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ORIGINAL ARTICLE

Switching-on of serotonergic calcium signaling in activated hepatic stellate cells

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Abstract

AIM: To investigate serotonergic Ca^{2+} signaling and the expression of 5-hydroxytryptamine (5-HT) receptors, as well as Ca^{2+} transporting proteins, in hepatic stellate cells (HSCs).

METHODS: The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of isolated rat HSCs was measured with a fluorescence microscopic imaging system. Quantitative PCR was performed to determine the transcriptional levels of 5-HT receptors and endoplasmic reticulum (ER) proteins involved in Ca^{2+} storage and release in cultured rat HSCs.

RESULTS: Distinct from quiescent cells, activated HSCs exhibited $[Ca^{2+}]_i$ transients following treatment with 5-HT, which was abolished by U-73122, a phospholipase C inhibitor. Upregulation of 5-HT_{2A} and 5-HT_{2B} receptors, but not 5-HT_3, was prominent during trans-differentiation of HSCs. Pretreatment with ritanserin, a 5-HT_2 antagonist, inhibited $[Ca^{2+}]_i$ changes upon application of 5-HT. Expression of type 1 inositol-5'-triphosphate receptor and type 2 sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase were also increased during activation of HSCs and serve as the major isotypes for ER Ca^{2+} storage and release in activated HSCs. Ca^{2+} binding chaperone proteins of the ER, including calreticulin, calnexin and calsequestrin, were up-regulated following activation of HSCs.

CONCLUSION: The appearance of 5-HT-induced $[Ca^{2+}]_i$ response accompanied by upregulation of metabotropic 5-HT_2 receptors and Ca^{2+} transporting/chaperone ER proteins may participate in the activating process of HSCs.

Key words: Hepatic stellate cells; 5-hydroxytryptamine; Intracellular Ca^{2+} transient; Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; Inositol-5'-triphosphate receptor; Endoplasmic reticulum chaperone

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INTRODUCTION

Hepatic stellate cells (HSCs), also known as "Ito cells" or "fat-storing cells", localize between hepatocytes and sinusoids (space of Disse) in mammalian livers. In their healthy state, HSCs control retinoid homeostasis, sinusoidal blood flow, macromolecule transport, and potentially act as antigen-presenting cells in the liver[1,2]. However, in response to hepatic injury, HSCs undergo gross morphological and functional changes, transforming to a myofibroblast-like phenotype in a process called "activation" or "trans-differentiation"[3,4]. Manifestations of activated HSCs include: (1) the expression of contractile cytoskeletal proteins such as α-smooth muscle actin (α-SMA)[5,6]; (2) enhanced extracellular matrix synthesis[7,8]; (3) increased cell size and proliferation[9]; (4) decreased size of lipid droplets[8,10]; and (5) well developed endoplasmic reticulum (ER), Golgi bodies, and compacted microfilaments[11,12]. In particular, the deposition of cross-linked collagen during the activation process may result in cirrhotic changes accompanied by life-threatening hepatic dysfunction.

Serotonin [5-hydroxytryptamine (5-HT)] is a neurotransmitter that also acts as a multifunctional hormone in various tissues[13], where it modulates proliferation and differentiation of muscle, neurons, and mammary glands[14-16]. Serotonin released from platelets at sites of injury plays an important role in liver regeneration and fibrosis[17]. It has been reported that patients with cirrhosis of the liver and portal hypertension have increased plasma serotonin levels[18]. The expression levels of 5-HT_{2A} and 5-HT_{2B} are increased in the liver after hepatectomy as well as in activated HSCs[2,17]. Moreover, 5-HT_2 receptor antagonists suppress cell proliferation and expression of key fibrogenic factors in activated HSCs[2,19]. Among the mammalian 5-HT receptors (5-HT_1 to 5-HT_7), the 5-HT_2 receptor family is coupled to the $G_{q/11}$ protein and increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) mobilized from ER reservoirs[20].

As the major intracellular calcium storage site, the ER possesses various kinds of calcium regulatory proteins that participate in: (1) pumping Ca^{2+} into the ER lumen, such as the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA); (2) releasing Ca^{2+} into the cytosol, such as IP_3 or ryanodine receptors; and (3) buffering Ca^{2+}, such as calreticulin and calnexin, which are also known as chaperones. ER Ca^{2+} homeostasis is maintained by a balance between Ca^{2+} release and replenishment[21]. The free Ca^{2+} concentration in the ER ($[Ca^{2+}]_{ER}$) ranges from 60-400 μmol/L, and disturbances in $[Ca^{2+}]_{ER}$ homeostasis can affect many of the functions of the ER including protein synthesis, secretion[22], protein folding[23], and sensitivity of cells to apoptosis[24]. Further, $[Ca^{2+}]_{ER}$ homeostasis might be critically required for the activation process of HSCs in order to keep up with accelerated protein synthesis. However, until now, the compensatory changes in ER protein expression involved in Ca^{2+} homeostasis and chaperone function have not been clearly elucidated.

$[Ca^{2+}]_i$ may be important for the activation of HSCs, primarily because $[Ca^{2+}]_i$ regulates the transcription of genes critical for cell function[25], and secondly because contractile elements such as α-SMA respond sensitively to $[Ca^{2+}]_i$[26]. We hypothesized that serotonin, acting as an autocrine or paracrine mediator, can elicit a Ca^{2+} signal, and this signal might be involved in the activation of HSCs. Moreover, there may be an alteration in the ER function of HSCs such as Ca^{2+} release and protein folding. In this study, we isolated and cultured rat HSCs on plastic dishes *in vitro*, which has been widely accepted as an appropriate model for the study of activated HSCs[8,27]. Appearance of $[Ca^{2+}]_i$ transients induced by 5-HT and the upregulation of 5-HT_2 receptors and ER proteins were observed during HSC activation. These observed changes may participate in an activation signal as well as adaptive changes during the trans-differentiation of HSCs.

MATERIALS AND METHODS

Isolation of rat HSCs

HSCs were isolated from male Sprague-Dawley rats (150-250 g) by means of a collagenase/pronase perfusion and Nycodenz-gradient centrifugation, as previously described[28,29]. HSCs were cultured with DMEM containing fetal bovine serum (10%) and antibiotics-antimycotics (Invitrogen, Carlsbad, CA, USA) in a humidified incubator (5% CO_2, 37℃). The purity of HSCs was > 95% as assessed by their typical microscopic morphology and positive immunocytochemical staining for desmin at 24 to 48 h after seeding.

Quantitative reverse transcription-polymerase chain reaction analysis

Total cellular RNA was isolated and purified from HSCs at different culture periods, and reverse transcription (RT) was performed with random hexamers. Quantitative real time PCR using SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA) was performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Sequence specific oligonucleotide primers for the genes of interest were designed based on rat sequences deposited in the GenBank database (Tables 1 and 2), and the amplification program included the activation of AmpliTaq Gold at 95℃ for 10 min, followed by 45 cycles of a two-step PCR reaction with denaturation at 95℃ for 15 s and annealing/extension at 60℃ for 1 min. The constitutively expressed housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was selected as an endogenous control to correct for potential variation in RNA loading and efficiency of amplification reactions.

Fluorescent $[Ca^{2+}]_i$ measurement

HSCs at 3 d or 2 wk after isolation were seeded on glass

Table 1 Primers for reverse transcription-polymerase chain reaction

Name	Sequence	Accession code	Position	Product (bp)
5-HT_{1A}				
(+)	5'-TCAGCTACCAAGTGATCACC-3'	NM_012585.1	98-117	211
(-)	5'-GTCCACTTGTTGAGCACCTG-3'		308-289	
5-HT_{1B}				
(+)	5'-TACACGGTCTACTCCACGGT-3'	NM_022225.1	610-629	258
(-)	5'-TCGCACTTTGACTTGGTTCAC-3'		867-847	
5-HT_{2A}				
(+)	5'-GTGTCCATGTTAACCATCCT-3'	NM_017254	446-465	376
(-)	5'-GTAGGTGATCACCATGATGG-3'		821-802	
5-HT_{2B}				
(+)	5'-CATGCATCTCTGTGCCATTTC-3'	NM_017250	652-672	352
(-)	5'-TGTTAGGCGTTGAGGTGGC-3'		1003-985	
5-HT_{3A}				
(+)	5'-TCCTCAACGTGGATGAGAAG-3'	NM_024394.1	553-572	352
(-)	5'-ATGTTGATGTCCTGGATGGT-3'		904-885	
5-HT_{3B}				
(+)	5'-AAGCCCATCCAGGTGGTCTC-3'	NM_022189.1	459-478	428
(-)	5'-GACATGTTGACCCTGAAGAC-3'		886-867	
5-HT_{4}				
(+)	5'-TCATGGTGCTGGCCTATTAC-3'	NM_012853.1	640-659	377
(-)	5'-CTCATCATCACAGCAGAGGA-3'		1016-997	
5-HT_{5A}				
(+)	5'-GAACAGGAGGAAGGAAGAGA-3'	NM_013148	1535-1554	109
(-)	5'-TAAGTCTCCTTGGTGTGAGG-3'		1643-1624	
5-HT_{5B}				
(+)	5'-TTCACCGTACTCGTGGTAAC-3'	L10073.1	453-472	132
(-)	5'-GGTCGAGGCTACCAAGTTAT-3'		584-565	
5-HT_{6}				
(+)	5'-CCTGAGAGTGTGCTGAATTG-3'	NM_024365.1	1716-1735	129
(-)	5'-AGCCACACTACACAAGCAAC-3'		1844-1825	
5-HT_{7}				
(+)	5'-GTGTGTCCACTGTCAAATCC-3'	NM_022938	2072-2091	148
(-)	5'-TCACTCATCTCCAGTTACCG-3'		2219-2200	

5-HT: 5-hydroxytryptamine.

coverslips and loaded with fura-2/AM (5 μmol/L) in a dark room for 30 to 60 min at room temperature. Dye-loaded cells were then washed and transferred to a perfusion chamber on a fluorescence microscope (IX-70, Olympus, Tokyo, Japan). The HSCs were alternately excited at 340 and 380 nm by a monochromatic light source (LAMDA DG-4; Sutter, Novato, CA, USA), and fluorescence images were captured at 510 nm with an intensified CCD camera (Cascade; Roper, Duluth, GA, USA). Images were analyzed using the Metafluor 6.1 software package (Universal Imaging Corporation, Downingtown, PA, USA).

Immunocytochemistry

HSCs cultured on coverslips were fixed in 4% paraformaldehyde and immunocytochemical staining was performed using an antibody for α-SMA (Sigma Chemical Co., St Louis, MO, USA). After incubating with a biotinylated secondary antibody, an avidin-conjugated peroxidase complex was added to the slides and 3-amino-9-ethylcarbazole (AEC) was used as the chromogen.

Electrophysiology

Whole-cell membrane currents were recorded using the gramicidin-perforated patch-clamp technique as described previously[28]. All experiments were performed at room temperature (20-24℃). The internal solution for the perforated patch clamp contained (in mmol/L): 140 KCl, 5 EGTA, 10 HEPES, 0.5 $CaCl_2$, 5 NaCl, and gramicidin (50 μg/mL) (pH 7.2). The external solution contained (in mmol/L): 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, and 10 glucose (pH 7.4).

Statistical analysis

Quantitative data are expressed as the mean ± SE. Statistical comparisons were made by the two-tailed Student's *t*-test and ANOVA. Differences with $P < 0.05$ were considered to be significant. PCR from each cDNA sample was done in triplicate and *n* indicates the number of experiments. For quantitative comparisons, the expression level of each gene was normalized to that of GAPDH and presented as relative expression ratio (target/GAPDH) by applying the formula $2^{-\Delta\Delta Ct}$[30].

RESULTS

Serotonergic signaling and receptor expression during HSC activation

We isolated HSCs using density gradient-based separation with Nycodenz. Most of the harvested cells (> 95%)

Table 2 Primers for quantitative reverse transcription-polymerase chain reaction

Name	Sequence	Accession code	Position	Product (bp)
5-HT2A				
(+)	5'-GGGTACCTCCCACCGACAT-3'	NM_	234-252	101
(-)	5'-TTTCCAGCAATGGTGAGAATAATC-3'	17254	334-311	
5-HT2B				
(+)	5'-CGCCATCCCAGTCCCTATTA-3'	NM_	781-800	101
(-)	5'-AGAGCATGAAACTGCCAAAGC-3'	17250	881-861	
IP3R 1				
(+)	5'-GCAGAAGCAGATTGGCTATG-3'	NM_	2072-2091	261
(-)	5'-GTCTCAATCAGGATGTCAGC-3'	1007235	2332-2313	
IP3R 2				
(+)	5'-CAAGAAGTTCAGAGACTGCC-3'	NM_	396-415	295
(-)	5'-ACGCATGGCATTCTTCTCCA-3'	31046.3	690-671	
IP3R 3				
(+)	5'-GATGTGGTGTTGCTGCAGAA-3'	NM_	390-409	137
(-)	5'-TTGTTGCTCTTCATGTGCAG-3'	13138.1	526-507	
RyR 1				
(+)	5'-CTGAATGTCTGCTCTCCAAG-3'	AC	35577-35596	112
(-)	5'-GAAGGGCAGAGAGACAAGAT-3'	165142.3	35688-35669	
RyR 2				
(+)	5'-ATGTAGGCTTCTTCCAGAGC-3'	XR_	11405-11414	136
(-)	5'-TGCAGTACCTTCTCTCCTGA-3'	8338.1	11540-11521	
RyR 3				
(+)	5'-TACCTTGCCTGGTACACAAC-3'	XM_001080527.1	13957-13976	123
(-)	5'-AGTCACAGATGACAGGATCG-3'		14079-14060	
SERCA 1				
(+)	5'-CCAAGGAGCCTCTTATCAGT-3'	NM_017254	2516-2535	111
(-)	5'-CCTCTGCATACAAGAACCAC-3'		2626-2607	
SERCA 2				
(+)	5'-AGTTCATCCGCTACCTCATC-3'	M 23114	2297-2316	119
(-)	5'-CACCAGATTGACCCAGAGTA-3'		2415-2396	
SERCA 3				
(+)	5'-CTCATGCAGAAGGAGTTCAC-3'	NM_172812	1563-1582	140
(-)	5'-CGCTCAATTACACTCTCAGG-3'		1702-1683	
Calreticulin				
(+)	5'-AGAAGACTGGGATGAACGAG-3'	NM_	683-701	109
(-)	5'-GTCCTCAGGCTTCTTAGCAT-3'	22399.1	791-772	
Calsequestrin-2				
(+)	5'-CAGATGGCTATGAGTTCCTG-3'	NM_	988-1007	118
(-)	5'-CAGTAAGCAACAAGCAGAGG-3'	17131.2	1105-1086	
Calnexin				
(+)	5'-GTGTTTGCTACTGGTCCTTG-3'	NM_	21-40	146
(-)	5'-ATGGAGGAGTGCTGGTATCT-3'	172008.1	166-147	
TGF-β type 1 R				
(+)	5'-ACCAGCTATTGCCCATAGAG-3'	L 26110	1011-1030	106
(-)	5'-GGCAGAATCATGTCTCACAG-3'		1116-1097	
α-SMA				
(+)	5'-GCAGAGCAAGAGGGATCCT-3'	X 06801	222-242	73
(-)	5'-CATGTCGTCCCAGTTGGTGAT-3'		294-274	
Cav1.2 (α1c)				
(+)	5'-GACCCGTAGGAGCACGTTTG-3'	NM_012517	2327-2346	71
(-)	5'-CCTCCCCGGTCAGGATCT-3'		2397-2380	

5-HT: 5-hydroxytryptamine; SERCA: Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; α-SMA: α-smooth muscle actin; RyR: Ryanodine receptor; TGF: Transforming growth factor.

exhibited positive intra-cytoplasmic staining for desmin and glial fibrillary acidic proteins (GFAP). Expression of HSC trans-differentiation markers was tested at 1 d, 1 wk and 2 wk after isolation. In activated HSCs (2 wk after isolation), bundles of α-SMA were clearly observed as cytoskeletal fibers in immunocytochemical staining (Figure 1A), which was not evident in quiescent cells. In a voltage-clamp mode, nimodipine (10 μmol/L)-sensitive L-type Ca^{2+} currents were recorded only for activated HSCs (Figure 1C). The expression level of α-SMA and the L-type Ca^{2+} channel (Cav1.2) were proportional to the activation period elicited by culturing cells on plastic dishes (Figure 1B and D). Transforming growth factor-β1 (TGF-β1), an abundant isoform of TGF in both normal and cirrhotic liver, is known as the main profibrogenic cytokine[31]. We observed that the type I receptor for TGF-β1 (Tβ-RI) was also upregulated during activation (Figure 1E), while the expression of 28S RNA as well as

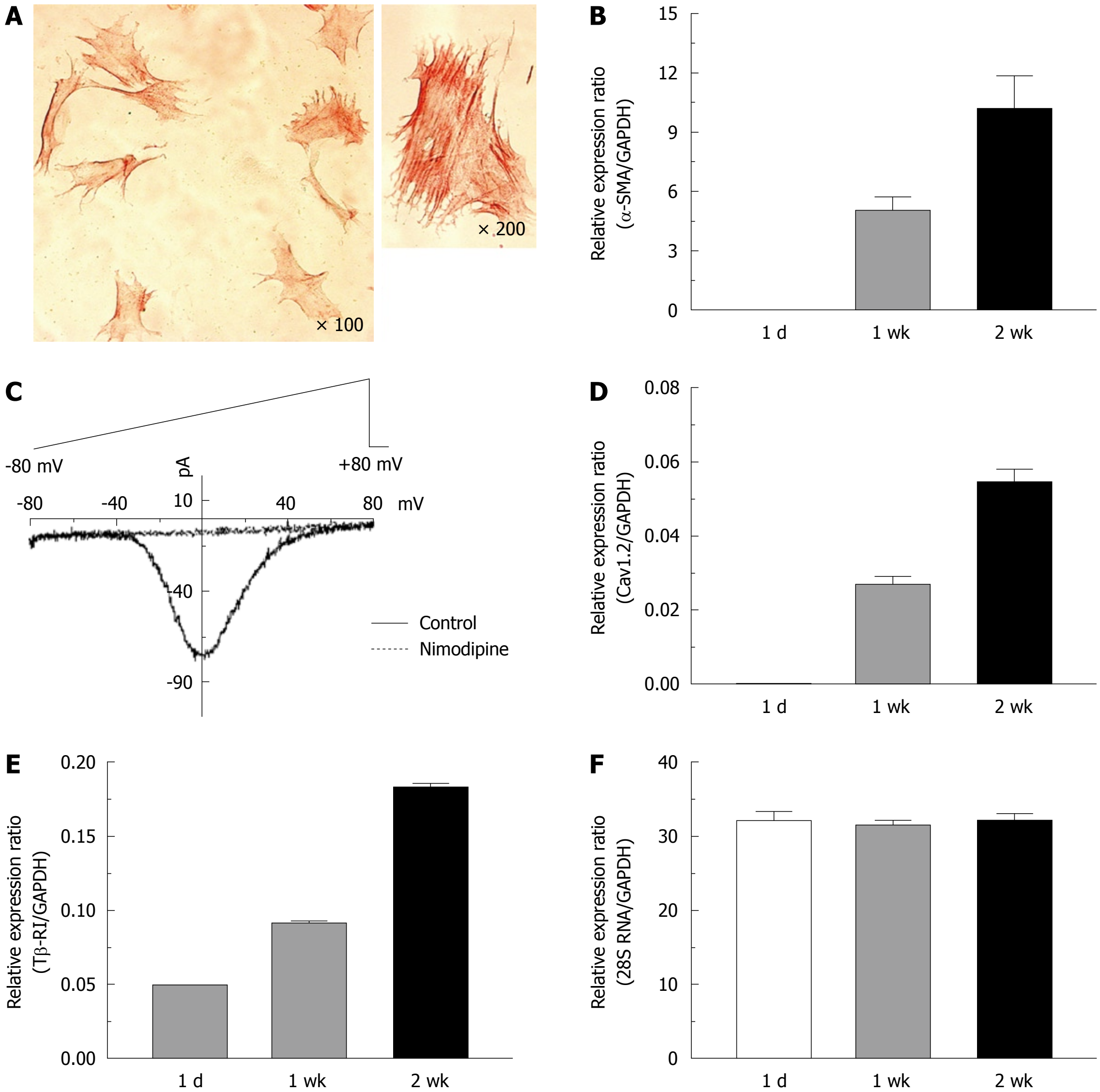

Figure 1 Expression of α-smooth muscle actin, L-type calcium channels and type 1 transforming growth factor-β receptors in activated rat hepatic stellate cells. A: Immunocytochemical staining for α-smooth muscle actin (α-SMA) was performed on hepatic stellate cells (HSCs) cultured for 1 wk; C: Whole cell Ca^{2+} currents in a voltage-clamp mode were recorded from 2 wk-cultured HSCs, and were completely blocked by nimodipine (10 μmol/L); Changes in the transcript levels of α-SMA (B), the α1c subunit of the L-type Ca^{2+} channel (Cav1.2) (D), the type 1 receptor of transforming growth factor-β (Tβ-RI) (E), and 28S RNA (F) during HSC culturing (1 d, 1 wk and 2 wk) were measured by quantitative real-time reverse transcription-polymerase chain reaction analysis. Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as a relative expression ratio (target/GAPDH). Data are presented as the mean ± SE (n = 3).

GAPDH was not changed during the activation process of HSCs (Figure 1F).

Serotonergic signaling has been suggested as a candidate for triggering activation of HSCs[2,17]. We focused on $[Ca^{2+}]_i$ signaling in HSCs, which has been emphasized by previous work as having an important role in the activation process[26,32]. As shown in Figure 2A and B, strong $[Ca^{2+}]_i$ transients followed by a slow plateau increase were recorded in response to 5-HT (10 μmol/L) application only from most of the activated HSCs (2 wk after isolation; 81 cells out of 92 cells), but not from quiescent cells (3 d after isolation; 0 out of 11 cells). The 5-HT-induced $[Ca^{2+}]_i$ increase was dose-dependent in activated HSCs (Figure 2C). Consistent with a previous report[33], ATP also evoked $[Ca^{2+}]_i$ transients in activated HSCs while acetylcholine did not (Figure 3).

Among the 5-HT receptors, 5-HT_2 is known to release Ca^{2+} from the ER while 5-HT_3 acts as a ligand-gated cation channel[20]. We estimated the steady-state mRNA levels of 5-HT receptor isotypes (5-HT_1 to 5-HT_7) using reverse transcription-polymerase chain reaction (RT-PCR) and found that the 5-HT_{2A} and 5-HT_{2B} receptors, but not 5-HT_3, were abundantly transcribed (Figure 2D). Consistent with the observed changes in $[Ca^{2+}]_i$, the expression of 5-HT_{2A} was increased by about 17-fold after 2 wk of isolation (5-HT_{2A}/GAPDH; from 0.004 at 1 d to 0.067 at 2 wk). 5-HT_{2B} was also found to be upregulated in activated HSCs (from 0.003 to 0.008) using quantitative RT-PCR (Figure 2E).

It has been recognized that 5-HT_2 receptors are coupled with the $G_{q/11}$-phospholipase C pathway. Figure 4A and B show that the 5-HT-induced $[Ca^{2+}]_i$ changes were abolished

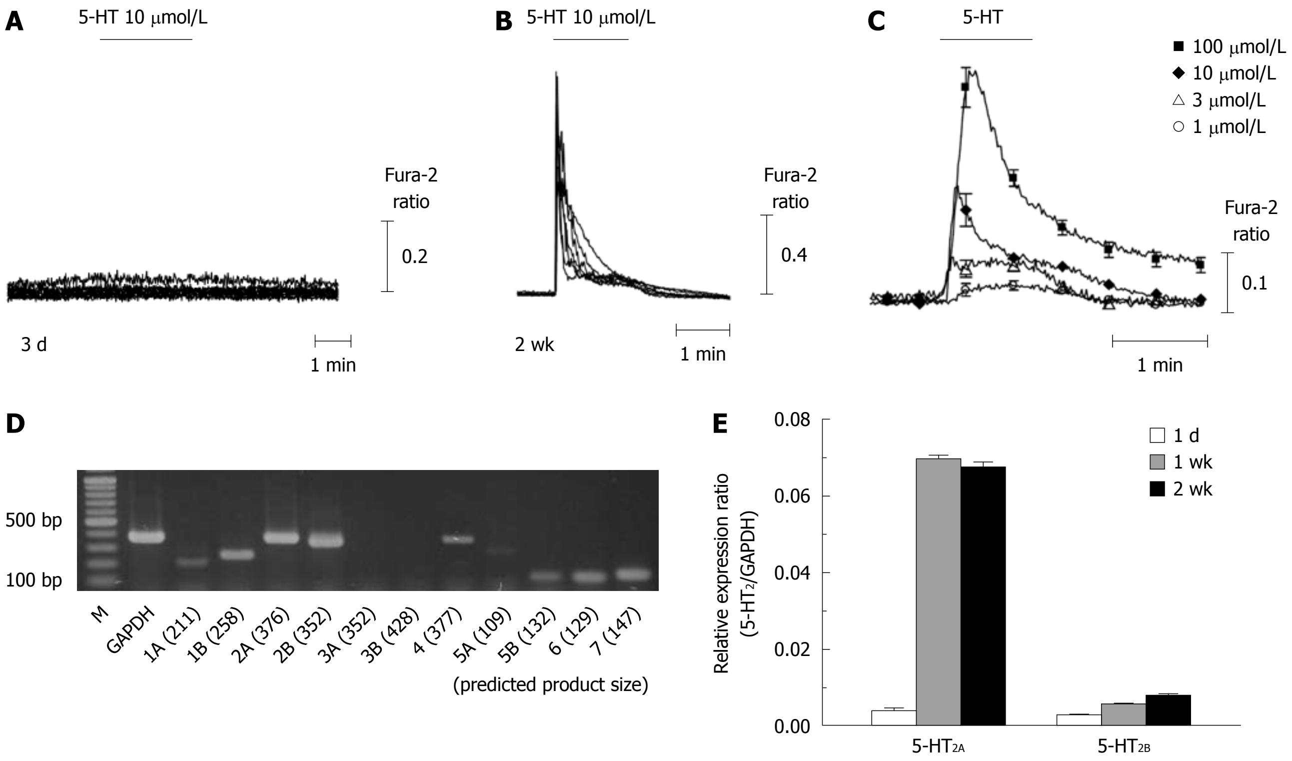

Figure 2 5-hydroxytryptamine-induced intracellular Ca^{2+} concentration changes and the expression of 5-hydroxytryptamine$_2$ receptors in quiescent and activated hepatic stellate cells. A, B: 5-hydroxytryptamine (5-HT)-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) transients were recorded from hepatic stellate cells (HSCs) at 3 d (A) and 2 wk (B) after isolation; C: Averages of $[Ca^{2+}]_i$ changes (from 13-40 cells/each trace) in response to 5-HT (1-100 μmol/L) application to 2 wk-cultured HSCs are shown; D: Steady-state mRNA levels of the 5-HT receptor isotypes in 2 wk-cultured HSCs were compared using reverse transcription-polymerase chain reaction (RT-PCR); E: Using quantitative RT-PCR, the transcriptional changes in 5-HT_2 receptors among 1 d-, 1 wk- and 2 wk-cultured HSCs were compared. Expression levels were normalized to GAPDH and expressed as a relative expression ratio (target/GAPDH, *n* = 3). Data are presented as the mean ± SE.

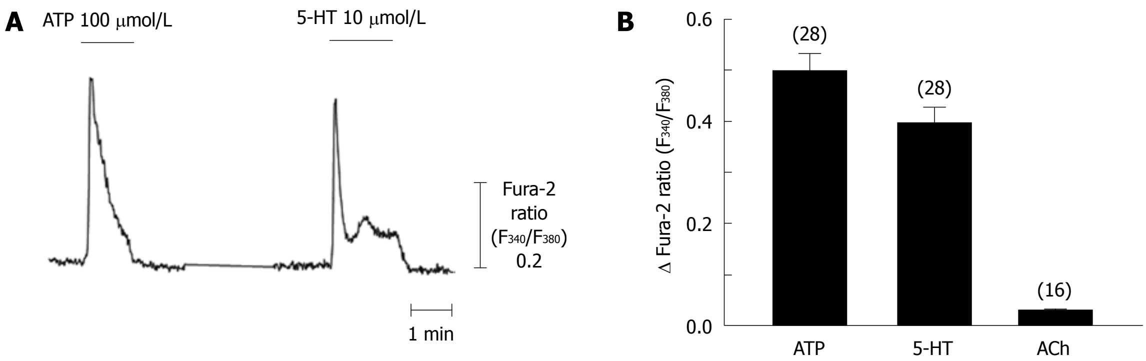

Figure 3 Comparison of intracellular Ca^{2+} concentration responses to various metabotropic receptor agonists in activated hepatic stellate cells. Intracellular Ca^{2+} concentration changes following application of ATP (100 μmol/L), 5-hydroxytryptamine (5-HT) (10 μmol/L), or acetylcholine (ACh, 10 μmol/L) were measured in 2 wk-cultured hepatic stellate cells (*n* = 3-6, 16-28 cells). Data are presented as the mean ± SE.

by pretreatment with 1 μmol/L U73122, a phospholipase C inhibitor (0.05 ± 0.05 peak changes of Fura-2 ratio from 0.66 ± 0.12, *n* = 13). We also observed that $[Ca^{2+}]_i$ transients induced by 5-HT were not altered in extracellular Ca^{2+}-free conditions (data not shown). These results suggest that 5-HT activates phospholipase C to produce IP_3, which induces Ca^{2+} release from ER in activated HSCs. To confirm the receptor subtype, we tested blocking effects of a universal 5-HT_2 antagonist, ritanserin, which does not discriminate among 5HT_2 isotypes. 5-HT-induced $[Ca^{2+}]_i$ responses were attenuated by pretreatment with 10 μmol/L ritanserin by 46.3% (0.34 ± 0.08 from 0.89 ± 0.10, *n* = 11).

Upregulation of calcium transporting and binding proteins in the ER

In mammalian cells, there are three major subtypes of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA1, 2, and 3) which pump Ca^{2+} into the ER lumen. We observed SERCA2 to be the dominant subtype in HSCs. SERCA2, especially SERCA2b, is considered to be a house-keeping protein expressed constitutively

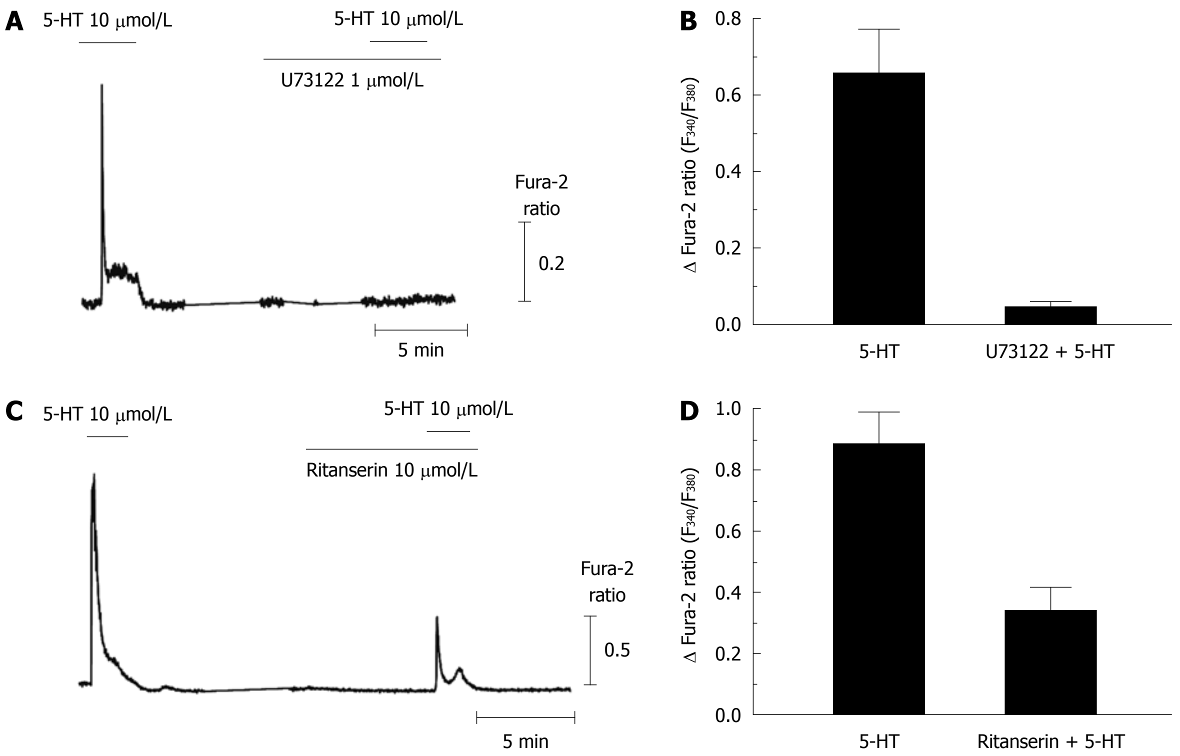

Figure 4 5-hydroxytryptamine-induced intracellular Ca^{2+} concentration transients *via* metabotropic 5-hydroxytryptamine$_2$ receptor in activated hepatic stellate cells. A, B: 5-hydroxytryptamine (5-HT)-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) transients were completely abolished by pretreatment with U73122 (1 μmol/L), a phospholipase C blocker (*n* = 3, 11 cells); C, D: Ritanserin (10 μmol/L), a 5-HT$_2$ antagonist, inhibited the $[Ca^{2+}]_i$ responses to 5-HT in activated hepatic stellate cells (2 wk-cultured cells; *n* = 3, 13 cells). Data are presented as the mean ± SE.

in most kinds of cells; however, in HSCs, the expression of SERCA2 tends to increase during activation. Specifically, the relative expression ratio of SERCA2 (SERCA2/GAPDH) at 1 d after isolation was 0.058, and increased to 0.106 after 1 wk in culture and 0.164 after 2 wk in culture *in vitro* (Figure 5A). The expression of SERCA3 was also increased during culture (SERCA3/GAPDH; 0.4 × 10^{-3} at 1 d and 6.9 × 10^{-3} at 2 wk).

Among the three isoforms (types 1 through 3) of the IP_3 receptor, the type 1 IP_3 receptor was the main subtype expressed in activated HSCs. We observed that the expression of the type 1 IP_3 receptor increased by about 7-fold (IP_3R 1/GAPDH = 0.037) after 1 wk of culture, and 20-fold (0.100) after 2 wk of culture compared to (0.005) levels 1 d after isolation (Figure 5B). In contrast, the expression level of ryanodine receptors, which are a family of Ca^{2+}-releasing channel proteins expressed in the ER, either did not change or was decreased during the activation of HSCs (Figure 5C).

We investigated whether Ca^{2+} binding chaperones of the ER could be up-regulated following the activation process of HSCs. There were similar increases in the expression levels of calreticulin (calreticulin/GAPDH; from 0.204 at 1 d to 0.655 at 2 wk), calnexin (calnexin/GAPDH; from 0.240 at 1 d to 0.750 at 2 wk), and calsequestrin in HSCs. In the case of calsequestrin, the expression level in HSCs at 1 d after isolation was undetectable, but was markedly increased (calsequestrin/GAPDH; 0.217) after 2 wk of culturing (Figure 5D).

DISCUSSION

Trans-differentiation of HSCs is accompanied by marked increases in protein synthesis, including collagen, elastin, and glycoproteins[7]. It is well known that Ca^{2+} homeostasis in the ER is critical for the synthesis, folding, and secretion of protein[22,23]. In HSCs, the depletion of ER Ca^{2+} stores inhibits protein synthesis and increases intracellular degradation of collagen[34]. Maintaining a high Ca^{2+} gradient across the ER membrane (around 1000-fold) is accomplished by active Ca^{2+} transport by SERCAs. Among the three different isoforms of SERCAs, SERCA2 is considered to be a house-keeping protein expressed in the ER of most cell types, including HSCs[34]. We observed that SERCA2 was the main isotype in quiescent and activated HSCs (Figure 5A). During activation, the expression of SERCA2 (and also SERCA3) was increased, which likely helped to maintain appropriate ER Ca^{2+} concentrations.

Chaperone proteins in the ER facilitate the folding of newly synthesized proteins and glycoproteins. In particular, calreticulin and calnexin are important chaperones involved in a "quality control" system for protein synthesis[35]. In addition, these chaperones act as Ca^{2+} binding proteins in the ER. Overexpression of calreticulin increases the total amount of Ca^{2+} in intracellular stores, whereas calreticulin-deficient cells have reduced ER Ca^{2+} storage capacity[36]. Impaired collagen synthesis has been observed in cells derived from mice possessing genetic defects in ER chaperone proteins[37]. In this study, we observed for

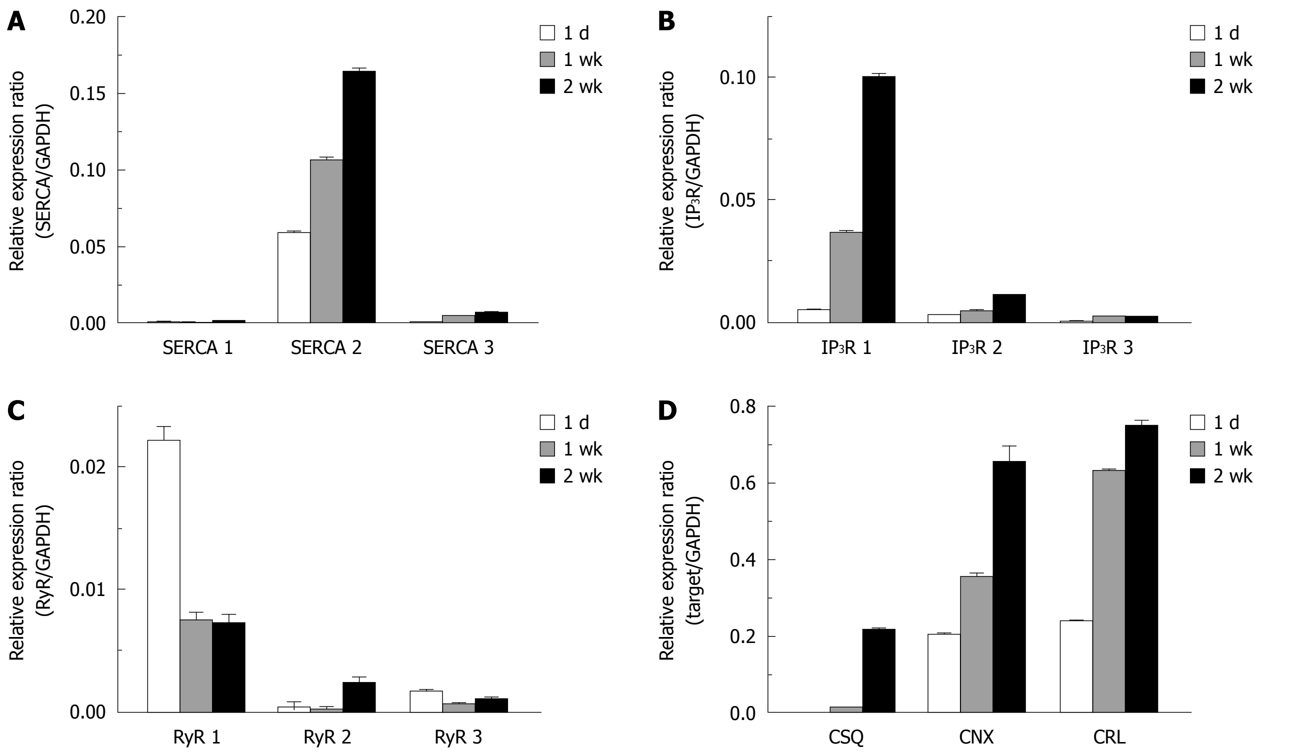

Figure 5 Up-regulation of endoplasmic reticulum Ca^{2+} transporting and binding proteins in activated hepatic stellate cells. Changes in the expression level of 3 isoforms of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (A), inositol triphosphate receptor (IP_3R) (B), ryanodine receptor (RyR) (C) and Ca^{2+} binding chaperones (D) during the culture periods (1 d, 1 wk and 2 wk) were measured by quantitative real-time reverse transcription-polymerase chain reaction analysis. Expression levels were normalized to GAPDH and expressed as a relative expression ratio (target/GAPDH). Data are presented as the mean ± SE (n = 3). CSQ: Calsequestrin, CNX: Calnexin, and CRL: Calreticulin.

the first time that the expression of ER Ca^{2+} binding proteins was markedly increased during the activation process of HSCs, which might be an important adaptive change for trans-differentiation.

Upon stimulation from the extracellular space, ER Ca^{2+} is the main source for releasing Ca^{2+} and is responsible for enabling biologic signaling mediated by Ca^{2+}. In addition, Ca^{2+} release from the ER stimulates store-operated Ca^{2+} entry into the cytosol, which eventually increases the refilling of the ER Ca^{2+} reservoir. It has been shown that cytosolic Ca^{2+} signaling is important for proliferation and differentiation of HSCs[25]. Similar to myofibroblast-like cells, activated HSCs can have a contractile response to $[Ca^{2+}]_i$ changes, which may increase vascular resistance leading to portal hypertension *in vivo*[32]. During trans-differentiation, the expression of L-type calcium channels increases, which may contribute to cytosolic Ca^{2+} signaling in HSCs[26,38]. In the present study, we observed that 5-HT increased $[Ca^{2+}]_i$ only in activated HSCs *via* a serotonergic receptor. Until now, 5-HT-induced $[Ca^{2+}]_i$ changes have not been reported in HSCs. Physiologic concentrations of 5-HT in plasma are known to be less than 100 nmol/L, but those in cirrhotic patients are significantly elevated (3-4 fold) compared to controls[39]. Moreover, intrahepatic neighboring cells secrete 5-HT to act as an autocrine/paracrine regulator[40]. Thus, we hypothesize that local 5-HT concentration close to the releasing cells might be higher than the plasma level and repetitive exposure may have additive effects on $[Ca^{2+}]_i$-mediated changes in the process of HSC activation.

We observed that 5-HT elicited a $[Ca^{2+}]_i$ response *via* the metabotropic 5-HT_2 receptor in activated HSCs. This was demonstrated by the findings that 5-HT-induced $[Ca^{2+}]_i$ transients were (1) completely blocked by a PLC inhibitor; (2) not altered by nominally Ca^{2+} free conditions; and (3) reduced by a 5-HT_2 blocker. 5-HT_{2A} is known to mediate mitogenic effects in fibroblasts[41], while 5-HT_{2B} is involved in the development of the heart and enteric nervous system[42]. However, we did not discriminate whether the 5-HT_{2A} and/or 5-HT_{2B} receptor mediated the serotonergic Ca^{2+} signaling in activated HSCs. We also observed that the type I IP_3 receptor (IP_3R 1) is the main isoform expressed in activated HSCs, which is consistent with a recent report by Kruglov *et al*[32]. The expression level of IP_3R 1 was increased during the activation process (Figure 5B).

Various ligands for $G_{q/11}$-coupled metabotropic receptors could be important extracellular stimuli, as they generate IP_3 by activating phospholipase-C. Interestingly, it has been reported that the expression of the P2Y metabotropic purinoceptor (P2Y6) is rapidly upregulated following activation of HSCs, with a similar increase in ATP-induced $[Ca^{2+}]_i$ transients[33]. The same study also reported that extracellular UDP increases the transcription of procollagen in activated HSCs *via* activation of the P2Y receptor, and this effect is partially inhibited by a P2Y receptor blocker. These results add further support to the hypothesis that Ca^{2+} signaling released from ER stores is associated with HSCs undergoing the process of activation. We also observed that ATP increased $[Ca^{2+}]_i$, which might be mediated by the metabotropic P2Y receptor (Figure 3). However, acetylcholine did not induce calcium changes, indicating that muscarinic acetylcholine receptors do not functionally exist in activated HSCs, even in the presence of machinery for ER Ca^{2+} release.

In this study, we observed the pronounced increase in serotonergic $[Ca^{2+}]_i$ response related to the upregulation of metabotropic 5-HT_2 receptors, type 1 inositol-5'-triphosphate receptor, type 2 sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase, and Ca^{2+} binding ER chaperone proteins following trans-differentiation of HSCs. These changes may be involved in the pathophysiologic (profibrotic) process of rat HSCs as well as being a compensatory mechanism for maintaining ER Ca^{2+} homeostasis and protein synthesis/maturation. Switching on and off of the serotonergic signaling pathway might be implicated in potential treatment for portal hypertension. Yet, the biological relevance of a 5-HT-induced $[Ca^{2+}]_i$ transient in HSCs remains to be clarified. Moreover, it is not obvious whether simply switching-off this serotonergic signaling is an ideal target for developing treatments for liver cirrhosis. While there is evidence to suggest that 5-HT_2 antagonists reduce proliferation and increase cell death of isolated HSCs[2,19], a recent study found that fibrotic changes induced by CCl_4 are not ameliorated by a 5-HT_2 antagonist[29,43]. Further studies to elucidate the detailed role of serotonergic signaling in HSCs are needed in order to develop therapeutic approaches to hepatic fibrosis.

COMMENTS

Background

Hepatic stellate cells (HSCs) are known to initiate hepatic fibrosis by trans-differentiating into myofibroblast-like cells. Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) have been suggested as a stimulus for the activation of HSCs.

Research frontiers

Recent data showed that activated HSCs responded to 5-hydroxytryptamine (5-HT) in a profibrogenic manner, which can be suppressed by 5-HT_2 antagonists. In this study, the authors demonstrated that 5-HT generated $[Ca^{2+}]_i$ transients released from endoplasmic reticulum (ER) in trans-differentiated HSCs, which was consistent with the upregulation of 5-HT_2 receptors.

Innovations and breakthroughs

Serotonergic $[Ca^{2+}]_i$ signaling has not been reported in HSCs, until now. It is also a novel finding that the expression of ER Ca^{2+} binding proteins was markedly increased during the activation process of HSCs.

Applications

The identification of $[Ca^{2+}]_i$ signaling and the expressional changes of Ca^{2+} handling proteins in the process of HSC activation could help us to understand the pathophysiology and develop therapeutic approaches to hepatic fibrosis.

Terminology

IP_3 receptor and sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase are ER proteins involved in Ca^{2+} release from, and refilling into, ER. Calsequestrin, calnexin, and calreticulin are ER Ca^{2+} binding chaperone proteins. Upregulation of all these proteins is important not only for $[Ca^{2+}]_i$ signaling but also for maintaining ER Ca^{2+} levels needed for protein synthesis/maturation.

Peer review

The manuscript by Park *et al* reports the results of investigations on the serotonergic Ca^{2+} signaling, and the expression of 5-HT receptors and Ca^{2+} transporting proteins in rat HSCs. By employing reverse transcription-polymerase chain reaction, and fluorescent (fura-2) and electrophysiological techniques, as well as immunocytochemistry, the authors conclude that the increase in serotonergic $[Ca^{2+}]_i$ responses accompanied by the upregulation in 5-HT_2 receptors and Ca-transport proteins attests to their role in HSC activation. It is worthy of publication.

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