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Succinate is a paracrine signal for liver damage☆

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***Background/Aims*: A G-protein-coupled succinate receptor has recently been identified in several tissues, including the liver. The objectives of this work were to determine the hepatic cell types that express this receptor and to determine its physiological role.**

***Methods*: Expression and distribution of the succinate receptor was determined by RT-PCR and confocal immunofluorescence. Biochemical assays were used to measure succinate and cAMP. Cytosolic Ca^{2+} was monitored in single cells by time-lapse imaging. Western blot was used to study the effect of succinate on activation of hepatic stellate cells.**

***Results*: The succinate receptor was expressed in quiescent hepatic stellate cells, and expression decreased with activation. Ischemia induced release of succinate in isolated perfused livers. In contrast to what is observed in cell expression systems, succinate did not inhibit cAMP production or increase cytosolic Ca^{2+} in primary hepatic stellate cells. However, succinate accelerated stellate cell activation.**

***Conclusions*: Hepatic stellate cells express the succinate receptor. Succinate may behave as a paracrine signal by which ischemic hepatocytes trigger stellate cell activation.**

***Keywords*: GPR91; Succinate receptor; Stellate cells; Liver; Ischemia; Signal transduction**

1. Introduction

Succinate is an important metabolic molecule that constitutes one of the intermediates of the citric acid cycle. Recently, a succinate receptor was identified that suggests succinate may also act as a signaling molecule [1]. This newly described receptor is the former orphan G-protein-coupled receptor GPR91. This receptor is expressed in kidney, liver, spleen and to a lesser extent small intestine [1]. The distribution and function of the succinate receptor within the kidney has been examined, but nothing is known about the receptor in the liver. The principal signaling actions of succinate that are mediated by the receptor GPR91 in kidney are to increase Ca^{2+} via Gq and to decrease cAMP via Gi. The principal downstream effect that has been identified is an increase in renin release, which produces a rise in arterial blood pressure [1]. Given the important role of succinate as an intermediary of the central cross-road of metabolism – the citric acid cycle – and its novel signaling properties, an attractive hypothesis is that GPR91 might serve as a sensor of metabolic activity in organs or tissues. Of special interest is the fact that succinate levels rise in response to ischemia [2,3], because GPR91 could be responsible for mediating the adaptation of organs and tissues to hypoxic conditions, including regulating

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secretion or repair and proliferation. Because the liver is the central metabolic organ and is particularly susceptible to ischemia, we examined the intrahepatic distribution and functional role of the succinate receptor.

2. Materials and methods

2.1. Animals and materials

Male Sprague–Dawley rats (200–250 g, Charles River Laboratories, Boston, MA) were used for all studies. Animals were maintained on a standard diet and housed under a 12-h light–dark cycle. Ultrapure succinic acid and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest quality commercially available.

2.2. Isolation of hepatic cell types

Hepatic stellate cells (HSC) were isolated from male Sprague–Dawley rats by Nycodenz gradient centrifugation, as described previously [4]. Primary cells were >95% pure. Cells were grown on glass coverslips in DMEM-F12 medium with 10% FCS. At day 1 after isolation HSC are phenotypically quiescent, while by day 7 they have become phenotypically activated. Portal fibroblasts were isolated from biliary trees as described previously [5], following *in situ* pronase/collagenase perfusion of male Sprague–Dawley rat livers. The biliary tree was minced and digested by collagenase, pronase and deoxyribonuclease, then filtered through 30 μm nylon mesh. The resulting cells were grown on tissue culture flasks in DMEM-F12 medium with 10% FCS. Hepatocytes and cholangiocytes were isolated by collagenase perfusion, according to protocols described previously [6,7]. Kupffer cells were isolated by collagenase perfusion followed by centrifugal elutriation [8] and their purity was confirmed by immunostaining with commercially obtained antibodies (Serotec, Düsseldorf, Germany) for the Kupffer cell marker F4/80 [9], while sinusoidal endothelial cells were isolated by collagenase perfusion followed by Percoll gradient centrifugation [10] and their purity was confirmed by staining with commercially obtained antibodies for endothelial nitric oxide synthase (eNOS) (BD Biosciences, San Jose, CA).

2.3. RT-PCR for GPR91

RNA was isolated from various tissues and cells using RNAqueous (Ambion, Austin, TX). RNA obtained from whole liver, hepatocytes, cholangiocytes, quiescent and activated hepatic stellate cells, quiescent and activated portal fibroblasts and kidney was reverse transcribed into cDNA by using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, La Jolla, CA) according to the manufacturer's instruction. The resulting cDNA was amplified by PCR using 45 μL of PCR SuperMix (Invitrogen, La Jolla, CA) and 250 nM of both sense and antisense primers for rat GPR91. The samples were subjected to 30 cycles consisting of: 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C. This was followed by a final extension at 72 °C for 10 min. The primers were designed using the software Primer3 [11] based on the sequence deposited in the NCBI Nucleotide Bank (AY612851). The forward primer sequence was 5′-TTACGCCA CTGGGAACTGGA-3′ and the reverse primer sequence 5′-TTGA TGGCCTTCTGGGAACA-3′. The primers for the GAPDH were designed based on the Rattus norvegicus sequence deposited in the NCBI nucleotide bank (NM_017008). The forward primer sequence was 5′-TGCCACTCAGAAGACTGTGG-3′ and the reverse primer sequence 5′-TTCAGCTCTGGGATGACCTT-3′. The PCR program for GAPDH consisted of 25 cycles of: 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C, followed by a final extension at 72 °C for 10 min. Both PCRs were performed in a PTC-100 automated thermocycler and the expected PCR products of 641 and 128 bp for the GPR91 and GAPDH, respectively, were subjected to agarose gel electrophoresis in TAE buffer, stained with ethidium bromide and visualized using a UV-light transilluminator.

2.4. Immunofluorescence

Confocal immunofluorescence histochemistry was performed on 4-μm-thick frozen sections of rat liver as described previously [6]. The frozen tissue sections were fixed and permeabilized by cold acetone. After blocking steps, specimens were labeled with primary antibodies for GPR91 (Chemicon International, Temecula, CA) and the stellate cell marker desmin (Sigma, St. Louis, MO), rinsed with phosphate-buffered saline, and incubated with fluorescent secondary antibodies (Invitrogen, Eugene, Oregon). Primary antibodies used were anti-desmin mouse monoclonal (1:50) and anti-GPR91 rabbit polyclonal (1:25). Secondary antibodies were Alexa 488 anti-rabbit (1:500) and Alexa 568 anti-mouse (1:500). Tissue also was labeled with the actin stain Alexa 647-conjugated phalloidin (Invitrogen) to facilitate identification of individual cells. For negative control studies, tissue was incubated with secondary antibodies, but primary antibodies were omitted. In single cell studies, freshly isolated stellate cells were double-labeled with GPR91 and a mouse monoclonal antibody directed against the quiescent stellate cell marker glial fibrillary acidic protein (GFAP, 1:100; Abcam, Cambridge, MA) [12]. Specimens were examined with a Zeiss LSM 510 META Laser Scanning Confocal Microscope equipped with argon and helium/neon lasers (Thornwood, NY). To ensure specificity of staining, images were obtained by using confocal machine settings (i.e., aperture, gain, and black level) at which no fluorescence was detectable in negative control samples labeled with secondary antibodies alone. Immunofluorescence images were obtained by excitation at 488 nm with observation at 505–550 nm to detect Alexa 488, by excitation at 543 nm and observed at 560–615 nm to detect Alexa 568, then by excitation at 633 nm with observation at >650 nm to detect Alexa 647. This approach eliminated bleed-through of Alexa 488 fluorescence into the longer wavelength detection channel. These confocal images used a Plan Apochromat 63×, 1.20 numeric aperture water immersion objective, argon (488 nm) and helium/neon (543 and 633 nm) laser lines, and a confocal pinhole adjusted to obtain a 1-μm depth of focus.

2.5. Isolated perfused rat liver studies

Liver perfusions were performed as described previously [13]. The pancreatoduodenal branch of the portal vein was ligated and the bile duct, portal vein and inferior vena cava were cannulated with the animal under pentobarbital anesthesia (50 mg/kg of body weight). The liver was transferred to a chamber maintained at 37 °C and perfused at 40 ml/min with Krebs–Ringer bicarbonate (KRB) buffer containing 5 mM glucose and gassed with 95% O_2/5% CO_2. Using a single-pass system, bile flow was measured gravimetrically in pre-tared tubes and perfusion pressure was monitored continuously before, during and after isolated perfused rat livers (IPRLs) were infused with succinate (100 μM). Viability of each IPRL was assessed by observing portal pressure and bile flow throughout each experiment. In addition, each liver was examined for evidence of mottling or other gross morphological changes, and trypan blue was added to the perfusate at the end of each experiment to assess the adequacy of vascular perfusion. Hepatic glucose release was measured throughout the course of experiments as an additional index of effects on hepatocytes.

2.6. Succinate assay

Succinate released from the liver was measured directly in IPRL effluent. The perfusion buffer was collected in plastic tubes after it passed through the liver and placed immediately on ice. The concentration of succinate in those samples was tested by using the Succinic Acid Assay Procedure (Megazyme, Bray, Ireland), which applies the method of succinyl-CoA synthase, pyruvate kinase, and lactate dehydrogenase, according to Beutler [14]. The principle of this method is that in the presence of adenosine-5′-triphosphate (ATP), succinate is converted to succinyl-CoA by the enzyme succinyl-CoA synthetase (SCS), with the concurrent formation of adenosine-5′-diphosphate (ADP) and inorganic phosphate (Pi). In the presence of pyruvate kinase, ADP reacts with phosphoenolpyruvate (PEP) to form pyruvate and ATP. The pyruvate produced is reduced to L-lactate by L-lactate dehydrogenase (L-LDH) in the presence of reduced nicotinamide adenine