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Loss of Inositol 1,4,5-Trisphosphate Receptors From Bile Duct Epithelia Is a Common Event in Cholestasis

KAZUNORI SHIBAO^{*}, KEIJI HIRATA^{*}, MARIE E. ROBERT[‡], and MICHAEL H. NATHANSON^{*}

^{*}Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut

[‡]Department of Pathology, Yale University School of Medicine, New Haven, Connecticut

Abstract

Background & Aims: Cholestasis is one of the principal manifestations of liver disease and often results from disorders involving bile duct epithelia rather than hepatocytes. A range of disorders affects biliary epithelia, and no unifying pathophysiologic event in these cells has been identified as the cause of cholestasis. Here we examined the role of the inositol 1,4,5-trisphosphate receptor (InsP3R)/Ca²⁺ release channel in Ca²⁺ signaling and ductular secretion in animal models of cholestasis and in patients with cholestatic disorders.

Methods: The expression and distribution of the InsP3R and related proteins were examined in rat cholangiocytes before and after bile duct ligation or treatment with endotoxin. Ca²⁺ signaling was examined in isolated bile ducts from these animals, whereas ductular bicarbonate secretion was examined in isolated perfused livers. Confocal immunofluorescence was used to examine cholangiocyte InsP3R expression in human liver biopsy specimens.

Results: Expression of the InsP3R was selectively lost from biliary epithelia after bile duct ligation or endotoxin treatment. As a result, Ca²⁺ signaling and Ca²⁺-mediated bicarbonate secretion were lost as well, although other components of the Ca²⁺ signaling pathway and adenosine 3',5'-cyclic monophosphate (cAMP)-mediated bicarbonate secretion both were preserved. Examination of human liver biopsy specimens showed that InsP3Rs also were lost from bile duct epithelia in a range of human cholestatic disorders, although InsP3R expression was intact in noncholestatic liver disease.

Conclusions: InsP3-mediated Ca²⁺ signaling in bile duct epithelia appears to be important for normal bile secretion in the liver, and loss of InsP3Rs may be a final common pathway for cholestasis.

Bile secretion is one of the primary functions of the liver, and disorders that result in cholestasis are among the most serious forms of liver disease. Over 20% of liver transplants in the United States are performed for chronic cholestatic conditions, and 50% of liver transplants among pediatric patients are performed for such disorders.¹ Most cholestatic liver disorders result from diseases affecting the epithelia that line the biliary tree.^{2,3} The most thoroughly characterized pathway for fluid and electrolyte secretion in bile duct epithelia is activated by secretin, mediated by adenosine 3',5'-cyclic monophosphate (cAMP), and links to chloride and bicarbonate secretion via the cystic fibrosis transmembrane conductance regulator (CFTR).^{4,5} Secretin and its second messenger cAMP also stimulate other aspects of secretion in bile duct epithelia, including targeting and

insertion of apical aquaporins⁶ and stimulation of exocytosis.⁷ However, components of these secretory pathways are not decreased in animal models of cholestasis,^{8,9} and there is no evidence that these pathways generally are defective in human cholestatic conditions. Therefore, the molecular basis for cholestasis in such conditions has been unclear.

There is also an alternative, Ca^{2+} -mediated pathway for secretion in bile duct epithelia.⁴ These cells express both basolateral M3 muscarinic acetylcholine (ACh) receptors¹⁰ and apical P2Y nucleotide receptors,^{11,12} and stimulation of either type of receptor leads to inositol 1,4,5-trisphosphate (InsP3)-mediated Ca^{2+} signaling.¹³ Increases in cytosolic Ca^{2+} (Ca_i^{2+}) in turn activate Ca^{2+} -dependent chloride channels and subsequently chloride-bicarbonate exchange, which ultimately results in net bicarbonate secretion into bile.^{14,15} Therefore, we examined whether Ca_i^{2+} signaling pathways are impaired in cholestasis. This was investigated in rats subjected to common bile duct ligation (BDL), which is a widely used and well-characterized animal model of cholestasis,³ and in patients with a range of common cholestatic disorders.

Materials and Methods

Animals and Materials

Male Sprague-Dawley rats (Camm Research Lab Animals, Wayne, NJ) weighing 250–300 g were used for all experiments. Animals were housed in temperature- and light-controlled rooms and maintained on a standard diet. ACh was purchased from Sigma Chemical (St. Louis, MO). The Ca^{2+} dye fluo-4 in acetoxymethyl ester form, the lipophilic membrane dye DiD, and rhodamine-conjugated phalloidin were from Molecular Probes (Eugene, OR). Lipopolysaccharide (LPS) from *Salmonella typhimurium* and α -naphthylisothiocyanate were from Sigma Chemical.

Antibodies

Type I InsP3 receptor (InsP3R) antibodies were from affinity-purified specific rabbit polyclonal antiserum directed against the 19 C-terminal residues of the mouse type I InsP3R,¹⁶ and were custom produced by Research Genetics (Huntsville, Alabama). Type II InsP3R antibodies were from affinity-purified specific rabbit polyclonal antiserum directed against the 18 C-terminal residues of the rat type II InsP3R,¹⁷ and were kindly provided by Richard J. Wojcikiewicz (State University of New York at Syracuse, Syracuse, NY). A monoclonal antibody from Transduction Laboratories (Lexington, KY) was used to label the N-terminal region of the human type III InsP3R.¹⁸ Monoclonal antibodies directed against the 19 C-terminal residues of $\text{G}\alpha_q/\alpha_{11}$ and the 20 C-terminal residues of PLC β 319 were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody M35 directed against the M3 muscarinic ACh receptor¹⁰ was obtained from Argene (Varilhes, France). Polyclonal antibody R3195 directed against CFTR²⁰ was kindly provided by Christopher R. Marino (University of Tennessee, Memphis, TN). Monoclonal antibody AC-15 directed against β -actin was obtained from Sigma. The secondary antibodies were Alexa 488 anti-rabbit and anti-mouse and Alexa 647 anti-rabbit immunoglobulin G (Molecular Probes).

Cell Isolation

Bile duct epithelia were isolated from rats under normal conditions, and 48 hours, 1 week, and 2 weeks after common BDL.^{13,21} Bile duct epithelia also were isolated from rats after treatment with LPS or α -naphthylisothiocyanate. Cholestasis of sepsis was induced by intraperitoneal injection of LPS (1 mg/kg) 24 hours before liver isolation and perfusion.²² Drug-induced cholestasis was induced by feeding a refined diet containing 0.1% α -naphthylisothiocyanate (Dyets, Bethlehem, PA) for 3 weeks, as described previously.²³ Briefly, rat livers were isolated and then perfused with buffer containing collagenase

(Boehringer Mannheim Biochemicals, Indianapolis, IN). The portal tissue residue was separated mechanically, then either cut into strips for Ca^{2+} measurements or processed further to obtain protein for immunoblots. For Ca^{2+} studies, DiD and fluo-4/acetoxymethyl ester were first co-injected into the common bile duct to facilitate identification of bile duct epithelia within portal strips.²¹

Immunoblot Analysis

All immunoblots were performed on bile duct cells that were prepared as described earlier. Protein concentration of cell homogenates was determined, and then proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes.^{21,24} Membranes were blocked overnight and probed for 2 hours with isoform-specific InsP3R primary antibodies,²¹ then washed, incubated for 1 hour with peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G secondary antibody, and revealed using an enhanced chemiluminescence kit from Amersham (Arlington Heights, IL). Immunoreactivity for each immunoblot was quantified using a BioRad GS-700 imaging densitometer (BioRad, Richmond, CA).

Confocal Immunofluorescence Microscopy

Immunocytochemistry was performed on 5- μm thick frozen sections of rat liver containing bile ducts and on paraffin-embedded sections of human liver biopsy specimens. Rat liver sections were fixed by cold acetone, blocked, labeled with primary antibody, rinsed with phosphate-buffered saline, and incubated with Alexa 488 – conjugated secondary antibody. Rat tissue also was labeled with rhodamine-conjugated phalloidin to facilitate identification of the plasma membrane²¹ because phalloidin labels submembranous actin. Paraffin-embedded sections were pretreated with 1 mmol/L citrate buffer at 100°C, then labeled with Alexa 488– and Alexa 647–conjugated secondary antibodies. Negative controls were performed under each experimental condition by incubating tissue with secondary antibodies but not anti-InsP3R (primary) antibodies. Only paraffin-embedded human liver specimens were available, so neither immunoblots nor Ca^{2+} signaling studies could be performed with cholangiocytes from these specimens. Specimens were examined using a Zeiss LSM 510 Laser Scanning Confocal Microscope (Thornwood, NY). To ensure specificity of staining, images were obtained using machine settings at which no fluorescence was detectable in negative control samples. Double-labeled rat liver specimens were excited serially at 488 nm and observed at greater than 515 nm to detect Alexa 488, then excited at 568 nm and observed at greater than 585 nm to detect rhodamine, whereas human liver specimens were excited serially at 488 nm and observed at greater than 515 nm to detect Alexa 488, then excited at 647 nm and observed at greater than 660 nm to detect Alexa 647.

Cytosolic Ca^{2+} Measurements

DiD (25 $\mu\text{mol/L}$) and fluo-4/acetoxymethyl ester (18 $\mu\text{mol/L}$) were co-injected into the common bile duct, then bile duct segments were isolated as described earlier, transferred to a perfusion chamber, and observed using a BioRad MRC 1024 confocal imaging system. Tissue was excited at 647 nm and observed at greater than 680 nm to identify individual DiD-labeled bile duct cells, then Ca_i^{2+} was monitored in these cells by exciting the specimen at 488 nm and detecting fluo-4 emission signals above 515 nm.²¹ Cells were observed with a spatial resolution of 0.20 $\mu\text{m}/\text{pixel}$ and a temporal resolution of 6 ms during line scanning.^{21,25} Increases in Ca_i^{2+} were expressed as percent increases in fluorescence intensity of fluo-4.^{13,21,25}

Isolated Perfused Rat Liver Studies

Liver perfusions were performed as described previously.¹⁵ Bivascular (i.e., combined portal venous and hepatic arterial) perfusions were performed in livers isolated from normal rats, whereas portal vein perfusions were performed in livers isolated after bile duct ligation. For bivascular perfusions, the liver was isolated and transferred to a temperature-controlled perfusion chamber, where both the hepatic artery and portal vein were perfused in a nonrecirculating fashion.¹⁵ Krebs-Ringer's bicarbonate buffer was infused at a speed of 30 mL/min into the portal vein and at 10 mL/min into the hepatic artery. For studies performed 2 weeks after common BDL, the isolated liver was perfused via the portal vein with Krebs-Ringer's bicarbonate at 40 mL/min. For all perfused liver studies, bile was collected gravimetrically and portal pressure was monitored continuously. Biliary bicarbonate was measured using a blood gas analyzer. Viability of each liver preparation was ascertained by monitoring perfusion pressure and O₂ consumption during the course of the experiment, and by determining trypan blue distribution on completion.¹⁵

Statistics

Data are presented as mean $\pm$ SEM. For statistical analysis, means between groups were compared by the Student *t* test. A *P* value <0.05 was considered significant.

Results

InsP3Rs Are Lost From Bile Duct Epithelia After BDL

The InsP3R is the only intracellular Ca²⁺ release channel in bile duct epithelia, and all 3 isoforms of the receptor are expressed in these cells.²¹ Immunoblot analysis was performed to compare the relative protein expression of the type I, II, and III isoforms in bile duct cells before and 2 weeks after BDL. Immunoblot analysis confirmed that all 3 isoforms were expressed in normal bile duct cells, but showed that expression of each was reduced markedly after BDL (Figure 1A). Densitometric analysis (Figure 1B) showed that the type I InsP3R was reduced to $23.8\% \pm 1.8\%$ of baseline (mean $\pm$ SEM), the type II isoform was reduced to $4.6\% \pm 8.7\%$ of baseline, and the type III isoform was reduced to $7.9\% \pm 0.4\%$ (*P* < 0.01 for each; *n* = 3). The type III InsP3R is the predominant isoform in cholangiocytes, accounting for over 80% of InsP3Rs in this cell type.²¹ Therefore, expression of this isoform was examined at earlier time points after BDL as well (Figure 1C). Expression of the type III InsP3R decreased progressively at 48 hours, and at 1 and 2 weeks after BDL. These data suggest that loss of InsP3Rs is a very early event in the development of cholestasis.

Each InsP3R isoform is distributed in a characteristic subcellular pattern in biliary epithelia,²¹ which may in turn affect the pattern of Ca_i²⁺ signals. Therefore, confocal immunofluorescence was performed to determine whether the subcellular distribution of the type I, II, and III InsP3Rs in bile duct cells is altered after BDL. Rat liver sections were labeled with isoform-specific InsP3R antibodies and colabeled with rhodamine-conjugated phalloidin to facilitate identification of bile duct landmarks (Figure 2). In bile ducts from normal rats, the types I and II InsP3Rs were distributed diffusely throughout the cytosol (Figure 2A and C), whereas the type III InsP3R was most concentrated in the apical region (Figure 2E), as has been described previously.²¹ Normal bile ducts expressed small amounts of the type I InsP3R (Figure 2A), and expression remained low 2 weeks after BDL (Figure 2B). The decrease in type II InsP3R expression after BDL was detectable more readily (Figure 2D), whereas the decrease in labeling of the type III isoform was most marked (Figure 2F). In particular, apical expression of the type III InsP3R was nearly absent after BDL. InsP3R expression also was examined in bile ducts of different sizes. In control animals, each size of cholangiocyte expressed all isoforms of the InsP3R. After BDL, expression of all 3 isoforms was decreased in each type of cholangiocyte, although the loss

was most dramatic in small cholangiocytes (data not shown). Taken together with the immunoblot results, these findings show that there is a marked loss of each InsP3R isoform in bile duct epithelia 2 weeks after BDL.

Ca²⁺ Signaling and Ca²⁺-Mediated Secretion Are Disrupted After BDL

The loss of InsP3Rs in bile duct epithelia suggests that BDL would impair Ca_i²⁺ signaling as well. To investigate this, we isolated intrahepatic bile duct segments from normal and bile duct–ligated rat livers, and then examined Ca_i²⁺ signaling in individual cells within the bile duct segments using confocal microscopy (Figure 3A).^{21,25} Bile ducts were stimulated with ACh, which causes an InsP3-mediated increase in Ca_i²⁺ in this cell type.¹³ In cells from normal rat liver, ACh (10 μmol/L) caused an apical-to-basal Ca_i²⁺ wave (Figure 3B and C), similar to what has been reported previously in this²¹ and other^{25,26} polarized epithelia. In contrast to what was observed in normal bile ducts, ACh increased Ca_i²⁺ in only 3 of 19 bile duct cells after BDL. Subsequent stimulation with the Ca²⁺-adenosine triphosphatase (ATPase) inhibitor thapsigargin (2 μmol/L) increased Ca_i²⁺ in 17 of those cells (Figure 3D), showing that Ca²⁺ stores nonetheless remain intact. Moreover, in the few cells that responded to ACh after BDL, the increase in Ca_i²⁺ was much slower than in normal bile duct cells (time to peak: 53.3 ± 2.6 sec vs. 9.1 ± 2.7 sec, *P* < 0.01; Figure 3E and F). Moreover, no clear initiation site for Ca_i²⁺ signals was observed in bile duct–ligated cells. However, there was no difference in peak fluorescence intensity between normal bile duct cells stimulated with ACh and those cells that responded to ACh after BDL (207.8% ± 31.9% vs. 257.9% ± 12.6 %, *P* = 0.191). These findings suggest that the loss of expression of InsP3Rs after BDL is associated with impaired Ca_i²⁺ signaling in biliary epithelia.

Ca_i²⁺ signaling regulates biliary bicarbonate secretion independent of cAMP.¹⁵ Therefore, biliary bicarbonate secretion was examined in the isolated perfused rat liver to determine the physiologic significance of the impaired Ca_i²⁺ signaling that occurs after BDL. Infusion of either the Ca²⁺ agonist ACh (10 μmol/L) or the cAMP agonist secretin (1 nmol/L) into the hepatic artery increased the biliary bicarbonate concentration under normal conditions (Figure 4A and B), consistent with previous reports.¹⁵ Much lower amounts of secretin are needed to obtain the same secretory effect as ACh, presumably because secretin is an endocrine hormone, whereas ACh is a neurotransmitter. After BDL, ACh or secretin instead was infused via the portal vein because adhesions and collateral vessel enlargement and proliferation around the hepatic artery made arterial perfusions impractical. Infusion of ACh no longer affected biliary bicarbonate, even though secretin-induced bicarbonate secretion was preserved and even enhanced (Figure 4C). Because secretin was able to reach cholangiocytes via the portal vein after BDL, it is likely that ACh was able to reach cholangiocytes by this route as well. Thus, these findings suggest that BDL results in selective impairment of Ca²⁺-mediated biliary bicarbonate secretion. Moreover, cAMP-mediated secretion increases after BDL, which may be a compensatory response to loss of Ca²⁺-mediated secretion.

Upstream Components of the Ca²⁺ Signaling Pathway Are Preserved After BDL

Several steps link ACh stimulation to activation of InsP3Rs to increase Ca_i²⁺ in bile duct cells. These steps include binding of ACh to the M3 muscarinic receptor, activation of an associated G protein, and then PLCβ activation.¹⁹ To determine whether any of these upstream signal transduction events also are affected by BDL, we examined expression of the M3 muscarinic ACh receptor, G_α/α₁₁ protein, and PLCβ3. Immunoblot analysis showed that M3 muscarinic receptor expression was not decreased after BDL (Figure 5A). In fact, densitometry showed that M3 muscarinic receptor expression actually was increased by 298% ± 9% relative to normal controls (*P* < 0.0005, Figure 5B), consistent with previous reports.⁸ The α subunits of the G_q and G₁₁ G proteins, which are coupled to the M3

muscarinic receptor,¹⁹ were studied in normal and bile duct–ligated biliary epithelia by the same technique. The amounts of the subunits were similar under both conditions (Figure 5C and D); cells after BDL expressed $93.2\% \pm 2.1\%$ of the amount of $G\alpha_q/\alpha_{11}$ expressed by normal bile duct cells ($P > 0.3$). PLC β 3 was studied as well because it is the member of the PLC β subfamily that is most sensitive to stimulation by $G\alpha_q$ subunits of G proteins²⁷ and would couple to both M3 and P2Y receptors on bile duct cells.¹⁹ Protein expression after BDL was $95.2\% \pm 7.1\%$ of the amount detected under normal conditions ($P > 0.25$). Thus, there was no significant difference in the amount of PLC β 3 in normal and bile duct–ligated bile duct cells (Figure 5E and F). Densitometric analyses for the M3 muscarinic ACh receptor, $G\alpha_q/\alpha_{11}$ protein, and PLC β 3 also were performed using β -actin as an internal control. Those data confirm that there is a significant increase in M3 muscarinic ACh receptor ($284.9\% \pm 51.6\%$, $P < 0.0005$) with no significant change in expression of either $G\alpha_q/\alpha_{11}$ ($38.6\% \pm 10.9\%$) or PLC β 3 ($55.3\% \pm 1.87\%$) after BDL. The decreased trend for $G\alpha_q/\alpha_{11}$ and PLC β 3 reflects the fact that β -actin expression increases slightly after BDL. Together, these findings show that the loss of responsiveness to ACh is owing to the selective loss of InsP3Rs, rather than to loss of upstream elements in the signal transduction pathway or loss of Ca^{2+} stores. In fact, an increase occurs in M3 receptor expression, which may represent a compensatory mechanism in response to decreased expression of InsP3R.

InsP3R Expression in Other Animal Models of Cholestasis

Expression of each InsP3R also was examined in 2 additional models of cholestasis. First, InsP3R expression was examined in rat cholangiocytes after treatment with LPS (Figure 6A and B) because inflammatory cytokines inhibit ductular secretion.²⁸ Here, expression of the type I isoform was $48.8\% \pm 8.0\%$ of control, whereas type III expression was $23.8\% \pm 13.1\%$ of baseline ($P = 0.019$ and $P < 0.005$, respectively; $n = 3$). Type II InsP3R was not reduced ($123.9\% \pm 13.1\%$ compared with control; $P = 0.27$, $n = 3$). These data suggest that decreased InsP3R expression occurs not only during chronic cholestasis but in acute cholestatic conditions as well. InsP3R expression in rat cholangiocytes after α -naphthylisothiocyanate feeding²³ also was examined in a single animal. Expression of types I and II InsP3R was not reduced, but type III InsP3R expression was 42.6% of untreated controls (Figure 6C). Thus, expression of the principal InsP3R isoform in cholangiocytes is decreased in multiple models of cholestasis.

InsP3Rs Are Lost in Human Cholestatic Diseases

To examine the relevance of our animal studies for human disease, InsP3R expression was examined in liver biopsy specimens from normal individuals plus adult and pediatric patients with a range of cholestatic disorders. Specimens were stained with an antibody for the type III InsP3R, which is the predominant isoform in rat bile ducts.²¹ Because bile duct epithelia are the only cell type within liver that express CFTR,²⁹ specimens were colabeled with an antibody for CFTR to facilitate identification of bile ducts. The type III InsP3R was expressed in human bile ducts and was concentrated in the apical region (Figure 7A), similar to what was observed in rats. InsP3R expression was decreased or absent in all patients studied who had chronic bile duct obstruction ($n = 5$; 3 cases from stone disease and 2 cases from malignancy; Figure 7B), also similar to what was observed in rats. Expression also was decreased or absent in all patients with biliary atresia ($n = 8$; age range, 4–16 wk; median, 10 wk; Figure 7C), a cholestatic condition that is the most frequent indication for liver transplant among pediatric patients. We also examined InsP3R expression in patients with primary biliary cirrhosis ($n = 5$; stage I: 2 cases; stage III: 3 cases; Figure 7D) and sclerosing cholangitis ($n = 5$; stage II, 1 case; stage III, 3 cases; stage IV, 1 case; Figure 7E), autoimmune cholestatic conditions of small and large intrahepatic bile ducts, respectively. Expression was decreased or absent in all patients tested with each of these conditions as well. Although InsP3R expression was diminished markedly in all 4 of the cholestatic

conditions examined, expression of CFTR by bile duct cells was preserved. Finally, we examined InsP3R expression in a group of patients with varying stages of hepatitis C infection, ranging from portal inflammation to cirrhosis. This chronic viral infection typically damages hepatocytes but not bile duct epithelia, and generally does not result in cholestasis. Expression of the type III InsP3R was normal in these patients ($n = 5$; stage I, 2 cases; stage II, 2 cases; stage IV, 1 case; Figure 7F), suggesting that loss of InsP3Rs is associated with cholestatic conditions, and is not merely a nonspecific response to hepatic inflammation or injury. Expression of type I (Figure 8) and type II (Figure 9) InsP3Rs also was examined in a limited number of patient specimens. Consistent with what has been reported in rats,²¹ expression of both of these 2 isoforms was low in human cholangiocytes (Figures 8A and 9A). Moreover, expression of both isoforms was decreased even further in both biliary obstruction (Figures 8B and 9B) and primary biliary cirrhosis (Figures 8C and 9C). Together, these findings lend further support to the hypothesis that loss of InsP3Rs is associated with cholestasis.

Discussion

Bile secretion is the result of bile formation by hepatocytes, followed by further conditioning by bile duct epithelia. Although genetic or acquired disorders of hepatocyte transporters account for some cases of cholestasis,² many instances instead are caused by diseases of the bile ducts.^{2,3} Perhaps the principal role of bile ducts is to regulate secretion of bicarbonate into bile.^{5,15} Positron emission tomography studies of bile formation in vivo suggest that hormone-induced bicarbonate secretion is reduced in patients with either bile duct obstruction or primary biliary cirrhosis, but is preserved in hepatocellular liver diseases.³⁰ It generally has been thought that the principal pathway for ductular bicarbonate secretion is mediated by cAMP and CFTR.^{3,5} However, this pathway is preserved in animal models of cholestasis such as BDL⁸ and treatment with α -naphthyl isothiocyanate.⁹ The current study raises the possibility that Ca^{2+} -mediated bicarbonate secretion is not just an alternative pathway, but may in fact be important for biliary bicarbonate secretion to occur under normal conditions. Previous work in isolated cholangiocytes suggests that signaling via Ca^{2+} and cAMP regulates secretion in a cooperative fashion.¹⁰ However, the importance of this cross-talk between signaling pathways may be limited in the intact liver.¹⁵ The current observation that ACh-induced bicarbonate secretion is lost after BDL while secretin-induced secretion is enhanced provides further evidence that these 2 signaling pathways can be dissociated in vivo.

The finding that the type III InsP3R is present and concentrated in the apical region of human bile duct epithelia has several implications. First, the type III InsP3R is concentrated in the apical region in a number of rodent epithelia,^{21,31,32} and this study shows that human epithelia may follow the same paradigm. Second, Ca_i^{2+} signals generally begin as apical-to-basal Ca^{2+} waves in polarized epithelia,³³ perhaps because the type III InsP3R is localized to the apical region.^{21,25,26} Biophysical properties of the type III isoform may enable it to trigger Ca_i^{2+} waves,^{18,34} although this is controversial.^{35,36} In any case, apical-to-basal Ca_i^{2+} waves are critical for secretion to occur in polarized epithelia such as bile duct cells because this type of Ca_i^{2+} wave mediates transport of fluid and electrolytes across the apical membrane.^{33,37} Third, unlike the type I InsP3R, the type III isoform is not inhibited by high local concentrations of Ca_i^{2+} ,¹⁸ so its presence in the apical region may permit the Ca_i^{2+} concentration there to reach 5–10 $\mu\text{mol/L}$.^{37,38} Such high concentrations are essential for local activation of synaptotagmin³⁹ and other events related to membrane fusion.^{40–42} These events in turn are necessary for proper targeting and insertion of anion channels and ion transporters into the apical membrane and for exocytosis. Fourth, loss of InsP3Rs causes resistance to apoptosis,⁴³ and the type III isoform may be particularly important for mediating apoptosis.⁴⁴ Because proliferation of bile ducts is a hallmark of cholestasis, and

agents that increase Ca_i^{2+} in duct cells inhibit bile duct proliferation,^{45,46} the loss of type III InsP3Rs from bile duct cells may in part be a defense mechanism that is responsible for bile duct proliferation. Together, these findings suggest that apical expression of the type III InsP3R may be important for proper biliary bicarbonate secretion, so that loss of this receptor would lead to bile duct proliferation and cholestasis.

A consistent finding here was the dramatic loss of InsP3R expression in cholangiocytes from both patients with cholestatic disorders and animal models of cholestasis. BDL and other cholestatic conditions generally increase bile acids within cholangiocytes, which in turn can activate nuclear bile acid receptors such as the farnesoid X receptor. Activation of the farnesoid X receptor decreases expression of certain hepatic proteins, such as the sodium-taurocholate cotransport polypeptide,⁴⁷ whereas the farnesoid X receptor instead transactivates the promoter of other liver proteins, such as the bile salt export pump.⁴⁸ It is not known whether the promoter regions of any of the InsP3R isoforms contain elements that are sensitive to the farnesoid X receptor, but retinoic acid decreases the activity of the promoter of the type I InsP3R.⁴⁹ Cholestatic conditions often are associated with an inflammatory infiltrate in the portal region, which raises the question of whether proinflammatory cytokines contribute to the decreased expression of InsP3Rs. Proinflammatory cytokines do not decrease expression of anion exchanger-2, CFTR, or the secretin receptor in cholangiocytes, and also do not impair Ca^{2+} -mediated secretion in these cells.²⁸ Consistent with this, interleukin 1 increases rather than decreases transcription of the type I InsP3R gene.⁵⁰ Alternatively, the loss of InsP3Rs observed in our study could be caused by increased degradation rather than decreased production. Indeed, stimulation of the InsP3R by secretagogues in pancreatic acinar cells leads to rapid ubiquitination and proteosomal degradation of the receptor.⁵¹ Thus, previous studies suggest that decreased production, increased degradation, or both, may contribute to the loss of InsP3Rs that occurs in cholestatic cholangiocytes.

Our findings also have implications for understanding the therapy of cholestasis. Currently, the only treatment of proven efficacy for a number of cholestatic disorders is ursodeoxycholic acid. This hydrophilic bile acid improves serum liver chemistries in both primary biliary cirrhosis⁵² and sclerosing cholangitis,⁵³ as well as in cystic fibrosis,⁵⁴ intrahepatic cholestasis of pregnancy,⁵⁵ and cholestasis induced by total parenteral nutrition.⁵⁶ Although the mechanism of action of ursodeoxycholic acid is controversial,⁵⁷ this bile acid increases Ca_i^{2+} in bile duct epithelia.⁴⁵ This action may in part be indirect because ursodeoxycholic acid induces secretion of ATP into bile,⁵⁸ and bile duct epithelia express apical P2Y receptors that link to InsP3-mediated Ca_i^{2+} signaling and bicarbonate secretion.¹¹ Stimulation of apical nucleotide receptors has been considered as a way to bypass defective CFTR in cystic fibrosis,^{59,60} but the current study suggests that Ca^{2+} -mediated secretion in the liver is important even in conditions in which CFTR expression is preserved. Thus, stimulation of Ca_i^{2+} signaling in bile ducts may provide a general strategy for treatment of cholestatic disorders. Treatment by this approach may be complex though because ACh and ATP both stimulate Ca^{2+} -mediated secretion,^{11,15} whereas direct application of ursodeoxycholic acid instead appears to inhibit secretion in a Ca^{2+} -dependent fashion.⁴⁵ Therefore, agonist-specific effects on Ca^{2+} and secretion must be understood better to design optimal therapies for cholestatic disorders.

The current findings may have broad significance beyond the field of liver disease. Ca_i^{2+} signaling provides a universal mechanism for cell regulation,⁶¹ and indeed InsP3R knock-out mice die in the perinatal period.⁶² Yet, there are few examples of human disease that result from molecular defects in Ca_i^{2+} signaling machinery.⁶³ Such examples include ryanodine receptor dysfunction in malignant hyperthermia,⁶⁴ defects in the Ca^{2+} -ATPase of the plasma membrane in certain hereditary forms of hearing loss,⁶⁵ and defects in the Ca^{2+} -

ATPase of the endoplasmic reticulum in certain inherited myopathies.⁶⁶ However, each of these disorders is rare. The current work provides evidence that a specific, acquired abnormality in Ca^{2+} signaling may be the final common pathway for a wide range of liver diseases that share a relatively frequent clinical presentation. Is loss of type III InsP3Rs necessary for cholestasis to occur? In the current work, loss of type III InsP3Rs was a very early event in several models of cholestasis, occurring as early as 24 hours after administration of endotoxin. Disruption of type III InsP3R expression in cholangiocytes would provide definitive evidence that this isoform is necessary for ductular secretion. However, to our knowledge no knockout animal for this protein exists. Further work therefore will be needed to confirm the role of InsP3R expression in cholestatic liver disease, and to determine whether altered InsP3R expression is responsible for secretory disorders of other organs as well.

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Abbreviations used in this paper

ACh	acetylcholine
BDL	bile duct ligation
Ca_i^{2+}	cytosolic Ca^{2+}
CFTR	cystic fibrosis transmembrane conductance regulator
InsP3	inositol 1,4,5-trisphosphate
InsP3R	inositol 1,4,5-trisphosphate receptor
LPS	lipopolysaccharide

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Figure 1.

Expression of each InsP3R isoform decreases after BDL in bile duct epithelia. (A) Western analysis using isoform-specific antibodies. Immunoblot using a polyclonal type I-specific InsP3R antibody identifies a single band in lysates from normal bile duct cells and very weak band in lysates from bile duct cells 2 weeks after BDL. Type II isoform-specific antibody CT2 identifies a strong band in lysates from normal bile duct cells and a weak band in lysates after BDL. A monoclonal type III isoform-specific InsP3R antibody also identifies a strong band in lysates from normal bile duct cells and weak band in lysates after BDL. Data were normalized by β -actin expression determined in the same blots. Amount of protein used was 100 μ g in each lane. (B) Densitometric analysis of immunoblots

normalized by β -actin expression. The expression of each InsP3 isoform was markedly decreased 2 weeks after BDL. Results are mean $\pm$ SEM of 3 independent experiments (* P < 0.01). (C) Type III InsP3R expression decreases progressively over time after BDL. InsP3R expression in cholangiocytes was 63.4% of baseline 48 hours after BDL, a value that was of marginal statistical significance (P = 0.07). InsP3R expression decreased to 27.2% and 7.9% of baseline 1 and 2 weeks after BDL, respectively (* P < 0.01). Results are mean $\pm$ SEM of 3 independent experiments.

Figure 2.

Subcellular distribution of each InsP3R isoform in bile duct epithelia before and after BDL, as determined by confocal immunofluorescence. Each image is double labeled with an isoform-specific antibody directed against the InsP3R (*green*), plus rhodamine-phalloidin (*red*) to identify actin beneath the plasma membrane. (A) A liver section obtained from normal rat liver, labeled with a monoclonal antibody directed against the type I InsP3R. Weak type I InsP3R labeling is seen throughout each bile duct cell. Scale bar, 10 μ m. (B) A liver section obtained 2 weeks after BDL, labeled with the same type I InsP3R antibody. This shows that type I InsP3R labeling is nearly absent. (C) Distribution of the type II InsP3R in normal rat bile duct epithelia. Type II InsP3R labeling is seen throughout each cell. (D) Distribution of the type II InsP3R 2 weeks after BDL. Labeling of this isoform now is almost absent from bile duct cells. (E) A section obtained from normal rat liver, labeled with a monoclonal antibody directed against the type III InsP3R. Note that type III InsP3R labeling is concentrated in the apical region. (F) A liver section obtained 2 weeks after BDL. Type III InsP3R labeling now is nearly absent, especially apically.

Figure 3.

Ca_i^{2+} signaling is impaired in bile duct cells after BDL. (A) Confocal image of a segment from a portal tract, excited at a wavelength to reveal loading with the Ca^{2+} dye fluo-4. The line used for confocal line scanning microscopy is shown in yellow. The line crosses the apical-to-basal axis of one of the cells. This and the subsequent image are pseudocolored according to the color scale shown. (B) Confocal line scanning image of the cell shown in the previous image. The tissue was stimulated with ACh (100 $\mu\text{mol/L}$) as confocal fluorescence along the line was collected every 6 ms. A Ca_i^{2+} wave crosses from the apical to the basolateral pole of the cell. (C) Tracing of the apical and basolateral components of the Ca_i^{2+} signal in the cell stimulated in the previous image. Inset shows the apical increase

in Ca_i^{2+} precedes the basolateral Ca_i^{2+} increase, reflecting an apical-to-basal Ca_i^{2+} wave. (D) After BDL, ACh (100 $\mu\text{mol/L}$) fails to increase Ca_i^{2+} , even though Ca_i^{2+} is increased by subsequent exposure to the Ca^{2+} -ATPase inhibitor thapsigargin (2 $\mu\text{mol/L}$). Result is representative of that seen in 16 of 19 bile duct cells. (E) The pattern of Ca_i^{2+} signaling is altered in the few cells that do respond to ACh after BDL. The Ca_i^{2+} signal reaches its peak gradually, during which time no clear apical-to-basal Ca_i^{2+} gradient is observed. (F) Time to peak intensity of Ca_i^{2+} signals is delayed markedly after BDL. The Ca_i^{2+} increase after BDL ($n = 3$) was much slower than in normal bile duct cells ($n = 8$; $*P < 0.01$).

Figure 4.

Ca²⁺-mediated bicarbonate secretion is impaired after BDL. (A) ACh (10 μmol/L) increases biliary bicarbonate concentration in normal rat liver. ACh was infused via the hepatic artery in the isolated bivascularly perfused normal rat liver. Values here and in subsequent panels are mean ± SEM of 4 separate experiments. (B) Secretin (1 nmol/L) increases biliary bicarbonate concentration in normal rat liver. Secretin was infused via the hepatic artery in the isolated bivascularly perfused normal rat liver. (C) BDL impairs ACh-induced but not secretin-induced bicarbonate secretion. Biliary bicarbonate secretion was measured in the isolated perfused rat liver 2 weeks after ligation of the common bile duct. Note that secretin-induced bicarbonate secretion actually is increased.

Figure 5.

Expression of proteins that are upstream of the InsP3R is preserved after BDL. (A) Immunoblot using M3 muscarinic ACh receptor-specific antibody identified a single band of the appropriate size in lysates from normal bile duct epithelia and a stronger band in lysates from cells isolated 2 weeks after BDL. In this and subsequent blots, 100 μ g of protein was used in each lane. (B) Densitometry of M3 muscarinic receptor expression. Expression is increased after BDL ($n = 3$; $*P < 0.01$). (C) Immunoblot using $G\alpha_q/\alpha_{11}$ protein-specific antibody identified a single band of the appropriate size in lysates from bile duct epithelia under normal conditions as well as 2 weeks after BDL (100 μ g each). (D) Densitometry of $G\alpha_q/\alpha_{11}$ protein expression. Protein expression is not altered significantly after BDL ($n = 3$ each). (E) Immunoblot using PLC β 3 protein-specific antibody identified a single band of the appropriate size in lysates from bile duct epithelia under normal conditions as well as 2 weeks after BDL. (F) Densitometry of PLC β 3 protein expression. Protein expression is not significantly altered after BDL ($n = 3$ each).

Figure 6.

InsP3R expression in animal models of cholestasis. (A) Endotoxin (LPS)-induced cholestasis. InsP3R isoform-specific immunoblots show that type I and III InsP3Rs decrease, whereas type II InsP3Rs do not. Cholestasis was induced by intraperitoneal injection of LPS (1 mg/kg) 24 hours before cholangiocyte isolation. (B) Densitometric analysis of immunoblots. The expression of type I and III InsP3 isoforms was decreased markedly 24 hours after LPS injection, whereas type II receptor expression was not. Results are mean $\pm$ SEM of 3 independent experiments (* $P < 0.01$). (C) Only the type III InsP3R decreases after α -naphthylisothiocyanate feeding. Western blot for each isoform of the InsP3R is shown.

Figure 7.

Expression of the type III InsP3R is decreased in the bile ducts of patients with cholestatic disorders. Confocal immunofluorescence images were obtained from paraffin-embedded liver biopsy specimens. Each tissue section was double-labeled with antibodies directed against the type III InsP3R (*green*) and CFTR (*red*). (A) Normal liver. Note that the InsP3R is localized to the apical region, but does not overlap with CFTR along the apical membrane. (B) Bile duct obstruction ($n = 5$; 3 cases from common duct stones and 2 cases from malignancy). Note that InsP3Rs are not detectable, even though CFTR expression is preserved. Bile duct proliferation can be seen as well, which is typical for this condition. (C) Biliary atresia ($n = 8$; age range, 4 -16 wk; median, 10 wk), (D) primary biliary cirrhosis (n

= 5, stage I: 2 cases; stage III: 3 cases), and (E) sclerosing cholangitis ($n = 5$; stage II: 1 case; stage III: 3 cases; stage IV: 1 case). As in biliary obstruction, InsP3Rs also are not detectable in any of these 3 liver biopsy specimens, even though CFTR expression is preserved. (F) Hepatitis C virus ($n = 5$; stage I: 2 cases; stage II: 2 cases; stage IV: 1 case). InsP3R expression is preserved in this disease of hepatocytes rather than bile duct cells, despite marked inflammation in the portal region. *Scale bar* = 30 μm .

Figure 8.

Expression of the type I InsP3R in normal and cholestatic human liver. Confocal immunofluorescence images were obtained from paraffin-embedded liver biopsy specimens. Each tissue section was double-labeled with antibodies directed against the type I InsP3R (red) and type III InsP3R (green). Images include both hepatocytes and cholangiocytes so that relative InsP3R expression in the 2 cell types can be compared. (A) Normal liver. Expression of the type I InsP3R is low in cholangiocytes relative to hepatocytes, whereas the type III InsP3R is localized to the apical region of cholangiocytes and is absent in hepatocytes. Note that the type I InsP3R is distributed diffusely in hepatocytes, as has been reported.⁶⁷ (B) Bile duct obstruction. Expression of type I InsP3R is preserved in hepatocytes, but neither type I nor type III InsP3R is detectable in cholangiocytes. Findings are representative of 3 separate biopsy specimens. (C) Primary biliary cirrhosis. Expression of type I InsP3Rs is preserved in hepatocytes, but neither type I nor type III InsP3Rs are detected in cholangiocytes. Findings are representative of 3 separate biopsy specimens. Each scale bar = 30 μ m.

Figure 9.

Expression of the type II InsP3R in normal and cholestatic human liver. Confocal immunofluorescence images were obtained from paraffin-embedded liver biopsy specimens. Each tissue section was double-labeled with antibodies directed against the type I InsP3R (red) and type III InsP3R (green). (A) Normal liver. Expression of the type II InsP3R is low in normal cholangiocytes compared with hepatocytes, and the type III InsP3R is concentrated in the apical region of cholangiocytes. Note that the type II InsP3R is concentrated in the canalicular region of hepatocytes, as has been reported.⁶⁷ (B) Bile duct obstruction. Neither the type II nor the type III InsP3R is detectable in cholangiocytes. Findings are representative of 3 separate biopsy examinations. (C) Primary biliary cirrhosis. Neither InsP3R isoform is detectable in cholangiocytes, even though hepatocytes continue to express the type II InsP3R. Findings are representative of 3 separate biopsy examinations. Scale bars = 30 μ m.