

Prolonged administration of secretin to normal rats increases biliary proliferation and secretin-induced ductal secretory activity

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Background and aim: Cholangiocyte proliferation is coordinately regulated by a number of gastrointestinal hormones/peptides, some of which display stimulatory effects and some have inhibitory actions on cholangiocyte proliferation. Enhanced biliary proliferation [for example after bile duct ligation (BDL) and partial hepatectomy] is associated with increased expression of secretin receptor (SR), cystic fibrosis transmembrane conductance regulator (CFTR) and Cl⁻/HCO₃⁻ anion exchanger 2 and secretin-stimulated ductal secretion, whereas loss/damage of bile ducts [for example after acute carbon tetrachloride (CCl₄) administration] is associated with reduced secretin-stimulated ductal secretory activity. There is growing information regarding the role of gastrointestinal hormones the regulation of biliary growth. For example, while gastrin, somatostatin and serotonin inhibit bile duct hyperplasia of cholestatic rats by downregulation of cAMP signaling, secretin has been shown to stimulate the proliferation of normal mice by activation of cyclic adenosine 3',5'-monophosphate (cAMP)-dependent signaling. However, no information exists regarding the stimulatory effects of secretin on biliary proliferation of normal rats. Thus, we evaluated the *in vivo* and *in vitro* effect of secretin on biliary proliferation, the expression of markers key of ductal secretion and secretin-stimulated ductal secretion.

Methods: Normal male rats were treated with saline or secretin (2.5 nmoles/kg BW/day by osmotic minipumps for one week). We evaluated: (I) intrahepatic bile duct mass (IBDM) in liver sections and PCNA expression in purified cholangiocytes; (II) SR and CFTR mRNA expression and secretin-stimulated cAMP levels in purified cholangiocytes; and (III) secretin-stimulated bile and bicarbonate secretion in bile fistula rats. *In vitro*, normal rat intrahepatic cholangiocyte lines (NRIC) were treated with BSA (basal) or secretin (100 nM) for 24 to 72 hours in the absence/presence of a PKA or a MEK inhibitor before evaluating proliferation by MTS assays.

Results: Prolonged administration of secretin to normal rats increased IBDM and PCNA expression in purified cholangiocytes compared to saline-treated normal rats. Also, secretin increased the expression of proteins (SR and CFTR) that are key in the regulating ductal secretion and enhanced secretin-stimulated cAMP levels and bile and bicarbonate secretion. *In vitro*, secretin increased the proliferation of NRIC, increase that was prevented by PKA and MAPK inhibitors.

Conclusions: We have demonstrated that secretin stimulates both *in vivo* and *in vitro* biliary proliferation and secretin-stimulated ductal secretory activity in normal rats. We suggest that the stimulatory effect of secretin on biliary proliferation and secretion may be important for preventing biliary dysfunction during ductopenic disorders.

Keywords: Biliary epithelium; bile flow; cyclic adenosine 3',5'-monophosphate (cAMP); cystic fibrosis transmembrane conductance regulator (CFTR); gastrointestinal hormones

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Introduction

Intrahepatic bile duct epithelial cells (i.e., cholangiocytes), which line the biliary ductal system, modify cholangiocyte bile into its final composition before reaching the small intestine (1-4). Ductal bile secretion is coordinately regulated by several gastrointestinal peptides/hormones including gastrin, endothelin-1, somatostatin and secretin that display inhibitory and stimulatory effects on bile and bicarbonate secretion (2,4-7). Among these factors, secretin stimulates water and bicarbonate secretion by interaction with basolateral secretin receptors (SR) that are expressed in the liver only by large bile ducts (1,8-11). The specific interaction of secretin with SR induces an increase in cyclic adenosine 3',5'-monophosphate (cAMP) levels (1,8,9,12,13), opening of the Cl⁻ channel [cystic fibrosis transmembrane conductance regulator (CFTR)] (14), which leads to the activation of the Cl⁻/HCO₃⁻ anion exchanger 2 (15) and bicarbonate ductal secretion (2,6).

In addition to modifying ductal bile (2,4-7), cholangiocytes (normal mitotically dormant) (16,17) are the capacity to markedly proliferate or undergo apoptosis in response to pathological maneuvers such as extrahepatic bile duct ligation (BDL), partial hepatectomy, acute carbon tetrachloride (CCl₄) or chronic administration of gamma-aminobutyric acid (16-19). While some of these maneuvers induce biliary proliferation (e.g., BDL and partial hepatectomy) (2,16,17), others cause bile duct damage (18,19). A number of studies have shown that enhanced biliary proliferation is associated with increased SR expression and secretin-stimulated ductal secretory activity (2,11,14,17). Conversely, damage of bile ducts is associated with reduced secretin-stimulated ductal secretion (18,19). The proliferative response of large cholangiocytes (the only liver cells expressing SR) (1,8) is regulated by activation of the cAMP-dependent PKA/ERK1/2 signaling (16,20,21). A previous study suggests that activation of the secretin SR axis stimulates cholangiocyte growth in mice, since knockout of the SR gene inhibits hyperplasia in cholestatic mice (22); however, no data exists regarding the trophic effect of secretin on normal rats. Thus, we performed studies in normal rats aimed to determine if secretin stimulates biliary proliferation and secretin-stimulated ductal secretory activity (functional indices of biliary proliferation) (1,2,16) by a cAMP-dependent signaling.

Methods and materials

Materials

Reagents were purchased from Sigma Chemical Co.

(St. Louis, MO, USA) unless otherwise indicated. Porcine secretin was purchased from Peninsula Laboratories (Belmont, CA, USA). The substrate for γ -glutamyltranspeptidase (γ -GT), N (γ -L-glutamyl)-4-methoxy-2-naphthylamide, was purchased from Polysciences (Warrington, PA, UK). The mouse anti-cytokeratin-19 (CK-19) antibody was purchased from Caltag Laboratories Inc. (Burlingame, CA, USA). The EIA kits for the measurement of intracellular cAMP levels in cholangiocytes were obtained from Cayman Chemical (Ann Arbor, MI, USA).

Animal models

Male Fisher rats (150-175 gm) were purchased from Charles River (Wilmington, MA, USA). The animals were maintained in a temperature-controlled environment (20-22 °C) with 12:12-hour light-dark cycles. All animal experiments were performed in accordance with protocols approved by the Scott and White and Texas A&M Health Science Center College of Medicine Institutional Animal Care and Use Committee. We used normal male treated with saline (0.9% NaCl) or secretin (3.12 nmol/kg body weight per day) (22,23) by intraperitoneally implanted Alzet[®] osmotic minipumps (Alzet, Cupertino, CA, USA) for seven days.

Freshly isolated cholangiocytes and normal intrahepatic cell lines

The *in vitro* studies were performed in freshly isolated cholangiocytes (6,24) and normal rat intrahepatic cholangiocyte lines (NRIC) (25). Virtually pure (by γ -GT histochemistry) (26) cholangiocytes were purified by immunoaffinity separation (6,24) by a monoclonal antibody, rat IgG_{2a} (provided by Dr. R. Faris, Brown University, Providence, RI, USA), against an antigen expressed by all rat cholangiocytes (24). NRIC, which display phenotypic and functional phenotypes similar to that of freshly isolated cholangiocytes, were cultured as described (25).

Evaluation of cholangiocyte proliferation and intrahepatic bile duct mass (IBDM)

In liver sections (4-5 μ m thick) from the two groups of animals, we measured IBDM (9) of cholangiocytes. The IBDM was measured as area occupied by CK-19 positive-bile ducts/total area \times 100. Proliferation was also measured by real-time PCR in total RNA (1 μ g) from purified

cholangiocytes. Blots were normalized by β -actin (9,27). RNA was extracted from cholangiocytes by the RNeasy Mini Kit (Qiagen Inc, Valencia, CA, USA), and reverse transcribed using the Reaction Ready™ First Strand cDNA synthesis kit (SuperArray, Frederick, MD, USA). These reactions were used as templates for the PCR assays using a SYBR Green PCR master mix and specific primers designed against the rat PCNA (NM_022381), and the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_008084), the housekeeping gene (27) (SuperArray, Frederick, MD, USA) in the real-time thermal cycler (ABI Prism 7900HT sequence detection system). A $\Delta\Delta$ CT (delta delta of the threshold cycle) analysis was performed using normal cholangiocytes as the control sample. Data are expressed as relative mRNA levels \pm SEM (n=3).

Measurement of mRNA expression of SR and CFTR and basal and secretin-stimulated cAMP Levels and bile and bicarbonate secretion

The mRNA expression of SR and CFTR, in purified cholangiocytes was evaluated by real-time PCR (27). The primers for rat SR and CFTR (SABiosciences) were designed according to the NCBI GenBank Accession numbers: NM_031115 (SR); XM_001059206 (CFTR); and GAPDH, NM_008084. A $\Delta\Delta$ CT analysis was performed using normal cholangiocytes as the control sample. Data are expressed as relative mRNA levels \pm SEM (n=3).

Basal and secretin-stimulated cAMP levels (a functional parameter of cholangiocyte growth) (17,27,28) were measured in purified cholangiocytes (after incubation for one hour at 37 °C to regenerate SR potentially damaged by enzyme digestion during cell isolation) (12) by commercially available EIA kits (3,29,30).

In the *in vivo* studies of biliary physiology, after anesthesia (50 mg/kg BW, IP) rats were surgically prepared for bile collection as described by us (2). After steady-state bile flow was reached (60-70 min from the intravenous infusion of Krebs-Ringer-Henseleit solution, KRH), the animals were infused with secretin (100 nM for 30 minutes) via a jugular vein followed by intravenous infusion of KRH for 30 min (2). Bicarbonate levels in bile were determined by a COBAS Mira Plus automated clinical chemistry analyzer (Bohemia, NY, USA).

In vitro effect of secretin on the proliferation of NRIC

NRIC were treated at 37 °C with: 0.2% BSA (basal) or

secretin (100 nM) for 24 to 72 hours in the absence or presence of pre-incubation (one hour) with H89 (a PKA inhibitor, 30 μ M) (31) or PD98059 (a MEK inhibitor, 10 nM) (30) before evaluating proliferation by CellTiter 96 AQueous One Solution Cell Proliferation Assay (27) (Promega Corp., Madison, WI, USA). Absorbance was measured at 490 nm on a microplate spectrophotometer (Versamax, Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

All data are expressed as mean \pm SEM. Differences between groups were analyzed by the Student's unpaired *t*-test when two groups were analyzed, and by ANOVA when more than two groups were analyzed, followed by an appropriate *post hoc* test.

Results

Evaluation of cholangiocyte proliferation and IBDM

The prolonged administration of secretin to normal rats increased IBDM compared to saline-treated normal rats (*Figure 1*). In purified cholangiocytes from secretin-treated normal rats there was an increase in mRNA PCNA expression compared to large cholangiocytes from saline-treated rats (*Figure 2A*).

Measurement of mRNA expression of SR and CFTR and basal and secretin-stimulated cAMP levels and bile and bicarbonate secretion

As shown in *Figure 2B,C*, there was enhanced mRNA expression of SR and CFTR in purified cholangiocytes compared to normal rats treated with saline.

Secretin increased cAMP levels at higher levels in cholangiocytes from rats treated with secretin compared to saline-treated rats (*Figure 3*). In agreement with previous studies (2,6), intravenous infusion of secretin did not increase significantly bile and bicarbonate secretion of rats treated with saline for one week (*Table 1*). When administered to rats treated with secretin for one week, secretin enhanced significantly bile and bicarbonate secretion compared to basal values (*Table 1*).

In vitro effect of secretin on the proliferation of NRIC

NRIC proliferate after the administration of secretin

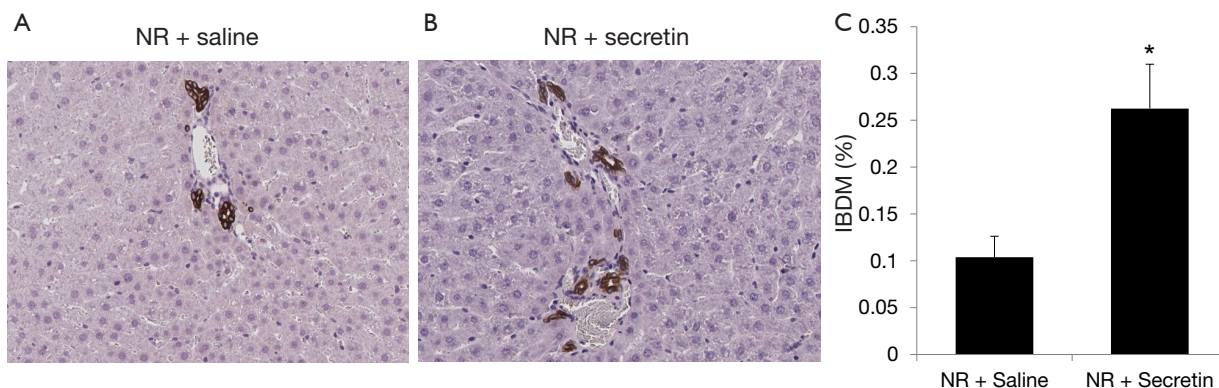


Figure 1 Evaluation of IBDM in normal rats treated with saline or secretin for one week. The prolonged administration of secretin to normal rats increased IBDM compared to saline-treated normal rats. Orig. magn., $\times 25$. *, $P < 0.05$ vs. saline-treated normal rats. IBDM, intrahepatic bile duct mass.

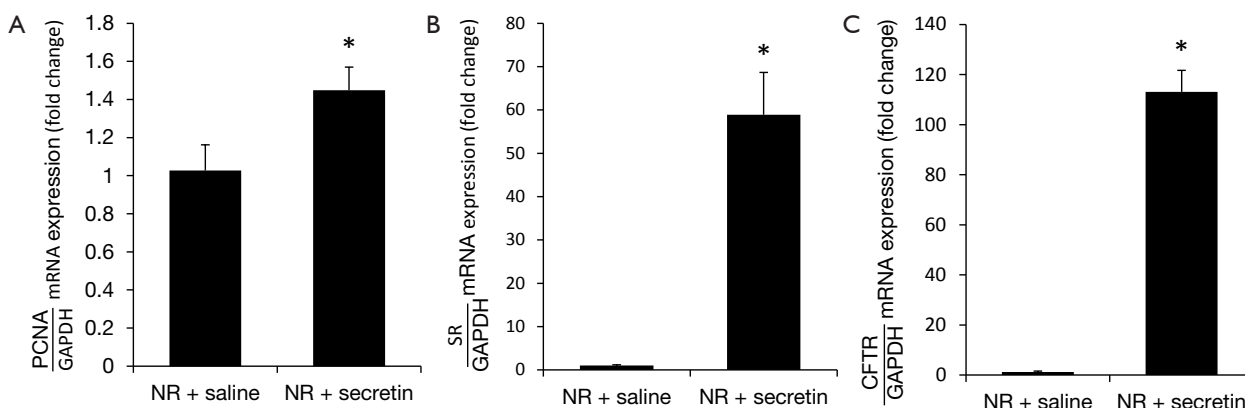


Figure 2 Measurement of mRNA expression of (A) PCNA, (B) SR and (C) CFTR in cholangiocytes from secretin-treated rats compared to normal rats treated with saline. There is enhanced mRNA expression of PCNA, SR and CFTR in purified cholangiocytes compared to normal rats treated with saline. *, $P < 0.05$ vs. cholangiocytes from saline-treated normal rats. Data are mean \pm SEM of three PCR reactions. SR, secretin receptor; CFTR, cystic fibrosis transmembrane conductance regulator.

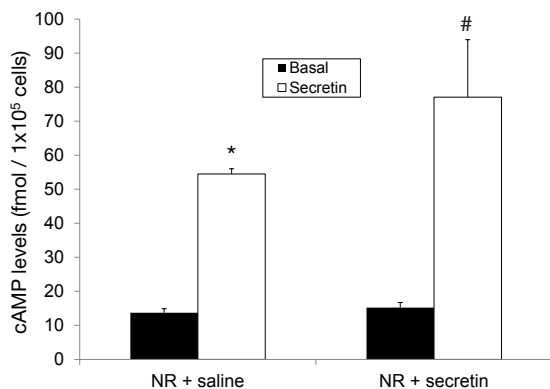


Figure 3 Secretin increased cAMP levels at higher levels in cholangiocytes from rats treated with secretin compared to saline-treated rats. *, $P < 0.05$ vs. cholangiocytes from saline-treated normal rats. #, $P < 0.05$ vs. secretin-stimulated cAMP levels from saline-treated normal rats. Data are mean \pm SEM of six evaluations.

for 6-24 hr. Secretin-stimulation of NRIC proliferation was blocked by H89 and PD98059 (Figure 4). The data suggests that secretin stimulates *in vitro* large cholangiocyte proliferation by PKA/ERK1/2-dependent mechanism.

Discussion

In the present study, we demonstrated that: (I) secretin increases the proliferation of cholangiocytes from normal rats; and (II) secretin-induced increase in biliary proliferation is associated with enhanced expression of SR and CFTR and secretin-stimulated cAMP levels and bicarbonate-rich choleresis, functional indices of biliary proliferation (2,8,11,14). Specifically, we treated normal rats with secretin for one week and demonstrated an increase in IBDM in liver sections and PCNA expression in purified

Table 1 Measurement of basal and secretin-stimulated bile flow, bicarbonate concentration and secretion in normal rats treated *in vivo* with saline or secretin for one week

Treatment	Bile flow ($\mu\text{L}/\text{min}/\text{kg}$ body weight)	Bicarbonate concentration (mEq/Liter)	Bicarbonate secretion ($\mu\text{Eq}/\text{min}/\text{kg}$ body weight)
Normal + saline (n=4)			
Basal	72.24 \pm 3.0	32.9 \pm 0.9	2.38 \pm 0.04
Secretin	71.26 \pm 2.9	35.4 \pm 0.86	2.52 \pm 0.16
Normal + secretin (n=7)			
Basal	71.26 \pm 2.9	31.0 \pm 0.6	2.29 \pm 0.09
Secretin	83.66 \pm 4.2 ^a	36.5 \pm 1.4 ^b	3.06 \pm 0.17 ^c

^{a,b,c}, $P < 0.05$ vs. the corresponding basal bile flow and bicarbonate concentration and secretion.

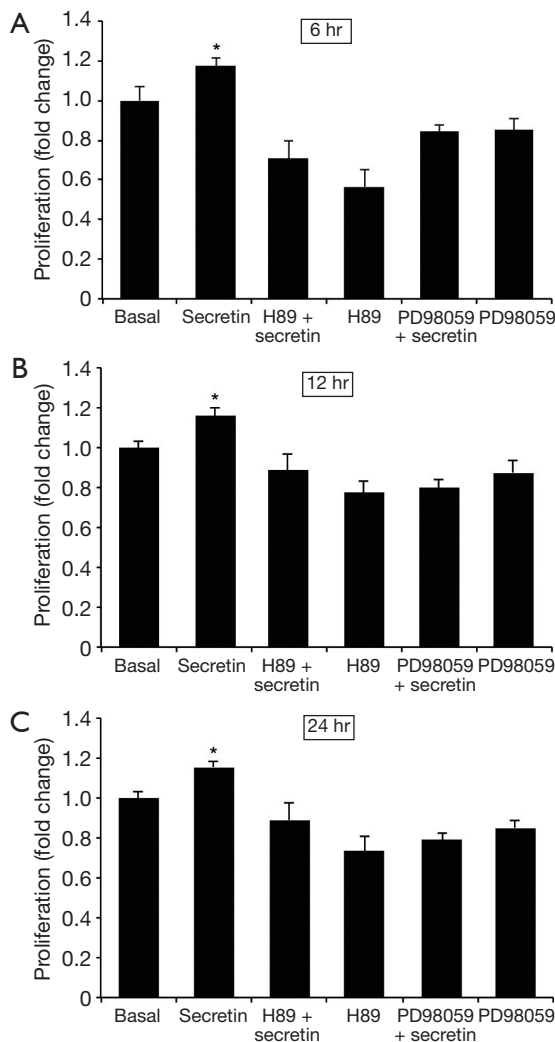


Figure 4 Effect of 0.2% BSA (basal) or secretin (100 nM) for 24 to 72 hours on the proliferation (by MTS assays) of NRIC. NRIC proliferate after the administration of secretin. Secretin-stimulation of NRIC proliferation was blocked by H89 and PD98059. *, $P < 0.05$ vs. basal values. Data are mean \pm SEM of three experiments. NRIC, normal rat intrahepatic cholangiocyte lines.

cholangiocytes compared to saline-treated normal rats. We also demonstrated that prolonged administration of secretin increased the expression of proteins (SR and CFTR) that are key in the regulating ductal secretory activity (1,2,8,16) and enhanced secretin-stimulated cAMP levels and bile and bicarbonate secretion. In *in vitro* studies, we demonstrated that secretin increased the proliferation of NRIC, increase that was prevented by PKA and MAPK inhibitors.

There is growing information regarding the role of gastrointestinal hormones/peptides in the regulation of biliary proliferation/damage (1,5,16,28,32,33). For example, studies have demonstrated that gastrin (by interaction with CCK-B/gastrin receptors) inhibits biliary hyperplasia in cholestatic rats by downregulation of Ca^{2+} -dependent PKC isoforms (28,33). Also, the gastrointestinal hormone, somatostatin, has been shown to inhibit biliary proliferation by interaction with SSTR_2 receptor (the only receptor subtype expressed by cholangiocytes) (5) by decreased expression of cAMP signaling (16). Prolonged administration of serotonin 1A and 1B receptors has also been shown to inhibit bile duct hyperplasia of cholestatic rats (32). Prolonged administration of gamma-aminobutyric acid induces the damage of large cholangiocytes and the *de novo* proliferation of small cholangiocytes and their differentiation into large cholangiocytes (19). Glucagon-like peptide-1 and Its receptor agonist, exendin-4, stimulates biliary proliferation of both normal and cholestatic rodents (34). While H3 histamine receptor agonists inhibit biliary proliferation of cholestatic rats (20), H1 histamine receptor agonists increase the proliferation of small cholangiocytes (27). Furthermore, melatonin has been shown to inhibit cholangiocyte proliferation in cholestatic rats by interaction with specific interaction with MT1 melatonin receptors (35). Furthermore, we have shown that follicle-stimulating hormone increases biliary growth by cAMP-dependent phosphorylation of

ERK1/2 and Elk-1 (36). Our findings provide new evidence that secretin stimulates biliary proliferation of normal rats, a phenomenon that was associated with increased expression of secretin-induced signaling and enhanced responsiveness to secretin. Although a number of studies suggest that the increased ductal secretory may be due to enhanced cholangiocytes proliferation (2,11,14,17), our findings introduce the novel concept that prolonged administration of secretin may increase the expression of SR and CFTR and cAMP levels and choleric effect in response to secretin, effects that may be independent from the enhanced proliferation of cholangiocytes. Since SR are only expressed by large cholangiocytes (1,8) and cAMP signaling is restricted to large cholangiocytes (1,8,30), we propose that secretin mainly target large cholangiocytes as demonstrated by enhanced large IBDM. In support of this concept, we have shown that secretin increased the proliferation of NRIC, increase that was prevented by PKA and MAPK inhibitors. Indeed, the cAMP-dependent signaling is an important regulator for the proliferation of large cholangiocytes (1,6,8,12,14,17). This also supported by a previous study showing that activation of adenylyl cyclase activity stimulates normal cholangiocyte proliferation by activation of the PKA/Src/MEK/ERK1/2 pathway (30). Furthermore, maintenance of cAMP levels prevents the functional damage of bile ducts induced by vagotomy (37).

In contrast to the stimulatory effect of secretin on biliary proliferation observed in the present and other studies (22), we have demonstrated that secretin inhibits cholangiocarcinoma growth (38). The different effect of secretin on the growth of cholangiocarcinoma may be due dysregulation in the coupling of the SR to $G\alpha_s$ and loss of cAMP response that is normally present for SR that when is stimulated triggers the activation of adenylate cyclase resulting in increased intracellular cAMP levels (38). This concept is supported by the fact that secretin stimulates the proliferation of normal human cell lines (38).

In summary, we have demonstrated that prolonged administration of secretin to normal rats induces an increase in biliary proliferation and IBDM and enhanced expression of SR and CFTR and increased choleric response to secretin. The study has potential clinical relevance since secretin may be important for the management of ductopenic liver diseases.

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