. Succinate concentration in mouse hepatocytes and isolated hepatocytes of MCD diet fed mouse model of NAFLD. (A, B) Succinate is induced by treatment with MCD media in AML12 cells. AML12 cells were cultured in control or MCD media for 24 h and succinate concentration was measured in whole cell lysates (n = 3). (C, D) Succinate measured in primary hepatocyte cells isolated from mice fed the MCD or control diet for 4 weeks (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls.

Several studies have shown that GPR91 is expressed only in the apical membrane and not in the basolateral membrane of retinal pigment epithelium [23] or macula densa cells of juxtaglommerular apparatus [9], suggesting that the receptor is activated by locally accumulated rather than systemic succinate. Another study showed that succinate was elevated in spontaneously hypertensive rats (SHR), *ob/ob* mice, *db/db* mice, and *fa/fa* rats compared to their non-diseased controls but not in patients with hypertension or diabetes [24]. We found that succinate concentration was elevated in plasma and both isolated hepatocytes and HSCs of MCD diet fed mice compared to controls, suggesting both systemic and local action of succinate in HSC activation. Under physiological conditions, mitochondrial succinate is mainly involved in energy production in the citric acid cycle and the peripheral GPR91 remains inactive [6]. However, under stressful conditions such as hypoxia, hyperglycemia, or liver injury, succinate accumulates in the mitochondria and subsequently leaves the mitochondria and enters the blood system and binds to GPR91, which serves as a sensor for extracellular succinate in various tissues [6,25]. In the present study, succinate, MCD media, and palmitate activated GPR91 and α-SMA expression in LX2 cells, and MCD treatment increased succinate concentration in hepatocyte lysates and isolated mouse hepatocytes compared to controls. These results suggest that succinate itself directly activates LX2 cells, and that succinate which was released from hepatocytes may stimulate HSC activation.

Recent studies have demonstrated the downstream signaling of succinate is associated with events such as hypoxia-inducible factor-1 α (HIF-1α) accumulation, succinylation of target proteins, and GPR91 activation [26,27]. GPR91 activation induces calcium mobilization through a protein kinase A-dependent pathway in cardiomyocytes [28] and induces intracellular calcium mobilization and activates ERK1/2 in kidney cells [7]. In the retinal ganglion cells, GPR91 inhibits ERK1/2 and MAPK signaling, thereby releasing VEGF in response to high levels of glucose [12]. HIF-1α also activates HSCs by regulating autophagy [29]. In contrast to renal or other cells, administration of succinate does not induce an intracellular calcium response or cAMP production in HSCs [16]. The signaling

mechanism of GPR91 linked to HSC activation requires further elucidation.

These results have significant clinical implications. HSCs are generally considered a central player in hepatic fibrosis. Our findings indicate excessive succinate accumulation and GPR91 overexpression in HSCs with decreased SDH activity, and suggest that accumulated succinate and GPR91 expression are important pathological hallmarks of further hepatic fibrosis.

Additional studies are needed to further characterize the function of the identified GPR91 in hepatic fibrosis and NAFLD. The molecular mechanism of HSC activation by succinate-GPR91 signaling and the transporter system of succinate in the cellular membrane require further clarification. Our results suggest that GPR91 is a potential therapeutic target for protecting the liver from fibrosis by deactivating HSCs.

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