Molecular basis for calcium signaling in hepatic stellate cells

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Kruglov EA, Correa PR, Arora G, Yu J, Nathanson MH, Dranoff JA. Molecular basis for calcium signaling in hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol 292: G975–G982, 2007. First published January 4, 2007; doi:10.1152/ajpgi.00401.2006.—Progressive liver fibrosis (with the resultant cirrhosis) is the primary cause of chronic liver failure. Hepatic stellate cells (HSCs) are critically important mediators of liver fibrosis. In the healthy liver, HSCs are quiescent lipid-storing cells limited to the perisinusoidal endothelium. However, in the injured liver, HSCs undergo myofibroblastic transdifferentiation (activation), which is a critical step in the development of organ fibrosis. HSCs express P2Y receptors linking extracellular ATP to downstream cytosolic Ca2+ signals and that these receptors markedly upregulated collagen transcription by activated HSCs.

The molecular mechanisms regulating hormone-induced cytosolic Ca2+ release are increasingly well understood. Ligand binding to Gq protein-coupled receptors induces G protein oligomerization, activation of phospholipase C, and generation of inositol (1,4,5)-trisphosphate (IP3) (4). IP3 receptors (IP3Rs), located in the endoplasmic reticulum (ER), are linked to Ca2+ stores. Binding of IP3 to the IP3R opens Ca2+ stores, allowing localized increases near the site of the IP3R (23). Three different IP3R isoforms are found in eukaryotic cells, and each differs in its regulation by Ca2+ concentration and subcellular distribution (4). Thus, the localization and type of IP3R within the cell determine the spatiotemporal aspects of hormone-induced Ca2+ signals.

Because the molecular mechanisms of intracellular Ca2+ signals are of great potential interest in HSC physiology, we investigated the molecular and functional expression of IP3Rs in quiescent and activated HSCs. Here, we report that HSCs express only the type I IP3R and that the receptor shifts into the nucleus and cell extensions upon activation. These cell extensions, furthermore, express sufficient machinery to enable local application of ATP to evoke highly localized Ca2+ signals that induce localized contractions. These autonomous units of subcellular signaling and response reveal a new level of subcellular organization, which, in turn, establishes a novel paradigm for the local control of fibrogenesis within the liver.

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RESULTS

HSCs express only type I IP₃R. Expressions of IP₃R isoforms in rat HSCs were determined using RT-PCR and immunoblot analysis. HSCs expressed mRNA transcripts for type I and II IP₃Rs but not for type III IP₃R (Fig. 1A). HSCs expressed only type I IP₃R protein (Fig. 1B). These data demonstrate that day 1 (quiescent) and day 7 (activated) HSCs express type I but not type II or III IP₃R proteins.

HSCs shift the subcellular expression of type I IP₃R upon activation. Subcellular distributions of type I IP₃R in day 1 and 7 HSCs were determined using confocal immunofluorescence. In day 1 HSCs, type I IP₃R was localized to the perinuclear cytoplasm, where it overlapped with the distribution of autofluorescent lipid droplets characteristic of these cells (Fig. 2, A and B). In contrast, in day 7 HSCs, type I IP₃R was localized to multiple subcellular compartments, including the nucleus, cytoplasm, and cell extensions (Fig. 2, C–E). In day 7 HSCs, type I IP₃R colocalized with SMA microfilaments and desmin intermediate filaments. Neither day 1 nor day 7 (Fig. 2, F–J) HSCs expressed type II or III IP₃Rs. Taken together, these data demonstrate that HSCs shift the distribution of type I IP₃R from the perinuclear cytoplasm to multiple distinct subcellular compartments upon activation.

ER and Ca²⁺ stores are expressed in multiple subcellular compartments in activated HSCs. Because of the unique distribution of type I IP₃R in day 7 HSCs, the distribution of the ER resident protein calreticulin was compared with that of type I IP₃R in these cells. Calreticulin is a chaperone protein important for glycoprotein folding that is known to be limited to the ER; for these experiments, calreticulin was used as a specific ER marker (32). As seen in Fig. 3A, calreticulin and type I IP₃R fluorescence were completely colocalized within cell extensions and the HSC cytoplasm. However, calreticulin was absent from the nucleus and perinuclear cytoplasm, where type I IP₃R was also expressed. Since IP₃R function relies upon IP₃R-linked Ca²⁺ stores, the expression of Ca²⁺ stores in day 7 cells was investigated using live-cell confocal microscopy. Day 7 HSCs were labeled with the low-affinity Ca²⁺-sensitive fluorophore Mag-fluo-4 AM. Mag-fluo-4 fluorescence was noted in HSC nuclei, perinuclear cytoplasm, and cell extensions (Fig. 3C). Since IP₃Rs are expressed along ER mem-

J (not shown). HSCs at day 1 after isolation are phenotypically quiescent, whereas cells at day 7 are phenotypically activated (12). RT-PCR. RNA was isolated from HSCs using RNAature reagent (Ambion, Austin, TX). HSC cDNA was produced using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) on DNase-treated RNA. Specific oligonucleotide primers were designed based on cloned rat IP₃R isoforms type I, type II, and type III. These were used to amplify day 1 and 7 HSC cDNA using the following thermal cycling parameters: 94°C for 5 min; 30 cycles (94°C for 30 s, 60°C for 1 min, and 72°C for 1 min); and 72°C for 5 min. Products were evaluated using agarose gel electrophoresis.

Immunoblot analysis. Relative expressions of IP₃R isoforms in quiescent and activated HSCs were determined by immunoblot analysis. Protein was isolated from HSCs at day 1 and 7 days after isolation after osmotic lysis. Equal amounts of protein for each group were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA). The membrane was blocked with nonfat milk (5% in PBS with 0.05% Tween, or skim milk) and II IP₃Rs but not for type III IP₃R (Fig. 1A). HSCs expressed only type I IP₃R protein (Fig. 1B). These data demonstrate that day 1 (quiescent) and day 7 (activated) HSCs express type I but not type II or III IP₃R proteins.

Confocal immunofluorescence. Subcellular distributions of IP₃R isoforms in quiescent and activated HSCs were determined by confocal immunofluorescence. Cells were plated on glass coverslips and fixed in 3.7% formaldehyde in PBS. Cells were washed and stained with rabbit anti-IP₃R type I, type II, or type III antibody (1:500) and either mouse monoclonal anti-α-SMA (1:800, Sigma), anti-desmin (1:50, Sigma), or anti-calreticulin (2 μl/ml) for 45 min at 37°C. Washed, and then incubated with AlexaFluor 488-conjugated anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) and AlexaFluor 598-conjugated anti-mouse secondary antibody (Molecular Probes). Specimens were then stained with TOPRO (Molecular Probes) for 10 min at room temperature. Specimens were examined using a Zeiss LSM 510 confocal imaging system equipped with both a krypton/argon and helium/neon laser at ×400 magnification. Triple-labeled specimens were serially excited at 488 nm and observed at >515 nm to detect AlexaFluor 488, excited at 568 nm and observed at >585 nm to detect AlexaFluor 598 using the krypton/argon laser, and then excited at 633 nm and observed at >650 nm to detect TOPRO using the helium/neon laser. Positive control experiments were performed on 10-μm liver sections (type II IP₃R) or Mz-ChA-1 cells (type III IP₃R).

Live cell confocal microscopic determination of Ca²⁺ stores. Distributions of Ca²⁺ stores were determined with confocal microscopy performed using live day 7 HSCs grown on glass coverslips. HSCs were loaded with Mag-fluo-4 AM (Molecular Probes) for 30 min at room temperature. Cells were examined using a Bio-Rad MRC 1024 confocal imaging system equipped with a krypton/argon laser. Fluo-4 fluorescence was excited using a krypton/argon laser at 433 nm; emitted fluorescence at >515 nm was collected (8).

Two-photon microscopic determination of ER membranes. Distributions of ER membrane structures were determined with two-photon confocal microscopy performed using live day 7 HSCs grown on glass coverslips. HSCs were loaded with ER-tracker (Molecular Probes) for 30 min at room temperature. Cells were examined using a Bio-Rad MRC 1024 confocal imaging system equipped with an infrared laser. ER-tracker was excited at 790 nm by two-photon excitation. We Because of the unique distribution of type I IP₃R in day 7 HSCs, the distribution of the ER resident protein calreticulin was compared with that of type I IP₃R in these cells. Calreticulin is a chaperone protein important for glycoprotein folding that is known to be limited to the ER; for these experiments, calreticulin was used as a specific ER marker (32). As seen in Fig. 3A, calreticulin and type I IP₃R fluorescence were completely colocalized within cell extensions and the HSC cytoplasm. However, calreticulin was absent from the nucleus and perinuclear cytoplasm, where type I IP₃R was also expressed. Since IP₃R function relies upon IP₃R-linked Ca²⁺ stores, the expression of Ca²⁺ stores in day 7 cells was investigated using live-cell confocal microscopy. Day 7 HSCs were labeled with the low-affinity Ca²⁺-sensitive fluorophore Mag-fluo-4 AM. Mag-fluo-4 fluorescence was noted in HSC nuclei, perinuclear cytoplasm, and cell extensions (Fig. 3C). Since IP₃Rs are expressed along ER mem-

Confocal video microscopic determination of Ca²⁺ signals and morphological changes. Changes in cytosolic Ca²⁺ were determined with confocal video microscopy performed using day 7 HSCs grown on glass coverslips (7). HSCs were loaded with the Ca²⁺-sensitive fluorophore fluo-4 AM (Molecular Probes) for 10 min at 37°C and mounted on a specially designed stage for use on a confocal microscope. Cells were perfused with HEPES buffer. HEPES buffer containing ATP (100 μM) and fluorescein was loaded into a microinjection needle and then applied to the region adjacent to an HSC extension using an Eppendorf FemtoJet microinjection system (Eppendorf North America, Westbury, NY). Changes in fluo-4 fluorescence were monitored using a Zeiss LSM 510 (Zeiss, Thornwood, NY) confocal imaging system. Fluo-4 fluorescence was excited using a krypton/argon laser at 488 nm; emitted fluorescence at >515 nm was collected. Changes in fluorescence over time were expressed as peak fluorescence divided by initial fluorescence. For post hoc analysis of cell morphological changes, raw still images at fixed time points were pseudocolored such that all fluorescence above background (determined separately) was observed. Still images were pseudocolored with distinct hues and added using the “Layer Addition” function of Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA) so that overlap of all spectra would produce a white hue.

Statistical analysis. Data are expressed as means ± SD where appropriate. Comparisons between individual groups were made with two-tailed t-tests.
branes, ER distributions in day 7 HSCs were further investigated using live cell two-photon microscopy. Day 7 HSCs were labeled with the ER-avid lipid fluorophore ER-tracker. ER-tracker fluorescence was found in HSC nuclei, perinuclear cytoplasm, and cell extensions (Fig. 3D). Taken together, these data show that the unique distribution of type I IP3R in activated HSCs is consistent with the distribution of Ca2+ stores and ER structures in these cells. Furthermore, the presence of ER membranes and Ca2+ stores within nuclei demonstrate that HSCs express a nucleoplasmic reticulum (8).

Activated HSC extensions express the functional machinery for localized hormone-induced Ca2+ release. HSC extensions contain IP3Rs and Ca2+ stores, so we tested the hypothesis that localized hormonal signaling could induce Ca2+ signals limited to these extensions. P2Y receptors are Gq protein-coupled receptors for extracellular ATP and other nucleotides that induce IP3-mediated Ca2+ signals in a variety of cells (27). Because HSCs are known to express P2Y receptors (7), the activation of P2Y receptors via ATP was used for these experiments. ATP (100 μM) was microperfused into the extracellular space within 10 μm of an HSC extension, and serial changes in Ca2+ were monitored (Fig. 4A). Localized application of ATP induced Ca2+ signals that were limited to the nearby cell extensions and did not spread into other subcellular regions (Fig. 4, B and C). These experiments provide evidence supporting the concept that HSC extensions express sufficient machinery (P2Y receptors, IP3Rs, and Ca2+ stores) to produce hormone-induced Ca2+ signals that are limited to the cell extension itself.

Ca2+ signals within cell extensions induce localized contractions. HSCs are contractile cells (1, 29), and Ca2+ signals mediate cell contractility in HSC and other cell types (3, 4, 15, 33). Therefore, we assessed the effects of localized ATP-induced Ca2+ signals on HSC contractility. Changes in HSC morphology were determined by post hoc analysis. As seen in Fig. 4D, localized ATP-induced Ca2+ signals induced morphological changes in HSCs characteristic of cell contraction. Similar effects were noted in 5 of 5 experiments. These experiments demonstrate that localized subcellular contraction is a downstream functional effect of Ca2+ signals limited to cell extensions.

DISCUSSION

Despite great advances in the understanding of liver fibrosis, there are still gaps in the understanding of HSC physiology and cell biology. The transdifferentiation that HSCs undergo in liver injury from quiescent lipid-storing cells to myofibroblast-like cells has been studied extensively (5, 11), yet little is known about the changes in signaling mechanisms in HSC transdifferentiation. For example, Ca2+ agonist hormones such as endothelin (28), PDGF (9), and ATP (7) are known to induce Ca2+ signals in HSCs, yet the specific subcellular mechanisms that may mediate these Ca2+ signals have not been investigated previously. Here, we demonstrate that HSCs express the type I IP3R and show that the distribution of this receptor changes upon HSC activation, with important functional consequences.

Fig. 1. Hepatic stellate cells (HSCs) express the type I inositol (1,4,5)-trisphosphate receptor (IP3R) isoform. A: RT-PCR. Expression of IP3R isoform mRNA in rat HSCs were determined using RT-PCR. Day 1 and 7 HSC cDNA was probed using isoform-specific cDNA probes for the cloned rat IP3Rs. Day 1 and 7 HSC expressed mRNA for type I and II IP3Rs. No expression of type II or III IP3R protein was noted in HSCs, but positive controls were used (total liver for type II IP3R and Mz-ChA-1 cells for type III IP3R).
The expression of a single IP₃R isoform in HSCs was unexpected. Cells expressing IP₃Rs typically express multiple IP₃R isoforms, which is thought to account for the complex spatiotemporal Ca²⁺ signaling patterns (4). For example, hepatocytes express IP₃R isoforms types I and II (17) and bile duct epithelia express IP₃R isoforms types I, II, and III (16). Thus, the expression of a single IP₃R isoform in a primary cell is quite unique (outside of the central nervous system). The perinuclear distribution of type I IP₃R in quiescent HSCs is typical of the distribution of IP₃Rs in a variety of cells (4), including liver epithelia. However, the distribution of type I IP₃R in activated cells within nuclei and cell extensions has not been described previously and may have important physiological consequences. Because the type I IP₃R is most strongly expressed in the central nervous system, the expression of type I IP₃R in HSCs is consistent with the concept that HSCs have...
many similarities to cells of neural crest origin (31). Furthermore, localized calcium signals in peripheral cell structures have only been observed in neurons, in which calcium signals in dendritic spines regulate memory-related changes (21), while calcium signals in neural growth cones regulate neural outgrowth (18, 34). To our knowledge, the present findings provide the first example outside of the nervous system of hormonal stimuli inducing localized subcellular calcium signals with localized downstream effects.

The functional importance of IP3R expression in the nucleus is of great potential interest. Calcium agonist hormones can alter gene transcription (7, 24). The present findings provide evidence that activated HSCs express nuclear ER structures and calcium stores compatible with a nucleoplasmic reticulum, a distinct nuclear organelle that mediates hormone-induced calcium signals within the nucleus (8). Nuclear calcium signals may be of particular importance in the regulation of gene transcription and cell proliferation (6, 26). Thus, expression of the type I
IP$_3$R in the nucleus of activated HSCs may contribute to the fibrogenic and proliferative responses of these cells to Ca$^{2+}$/H$11001$ agonist hormones.

The functional importance of IP$_3$R expression in cell extensions is also of great potential interest. The role of peripheral Ca$^{2+}$ signals in cell contractility is well established (20) and is compatible with our findings demonstrating that Ca$^{2+}$ signals in cell extensions mediate HSC contraction. Additionally, HSC extensions may be critical mediators of intercellular communication via gap junctions (10), and gap junction communica-
Fig. 3. Activated HSCs contain endoplasmic reticulum (ER) and Ca\textsuperscript{2+} stores in multiple subcellular compartments. A: immunolocalization of calreticulin and type I IP\textsubscript{3}R in day 7 HSCs. Day 7 HSCs were stained for type I IP\textsubscript{3}R (green) and calreticulin (red) using immunofluorescence and counterstained with the nuclear dye TOPRO (blue). In cell extensions and the cytoplasm, there was complete colocalization of type I IP\textsubscript{3}R and calreticulin. However, type I IP\textsubscript{3}R was also present within nuclei and the perinuclear cytoplasm in regions where calreticulin was absent. B: zoomed image of a cell extension. A zoomed image from A showed an IP\textsubscript{3}R type I- and calreticulin-positive cell extension reaching from one HSC to a neighbor. C: confocal microscopic detection of Ca\textsuperscript{2+} stores in HSCs. Confocal microscopy was used to image live day 7 HSCs labeled with Mag-fluo-4. Raw images (A, C, and E) and images pseudocolored according to the scale below (B, D, and F) are shown for comparison. In the low-power images (A and B), Mag-fluo-4 fluorescence was seen in multiple subcellular compartments, including nuclei, the perinuclear cytoplasm, and cell extensions. Zoomed images are provided to show Mag-fluo-4 fluorescence in nuclei (arrows in C and D) and cell extensions (arrows in E and F).

Fig. 4. Local application of extracellular ATP induces localized Ca\textsuperscript{2+} signals and contraction. A: representative confocal fluorescence image. Day 7 HSCs were plated and loaded with fluo-4 AM. ATP (100 µM) was perfused in the region of a cell extension using a microinjection pipette. The pipette was coloaded with fluorescein to identify the perfused region, the tip was advanced to the region near a cell extension (large arrowhead), and ATP was then released in a low-flow fashion. Changes in fluo-4 fluorescence as a measure of cytosolic Ca\textsuperscript{2+} were monitored using confocal microscopy. Four regions were identified (nearby cell extension, 1; distant cell extension, 2; cytosol, 3; and nucleus, 4). B: representative tracing. Quantification of fluo-4 fluorescence changes over time demonstrated a fluorescence increase limited to region 1. C: aggregate data. Aggregates of all of the data from multiple experiments (n = 5) demonstrated that localized extracellular ATP release induced Ca\textsuperscript{2+} signals limited to nearby cell extensions. D: localized extracellular ATP release induced HSC contraction. Day 7 HSCs were plated and loaded with fluo-4 AM. Localized ATP (100 µM) was coloaded with fluorescein for identification and loaded into a microinjection needle as described above. Serial images of the cells examined over time were collected at 10-s intervals, and each image was pseudocolored with a single distinct color value. Images were digitally added in the composite image at the bottom. As seen in the digitally added images, the cell in the top left has retracted in a downward direction. Note that there was no change in position of either of the other cells seen in the photomicrograph.
tion is regulated by localized Ca\(^{2+}\) signals (30). Finally, exocytosis of stored vesicles is regulated by localized Ca\(^{2+}\) signals (2); and, in HSCs, this may be important for the local release of collagen, which is a critical step in fibrogenesis (11). Thus, these cell extensions in activated HSCs function as autonomous subcellular units that link extracellular stimuli to localized subcellular responses.

In summary, we demonstrated that the type I IP\(_3\)R, a necessary mediator of hormone-induced intracellular Ca\(^{2+}\) signals, is expressed in HSCs. In activated HSCs, type I IP\(_3\)R is expressed in a previously unreported subcellular distribution. Type I IP\(_3\)R expression is found both in the nucleus, where it may mediate proliferation and gene transcription, and in cell extensions, where it may mediate contraction, gap junction signaling, and exocytosis. Further studies to examine each of these phenomena are likely to greatly increase our understanding of liver fibrosis and perhaps will provide new pharmacological targets to prevent or treat cirrhosis in patients.

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