Somatostatin stimulates ductal bile absorption and inhibits ductal bile secretion in mice via SSTR2 on cholangiocytes

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Somatostatin stimulates ductal bile absorption and inhibits ductal bile secretion in mice via SSTR2 on cholangiocytes. Am J Physiol Cell Physiol 284: C1205–C1214, 2003; 10.1152/ajpcell.00313.2002.—With an in vitro model using enclosed intrahepatic bile duct units (IBDUs) isolated from wild-type and somatostatin receptor (SSTR) subtype 2 knockout mice, we tested the effects of somatostatin, secretin, and a selective SSTR2 agonist (L-779976) on fluid movement across the bile duct epithelial cell layer. By RT-PCR, four of five known subtypes of SSTRs (SSTR1, SSTR2A/2B, SSTR3, and SSTR4, but not SSTR5) were detected in cholangiocytes in wild-type mice. In contrast, SSTR2A/2B were completely depleted in the SSTR2 knockout mice whereas SSTR1, SSTR3 and SSTR4 were expressed in these cholangiocytes. Somatostatin induced a decrease of luminal area of IBDUs isolated from wild-type mice, reflecting net fluid absorption; L-779976 also induced a comparable decrease of luminal area. No significant decrease of luminal area by either somatostatin or L-779976 was observed in IBDUs from SSTR2 knockout mice. Secretin, a choleric hormone, induced a significant increase of luminal area of IBDUs of wild-type mice, reflecting net fluid secretion; somatostatin and L-779976 inhibited (P < 0.01) secretin-induced fluid secretion. The inhibitory effect of both somatostatin and L-779976 on secretin-induced IBDU secretion was absent in IBDUs of SSTR2 knockout mice. Somatostatin induced an increase of intracellular cGMP and inhibited secretin-stimulated cAMP synthesis in cholangiocytes; depletion of SSTR2 blocked these effects of somatostatin. These data suggest that somatostatin regulates ductal bile formation in mice not only by inhibition of ductal fluid secretion but also by stimulation of ductal fluid absorption via interacting with SSTR2 on cholangiocytes, a process involving the intracellular cAMP/cGMP second messengers.

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after bile duct ligation, suggesting a role for SSTR2 in the regulation of ductal bile formation.

The rationale of the current study was therefore to test the effects of somatostatin on ductal bile formation in mice and to evaluate the role of SSTR2 in this process. We characterized the effects of somatostatin, secretin, and a selective agonist of SSTR2, L-779976, on net fluid secretion and absorption across the biliary epithelial barrier with an in vitro model using enclosed intrahepatic bile duct units (IBDUs) isolated from wild-type and SSTR2 knockout mice. SSTR gene expression in cholangiocytes, as well as effects of somatostatin and secretin on intracellular cAMP and cGMP synthesis, were measured in isolated, purified cholangiocytes. We found that somatostatin stimulates ductal fluid absorption and inhibits secretin-stimulated bile secretion in mice, resulting in decreased bile flow, a process involving regulation of intracellular cAMP/cGMP synthesis and mediated by interaction with SSTR2 on cholangiocytes.

MATERIALS AND METHODS

Reagents. Somatostatin and secretin were purchased from Peninsula Laboratories (Belmont, CA). L-779976, a selective SSTR2 agonist (41), was provided by Dr. L. Koch (Merck Research Laboratories, Rahway, NJ), and the identification and characterization of this compound were described in detail in previous reports (41). Collagenase type II and trypsin were purchased from Worthington Biochemical (Lake-wood, NJ). EGTA, DNase, collagenase type XI, and hyaluronidase were purchased from Sigma-Aldrich (St. Louis, MO). A monoclonal anti-mouse cytokeratin-19 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal antibody to SSTR2/B was from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal rat anti-mouse epithelial cell adhesion molecule (EPCAM) antibody was generated from a G8.8 hybridoma cell line (Developmental Studies Hybridoma Bank, Iowa City, IA), and M-450 sheep anti-rat IgG Dynabeads were obtained from Dynal (Lake Success, NY). ELISA kits for the determination of intracellular cAMP and cGMP levels were purchased from Sigma-Aldrich.

Animals. Mice deficient in SSTR2 [homozygous SSTR2 (−/−) knockout] were generated by gene targeting in mouse embryonic stem cells as previously described (47, 55). Corresponding wild-type adult C57BL/129 mice (10–12 wk) were used as controls. Mice were maintained under controlled conditions at 25°C with food and water available ad libitum. All animal experimental protocols were approved by the Animal Use and Care Committee of the Mayo Foundation.

Solutions. The composition of isotonic (290 mosmol/kgH2O) HEPES-buffered saline (HBS) was (in mM) 140 NaCl, 5.4 KCl, 0.8 Na2HPO4, 25 HEPES, 2.5 glucose, 2 CaCl2, and 0.8 MgSO4, pH 7.4. The composition of isotonic HBS-buffered saline (HBS) was (in mM) 120 NaCl, 5.9 KCl, 1.2 Na2HPO4, 1 MgSO4, 1.25 CaCl2, 5 glucose, and 25 NaHCO3. The precise osmolality of solutions was determined with a freezing point osmometer (Osmette S; Precision System, Natick, MA).

Isolation of mouse cholangiocytes. Highly purified cholangiocytes were isolated from the livers of wild-type and SSTR2 knockout mice according to the method of Vierling and co-workers (13) with minor modifications. Briefly, mice were anesthetized with pentobarbital sodium (50 mg/kg ip). Livers were first perfused with HBS containing 0.02% EGTA through the portal vein to remove blood cells. Livers were then harvested, transferred to a temperature-controlled chamber at 37°C, and perfused for 10 min with HBS containing 0.05% collagenase type II (Worthington) and 2 mM CaCl2. Hepatocytes were removed by gentle, mechanical disruption of the Glisson’s capsule followed by agitation for 10 min at 4°C. The biliary tree segments were then digested with a solution of 0.02% DNase, 0.03% collagenase type XI, and 0.048% hyaluronidase (Sigma) for 30 min at 37°C and passed three times through a 19-gauge needle. After cells were incubated with 0.08% trypsin (Worthington) for 15 min, passing three times through a 22-gauge needle and filtering through a 70-μm Falcon cell strainer, cholangiocytes were immunomagnetically isolated from other cell types with a rat anti-EPCAM antibody. Cholangiocytes opsonized with anti-EPCAM were bound to Dynabeads bearing anti-rat IgG and isolated in a magnetic field. The viability of freshly isolated cholangiocytes was >90% by Trypan blue exclusion, and the purity was >95% by positive immunostaining to cytokeratin-19, a specific protein marker for cholangiocytes.

RNA extraction and reverse transcription-PCR. Total cellular RNA was extracted from freshly isolated mouse cholangiocytes with TRI-Reagent (Sigma). Isolated cholangiocytes were lysed in 1 ml of TRI-Reagent per 5 × 106 cells with 2.5 μl of Glyco-Blue (Ambion, Austin, TX) added as a coprecipitant and incubated at room temperature for 5 min. After addition of 0.2 ml of 1-bromo-3-chloropropane per 1 ml of TRI-Reagent, samples were mixed vigorously, incubated for 15 min at room temperature, and centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase was collected and transferred to a new tube; to this, 0.5 ml of isopropanol was added per 1 ml of TRI-Reagent used for the initial lysis. Samples were stored for 10 min at room temperature and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was removed, the RNA pellet was washed with 1 ml of 75% ethanol and repelleted by centrifugation at 12,000 g for 15 min at 4°C. RNA was resuspended in RNA Secure Solution (Ambion), and the concentration and purity were determined by spectrophotometry.

Total RNA (5 μg) was reverse-transcribed to cDNA by using a Moloney murine leukemia virus reverse transcriptase kit (MMLV-RT kit; Life Technologies, Rockville, MD). RNA was first incubated with a random hexamer as primer at 65°C for 6 min and then reacted with a mixture in a total volume of 50 μl at 37°C for 50 min and 95°C for 5 min. The mixture contained first-strand reaction buffer, dithiothreitol, deoxyribonucleotide triphosphates, RNase inhibitor, and MMLV-RT. After reverse transcription (RT), cDNA was amplified with the polymerase chain reaction (PCR) with gene-specific primers designed to amplify a portion of the coding sequences of each of the five mouse SSTR genes (Table 1). The PCR reaction consisted of 1 cycle of 10-min denaturation at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final extension step at 72°C for 10 min. Products of RT-PCR amplification were analyzed by 1.5% agarose gel electrophoresis, and the bands were visualized by ethidium bromide staining. Equal amounts of cDNA for each lane were analyzed, and all experiments were done in duplicate. Control PCR reactions were performed in parallel with template cDNA prepared from brain as positive control and using total RNA without RT as a genomic DNA contamination control. Sequencing was performed on all positive PCR products (Mayo Molecular Core Facility, Rochester, MN) to confirm the identity of amplified genes.

Isolation of IBDUs from mouse liver. The technique used to isolate IBDUs from mice was developed by us and has been previously described in detail (12). Briefly, wild-type and...
SSTR2 knockout mice (10–12 wk) were anesthetized with pentobarbital sodium (50 mg/kg ip). The liver was perfused with ice-cold saline through the portal vein. Subsequently, 2–3 ml of liquid Trypan blue agar was injected into the portal vein. The liver was then removed and immersed in an ice-cold, preoxygenated HBS buffer. After the hepatic capsule and surface hepatocytes were removed, intrahepatic bile ducts were dissociated under a dissection microscope by using the Trypan blue agar-filled portal vein as reference. The dissociated bile duct was digested by shaking at 37°C for 10 min in an enzyme solution (containing RPMI 1640 supplemented with 0.032% collagenase XI, 0.016% DNase, and 0.03% hyaluronidase). Further microdissection was performed at higher magnification to remove residual hepatocytes, components of the portal veins and hepatic arteries, and excess connective tissues. The isolated intrahepatic bile ducts with luminal diameters ranging from 25 to 100 μm were cut into 0.5-mm segments and transferred to eight-well culture chambers that were coated with poly-L-lysine hydrochloride. The segments were rinsed with culture media, adequate response to hormones, and capabilities for change in luminal area from basal values (without treatment). Characterizations of isolated IBDUs from mice were previously described by us (12). Those characterizations include retention of in situ morphology with a lumen surrounded by a single layer of viable epithelial cells with tight junctions between adjacent cells, an intact basolateral structure, adequate response to hormones, and capabilities for fluid absorption and secretion across biliary epithelial cells.

**Immunoblot of SSTR2 in IBDUs.** IBDUs isolated from both wild-type and SSTR2 knockout mice were cultured overnight and lysed with the M-PER mammalian protein extraction reagent (Pierce, Rockford, IL), and protein concentrations were determined with Bradford reagent according to the instructions of the supplier (Sigma-Aldrich). Twenty micrograms of lysate protein per lane was separated by SDS-polyacrylamide gel electrophoresis under reducing conditions and blotted onto nitrocellulose membranes. Membranes were sequentially incubated with the SSTR2 antibody (Santa Cruz Biotechnology) and then with 0.2 μg/ml of horseradish peroxidase-conjugated secondary antibody and revealed with enhanced chemiluminescence light substrate (ECL; Amersham).

**Determination of ductal fluid absorption and secretion of IBDUs in response to somatostatin, L-779976, and secretin.** Net ductal fluid absorption and secretion across biliary epithelial in response to a cholestatic agent (somatostatin and L-779976) and a choleretic agent (secretin) were determined with a quantitative image analysis technique based on the changes of luminal area of enclosed IBDUs, a methodology previously validated by us (39). Briefly, after 15-min incubation in an isotonic (290 mosmol/kgH2O) KRB buffer, enclosed and polarized IBDUs isolated from wild-type and SSTR2 knockout mice were treated with 30-min exposure to 10−7 M somatostatin, 2) 10−7 M L-779976, 3) 10−7 M secretin, 4) 10−7 M secretin plus 10−7 M L-779976 in an isotonic (290 mosmol/kgH2O) KRB solution. Somatostatin, secretin, and L-779976 at a concentration of 10−7 M showed most potent effects based on dose-response experiments and were selected for the studies. Serial photographs of the same enclosed IBDU before and after treatment were digitized, and the luminal area of IBDUs was measured with an image analysis software program (Fig. 1). The results were expressed as percent change in luminal area from basal values (without treatment).

**Table 1. RT-PCR primers used for SSTR expression**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (forward; reverse)</th>
<th>Amplicon Size, bp</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSTR1</td>
<td>5′-caccagacctactgtctgactg-3′</td>
<td>638</td>
<td>NM009216</td>
</tr>
<tr>
<td>SSTR2</td>
<td>5′-catagtgcagcctgtttccc-3′</td>
<td>1081(2A); 742(2B)</td>
<td>NM009217</td>
</tr>
<tr>
<td>SSTR3</td>
<td>5′-cttgccatcgcagctgcctag-3′</td>
<td>627</td>
<td>NM009218</td>
</tr>
<tr>
<td>SSTR4</td>
<td>5′-catgctgctctcccgtcctggtttg-3′</td>
<td>483</td>
<td>NM009219</td>
</tr>
<tr>
<td>SSTR5</td>
<td>5′-catagtagtcgactggttcctcc-3′</td>
<td>784</td>
<td>U82697</td>
</tr>
</tbody>
</table>

SSTR, somatostatin receptor.
both absorptive and secretory activities. No obvious morphological changes, such as decrease of cholangiocyte viabilities by Trypan blue staining or disruption of tight junctions or seals between adjacent cells by electron microscopy, was observed in IBDUs after incubation with somatostatin, secretin, or a combination (data not shown). To further confirm that somatostatin and L-779976 do not affect the function of tight junctions in cholangiocytes, an immortalized normal mouse cholangiocyte cell line (a gift from Dr. Y. Ueno, Tohoku University School of Medicine, Sendai, Japan) that expresses SSTR2 and has been fully characterized previously (25) was grown on 12-well inserts to confluence to form polarized monolayers. Transepithelial electrical resistance across the polarized cholangiocyte monolayers was assessed as described previously (7) before and after 30-min incubation with somatostatin (10⁻⁷ M) or L-779976 (10⁻⁷ M) added to the chambers. No significant change of transepithelial electrical resistance was detected in the monolayers before and after 30-min incubation with somatostatin (220.00 ± 7.21 and 222.00 ± 8.33 Ω·cm², respectively; P > 0.05) or L-779976 (210.67 ± 10.48 and 206.00 ± 10.58 Ω·cm², respectively; P > 0.05).

**RESULTS**

**Expression of SSTRs in isolated mouse cholangiocytes.** RT-PCR was carried out to identify transcripts encoding SSTRs in highly purified mouse cholangiocytes. Reverse-transcribed cDNA from highly purified mouse cholangiocytes was amplified by PCR with specific primers for the five cloned murine SSTRs. As shown in Fig. 2A, PCR amplification of reverse-transcribed mRNA prepared from highly purified cholangiocytes immunoisolated from wild-type mice produced strong, sharp bands corresponding to SSTR1, SSTR2A, SSTR2B, SSTR3, and SSTR4, whereas no SSTR5 signal was detected. Two bands for SSTR2 have been identified with primers that amplify SSTR2A and -2B. In the control experiments using cDNA derived from mouse brain tissue as the template (Fig. 2B), all five SSTRs were amplified, consistent with previous reports (8, 10, 53).

To confirm the complete deletion of SSTR2 message expression in SSTR2 knockout mice, RT-PCR for five SSTRs using the same primers was performed with commercially available ELISA kits (Sigma-Aldrich). The concentrations of cAMP and cGMP detected were then calculated from standard curves and expressed in femtomoles per 100,000 cells.

**Statistical analysis.** All data are expressed as means ± SE and represent three independent experiments with five to seven IBDUs each, unless otherwise stated. The unpaired Student’s t-test was used for statistical analysis. P < 0.05 was considered as statistically significant.

**Fig. 2.** Expression of somatostatin receptors (SSTRs) in cholangiocytes isolated from wild-type and SSTR2 knockout mice. A: total RNA from cholangiocytes isolated from wild-type and SSTR2 knockout mice was reverse transcribed with random primers and then PCR amplified with primers designed to amplify specific SSTR gene-encoding cDNA. Positive bands are shown for SSTR1, SSTR2A, SSTR2B, SSTR3, and SSTR4 in wild-type mice and SSTR1, SSTR3, and SSTR4 in SSTR2 knockout mice. B: total RNA from mouse brain was reverse transcribed and then amplified by PCR as positive controls. All 5 types of SSTRs and 2 isoforms of SSTR2 show positive bands. Total RNA preparations amplified by PCR without reverse transcription showed no bands (data not shown). C: a representative Western blot for SSTR2 in IBDUs from wild-type and SSTR2 knockout mice. β-Actin was also immunoblotted to ensure equal loading of proteins to each lane.
cholangiocytes isolated from SSTR2 homozygous knockout mice. As shown in Fig. 2A, although SSTR2A/2B was completely depleted in cholangiocytes isolated from SSTR2 knockout mice, SSTR1, SSTR3, and SSTR4 were expressed in these cholangiocytes whereas SSTR5 was absent. Total RNA amplified by PCR without RT showed no band (data not shown), confirming the absence of genomic DNA contamination in all RT-PCR studies. Equal amounts of all analyzed RNA were confirmed by ethidium bromide staining (data not shown).

To confirm SSTR2 protein expression in cholangiocytes in mice, cell lysates of IBDUs from wild-type and SSTR2 knockout mice were immunoblotted with an antibody that recognizes both SSTR2A and -2B (A-20; Santa Cruz Biotechnology). As shown in Fig. 2C, a single band of ~87 kDa was detected in the cell lysates of IBDUs from wild-type mice, consistent with previous reports showing SSTR2A and -2B with a similar molecular mass around 87 kDa (19, 33, 44). As expected, this band was absent in IBDUs from SSTR2 knockout mice. β-Actin was also immunoblotted to ensure equal protein loading to each lane.

Effects of somatostatin and L-779976 on ductal fluid absorption in IBDUs isolated from wild-type and SSTR2 knockout mice. In the absence of somatostatin, IBDUs isolated from wild-type mice showed no change in the luminal area after incubation in an isotonic (290 mosmol/kgH₂O) buffer for 30 min, indicating the absence of net fluid movement across the epithelial barrier. After incubation with 10⁻⁷ M somatostatin in an isotonic KRB buffer for 30 min, IBDUs isolated from wild-type mice displayed a significant decrease (16.72 ± 1.85%; P < 0.01) of luminal area (Fig. 3A), reflecting net fluid absorption from the lumen. To identify whether SSTR2 plays a role in somatostatin-mediated ductal fluid absorption, we investigated the effects of a novel nonpeptidyl selective agonist specific for SSTR2, L-779976, on bile fluid absorption across the biliary epithelial barrier. Exposure of IBDUs isolated from wild-type mice with 10⁻⁷ M L-779976 for 30 min resulted in a significant decrease (15.39 ± 1.83%; P < 0.01) of IBDU luminal area (Fig. 3B), similar to the somatostatin-stimulated decrease of luminal area in IBDUs. These data suggest that somatostatin, as well as its SSTR2-selective agonist, L-779976, directly induce net fluid absorption across the biliary epithelial barrier by interacting with SSTR2.

To further test the role of SSTR2 in somatostatin-mediated ductal bile absorption, somatostatin- and L-779976-induced bile fluid absorption was also characterized in IBDUs isolated from SSTR2 knockout mice. Incubation of IBDUs isolated from SSTR2 knockout mice with the same concentration of somatostatin for 30 min resulted in no significant decrease of luminal area compared with that in the wild-type mice (Fig. 3A). Moreover, no significant change of luminal area was detected in IBDUs isolated from SSTR2 knockout mice after incubation with L-779976 (Fig. 3B). The data suggest that SSTR2 plays a role in somatostatin-induced ductal bile absorption in mice.
in wild-type mice. All data are means ± SE from measurements of >6 IBDUs in each experimental group.

Fig. 4. Effects of somatostatin and L-779976 on secretin-induced ductal fluid secretion in IBDUs isolated from wild-type and SSTR2 knockout mice. IBDUs were incubated with an isotonic KRB buffer in the absence or presence of 10⁻⁷ M secretin or 10⁻⁷ M secretin + 10⁻⁷ M somatostatin (A) and 10⁻⁷ M secretin + 10⁻⁷ M L-779976 (B) at 37°C for 30 min. Changes of luminal area of IBDUs were assessed by a quantitative image analysis technique. In the absence of somatostatin or L-779976, IBDUs isolated from wild-type mice showed a significant increase of the luminal area after incubation with 10⁻⁷ M secretin. In the presence of somatostatin or L-779976, a significant decrease of secretin-induced ductal fluid secretion was detected in IBDUs isolated from SSTR2 knockout mice. When IBDUs isolated from SSTR2 knockout mice were incubated with 10⁻⁷ M secretin in an isotonic KRB buffer for 30 min, a significant increase (22.39 ± 3.27%) of luminal area was observed, which was comparable with that in wild-type mice (P > 0.05). However, incubation of IBDUs isolated from SSTR2 knockout mice in an isotonic KRB buffer containing the same concentration of secretin together with somatostatin or L-779976 for 30 min resulted in a similar increase (18.47 ± 2.21% and 21.35 ± 4.46%, respectively; P > 0.05 compared with secretin treatment alone) of luminal area (Fig. 4). These findings indicate that SSTR2 deletion in IBDU epithelia significantly abolished the inhibitory effect of somatostatin or L-779976 on secretin-stimu-

lated IBDU secretion, suggesting that SSTR2 plays a role in the inhibition of secretin-stimulated ductal fluid secretion by somatostatin in mice.

Effects of somatostatin and secretin on intracellular levels of cAMP and cGMP in cholangiocytes isolated from wild-type and SSTR2 knockout mice. To explore the molecular mechanisms by which somatostatin stimulates ductal bile absorption and inhibits secretin-induced ductal bile secretion, we observed the effects of somatostatin on intracellular levels of cAMP and cGMP in cholangiocytes in the presence or absence of secretin. Cholangiocytes isolated from wild-type mice showed a significant increase of intracellular cAMP level, but not cGMP, after incubation with secretin for 30 min (Fig. 5). A significant increase of cGMP level, but not cAMP, was detected in cholangiocytes of wild-type mice after a 30-min incubation with somatostatin (Fig. 5). In the presence of somatostatin, a significant inhibi-
ction of secretin-induced cAMP increase in cholangiocytes of wild-type mice was detected, suggesting inhibition of secretin-induced cAMP synthesis by somatostatin. However, no significant difference of intracellular cGMP levels was detected between cells exposed to somatostatin alone and cells treated with somatostatin plus secretin (Fig. 5).

To test the role of SSTR2 in somatostatin-induced synthesis of intracellular cGMP and inhibition of secretin-stimulated cAMP synthesis in cholangiocytes, intracellular levels of cAMP and cGMP in response to somatostatin and secretin in cholangiocytes isolated from SSTR2 knockout mice were also measured. Although a comparable increase of cAMP synthesis in response to secretin was also detected in cholangiocytes from SSTR2 knockout mice, no significant decrease of secretin-stimulated cAMP synthesis by somatostatin was found (Fig. 5A). Moreover, no significant increase of intracellular cGMP level was found in cholangiocytes from SSTR2 knockout mice in response to secretin. Furthermore, no significant inhibition of secretin-stimulated cAMP synthesis by somatostatin was found in cholangiocytes from SSTR2 knockout mice, or a combination (Fig. 5B). These data suggest that somatostatin induces synthesis of intracellular cGMP and inhibits secretin-stimulated cAMP synthesis in cholangiocytes mainly by interacting with SSTR2 on cholangiocytes, a process potentially involved in the increase of ductal bile absorption and inhibition of secretin-stimulated ductal bile secretion.

**DISCUSSION**

The major findings reported here relate to the molecular mechanisms by which somatostatin regulates ductal bile formation. Using an in vitro model of enclosed IBDUs isolated from wild-type and SSTR2 knockout mice, we found that 1) four of the five known subtypes of SSTRs (SSTR1, SSTR2A/2B, SSTR3, and SSTR4) are expressed in wild-type cholangiocytes; 2) somatostatin and L-77976, a selective somatostatin analog specific to SSTR2, both directly stimulate ductal fluid absorption and inhibit secretin-induced ductal fluid secretion in mouse IBDUs; 3) deletion of SSTR2 leads to diminished increase of ductal fluid absorption and attenuated inhibition of secretin-stimulated fluid secretion induced by somatostatin or L-77976; and 4) somatostatin induces intracellular cGMP synthesis and inhibits secretin-stimulated cAMP synthesis in cholangiocytes, and deletion of SSTR2 completely blocks these effects of somatostatin. Our results provide the first functional evidence that somatostatin regulates murine ductal bile formation through both stimulation of bile fluid absorption and inhibition of fluid secretion via interacting with SSTR2 on cholangiocytes, a process that may involve the intracellular cAMP/cGMP second messengers.

Ductal bile formation is the net result of the bidirectional movement of ion/water molecules across the biliary epithelial barrier, a process regulated by hormones (4, 49). Cholangiocytes express a wide array of transport, exchanger, channel, and receptor proteins on their apical and basolateral plasma membrane domains. Those located in or near the secretory pole (apical domain), such as the cystic fibrosis transmembrane regulator (CFTR) Cl⁻ channel, Cl⁻/HCO₃⁻ exchangers (4, 26, 34), and the recently identified sodium-coupled glucose (SGLT1; Refs. 21, 23) and bile salt (ASBT; Ref. 20) cotransporters, could be directly involved in the generation of driving forces for the osmotically induced movement of water into or out of the ductal lumen. Because genes encoding several proteins involved in hormone-mediated ductal bile formation (e.g., Cl⁻/HCO₃⁻ exchanger, CFTR, secretin and somatostatin receptors) are expressed only in cholangiocytes (1, 2, 16), enclosed IBDUs provide an excellent physiological model to test the molecular mechanisms of hormone-regulated ductal bile formation. Secretin stimulates ductal bile secretion by binding to its receptor on the basolateral domain of cholangiocytes, a ligand-receptor interaction that activates cAMP, thereby stimulating Cl⁻ secretion. The increase of ion/solute transport into the lumen generates an inward osmotic gradient and thus drives water secretion into the lumen (3, 17, 27, 37, 39, 46). Consistent with previous in vivo studies (28, 31, 36, 50), we found a significant inhibitory effect on secretin-stimulated ductal fluid secretion by somatostatin or its agonist L-77976 in IBDUs isolated from wild-type mice. A significant inhibition of secretin-stimulated cAMP synthesis in cholangiocytes by somatostatin was detected, supporting the notion that somatostatin, by blocking intracellular cAMP production (50), inhibits the secretin-stimulated transport of AQP1 water channels and the activation of the Cl⁻/HCO₃⁻ exchanger, thus inhibiting ductal bile secretion.

Interestingly, when enclosed IBDUs were incubated with somatostatin or the SSTR2-selective agonist L-77976 alone, a significant decrease of luminal area was detected, suggesting that somatostatin can directly induce bile fluid absorption, consistent with our previous studies using enclosed IBDUs isolated from rat liver (7). A decrease in bile flow by somatostatin has also been reported in in vivo studies in the rat, dog, and human (18, 28, 31, 36). One possible mechanism by which somatostatin may directly stimulate ductal fluid absorption is to induce absorption of Na⁺ and Cl⁻ from bile, thus generating an inward osmotic gradient to drive water absorption. Intravenous administration of somatostatin or its analogs induces a marked increase of both net Na⁺ and Cl⁻ absorption in the intestine in the rat, rabbit, and human (40, 43). Stimulation by somatostatin of Na⁺-Cl⁻ transport and consequent increases in ion and water absorption have also been reported to occur in the gallbladder (48). Absorption of other molecules via transporters/exchangers expressed on the apical membrane of cholangiocytes, such as glucose via SGLT1 and bile acids by ASBT, may also be involved. Indeed, our most recent studies (29) using a microperfused model of IBDUs isolated from rats showed that cholangiocytes absorb glucose from the lumen, resulting in net water absorption across the biliary epithelial barrier. Despite the fact that the intrahepatic biliary tree is primarily thought to be a

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secretory organ, our results provide new evidence that it can also absorb fluid in response to hormones such as somatostatin.

cGMP has been reported as a second messenger for somatostatin in many cell types (11, 30). In the present study, we found a significant increase of intracellular cGMP level in cholangiocytes in response to somatostatin, whereas somatostatin alone showed no effect on the intracellular level of cAMP in cholangiocytes, suggesting that cGMP may be the second messenger for somatostatin in cholangiocytes. However, the experimental data on the response of cholangiocytes to extracellular cGMP are quite controversial. Cholangiocytes or bile ducts from rat appear not to respond to extracellular cGMP (45, 52). Another report suggests that cell-permeant cGMP analogs may even stimulate CI− secretion in a human bile duct epithelial cell line. Cellular responses to extracellular cGMP vary in different species and under different experimental conditions (14, 15, 32). cGMP has opposite effects in the mucosal and serosal compartments of the rat jejunum, i.e., mucosal cGMP increases fluid secretion and serosal cGMP enhances fluid absorption (14, 15). Additional studies are currently under way to fully test the importance of intracellular cGMP in cholangiocytes in somatostatin-induced ductal bile absorption.

Somatostatin induces biological cell responses by interacting with specific receptors (SSTRs) expressed on the target cell membrane. Recent studies have shown that multiple SSTRs (SSTR1–SSTR5) are heterogeneously distributed in various tissues. For example, high levels of SSTRs are expressed in the brain, gastrointestinal tract, and pancreas (5, 18, 35). In the present study, we found that four of five known transcripts encoding SSTRs (SSTR1–4 but not SSTR5) were present in highly purified cholangiocytes immunolabeled from wild-type mice. Both of the two isoforms of SSTR2 (SSTR2A/2B), were identified in cholangiocytes as reported in other tissues (8, 53). No increase of intracellular cGMP was detected in cholangiocytes isolated from SSTR2 knockout mice after incubation with somatostatin. Deletion of SSTR2 also resulted in a much lower decrease of luminal area in IBDUs isolated from SSTR2 knockout mice after treatment with somatostatin compared with that in wild-type mice. Inhibition of secretin-stimulated cAMP synthesis by somatostatin was not detected in cholangiocytes isolated from SSTR2 knockout mice. Moreover, the inhibitory effect of both somatostatin and L-779976 on secretin-induced IBDU secretion was completely absent in IBDUs from SSTR2 knockout mice. Those findings strongly support the notion that somatostatin directly stimulates fluid secretion and inhibits fluid secretion via interacting with SSTR subtype 2 on cholangiocytes. Because there is still a small inhibitory effect seen with somatostatin in IBDUs of SSTR2 knockout mice (Fig. 3A), we cannot completely exclude the possibility of involvement of other SSTRs expressed on cholangiocytes. Moreover, the biliary tree is characterized by significant structural and functional heterogeneity of cholangiocytes including their receptor expression, ion transporting capabilities, and proliferative capacity. Previous reports demonstrated that, in rats, SSTR2 is expressed in large but not small bile ducts (1, 2). Further studies should address the heterogeneity of SSTR expression along the biliary tree and the role of somatostatin on other effects in cholangiocytes, such as proliferation (51).

In conclusion, with highly purified mouse cholangiocytes and an in vitro model using enclosed IBDUs isolated from wild-type and SSTR2 knockout mice, we have demonstrated that cholangiocytes express multiple SSTRs and that somatostatin regulates ductal bile formation through both stimulation of fluid absorption and inhibition of fluid secretion by interacting with SSTR2 on cholangiocytes in mice, a process that may be mediated by the intracellular cAMP/cGMP second messengers. Future studies will be necessary to define the molecular mechanisms of somatostatin-stimulated ductal bile absorption, for example, which ions or solutes are involved in generating osmotic gradients for water absorption.

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