Knockout of the neurokinin-1 receptor reduces cholangiocyte proliferation in bile duct-ligated mice

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Knockout of the neurokinin-1 receptor reduces cholangiocyte proliferation in bile duct-ligated mice. Am J Physiol Gastrointest Liver Physiol 301: G297–G305, 2011. First published May 19, 2011; doi:10.1152/ajpgi.00418.2010.—In bile duct-ligated (BDL) rats, cholangiocyte proliferation is regulated by neuroendocrine factors such as α-calcitonin gene-related peptide (α-CGRP). There is no evidence that the sensory neuropeptide substance P (SP) regulates cholangiocyte hyperplasia. Wild-type (WT) and NK-1 receptor (NK-1R) knockout (NK-1R−/−) mice underwent sham or BDL for 1 wk. Then we evaluated J) NK-1R expression, transaminases, and bilirubin serum levels; 2) necrosis, hepatocyte apoptosis and steatosis, and the number of cholangiocytes positive by CK-19 and terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling in liver sections; 3) mRNA expression for collagen Iα and α-smooth muscle (α-SMA) actin in total liver samples; and 4) PCNA expression and PKA phosphorylation in cholangiocytes. In cholangiocyte lines, we determined the effects of SP on cAMP and α-myo-inositol 1,4,5-trisphosphate levels, proliferation, and PKA phosphorylation. Cholangiocytes express NK-1R with expression being upregulated following BDL. In normal NK-1R−/− mice, there was higher hepatocyte apoptosis and scattered hepatocyte steatosis compared with controls. In NK-1R−/− BDL mice, there was a decrease in serum transaminases and bilirubin levels and the number of CK-19-positive cholangiocytes and enhanced biliary apoptosis compared with controls. In total liver samples, the expression of collagen Iα and α-SMA increased in BDL compared with normal mice and decreased in BDL NK-1R−/− compared with BDL mice. In cholangiocytes from BDL NK-1R−/− mice there was decreased PCNA expression and PKA phosphorylation. In vitro, SP increased cAMP levels, proliferation, and PKA phosphorylation of cholangiocytes. Targeting of NK-1R may be important in the inhibition of biliary hyperplasia in cholangiopathies.

HUMAN CHOLANGIOCYTES ARE the target cells in a number of cholestatic liver diseases (i.e., cholangiopathies) including primary sclerosing cholangitis and primary biliary cirrhosis (5, 28), which are characterized by an abnormal balance between biliary growth and damage (5, 28). In animal models of cholestasis, changes in biliary growth and damage are achieved by a number of pathophysiological maneuvers including extrahepatic bile duct ligation (BDL) (4, 15, 23), acute administration of carbon tetrachloride (CCl4) (31), cholinergic (30) denervation, and ablation of sensory innervation by knockdown of α-calcitonin gene-related peptide (α-CGRP) (23). In these models, small and large cholangiocytes (lining small and large bile ducts, respectively) (2, 6, 21) show a different biological response, in terms of proliferation, survival, and secretory activity (3, 15, 21, 30, 31). Relevant to this study, in the cholestatic BDL rodent model large but not small cholangiocytes undergo mitosis leading to enhanced large bile duct mass (3, 15, 31). Two second messengers, α-myo-inositol 1,4,5-trisphosphate (IP3) and cyclic adenosine 3′,5′-monophosphate (cAMP), regulate the proliferative, apoptotic, and secretory functions of small and large cholangiocytes (3, 15–17, 23, 30, 31). Although the IP3/Ca2+−dependent signaling modulates the function of small cholangiocytes (16, 31), large cholangiocyte hyperplasia following BDL is regulated by the activation of cAMP-dependent PKA signaling (3, 15, 17, 23, 30).

Two afferent nerve pathways are present in the liver: the vagal and the spinal afferent nerve pathways that run through the dorsal root ganglion (45). Sensory nerves also display an efferent function, which is mediated by the release of sensory neuropeptides such α-CGRP from their peripheral terminals in tissues that innervate modulating cellular functions (25). Substance P (SP), containing peptidergic nerves, is present in the spinal afferent nerve pathway. SP-positive innervation has been localized in the portal regions of guinea pig and human liver (43). SP is a member of the tachykinin peptide family that is formed by six members: SP, neurokinin A, neurokinin B, neuropeptide K, neuropeptide γ, and hemokinin (1). SP and neurokinin A are present in the central nervous system and primary sensory afferent neurons innervating peripheral tissues and are released from sensory nerve endings both at the level of the spinal cord and in peripheral tissues (1). The tachykinin receptor family consists of three types of seven transmembrane G protein-coupled receptors: neurokinin-1, -2,
and -3 receptors (NK-1R, NK-2R, and NK-3R). SP preferentially binds and signals via the NK-1R (1). Limited data exist regarding the role of sensory innervation in the regulation of biliary functions. Tachykinins are the main nonadrenergic and noncholinergic excitatory neurotransmitters in the common bile duct of guinea pigs (38). Sensory neuropathy has been associated in patients with primary biliary cirrhosis (13). Also, knockout of α-CGRP reduces cholangiocyte hyperplasia in cholestatic BDL mice by downregulation of cAMP signaling (23). No data exist regarding the role of SP in the regulation of cholestatic BDL mice by downregulation of cAMP signaling (13).

In many cell types, SP-induced activation of NK-1R (coupled to pertussis toxin-insensitive Gq/GII) activates phospholipase C and subsequent formation of IP3 and diacylglycerol mobilizing intracellular Ca2+ (26, 36). In other cells (27, 37), NK-1R also couple to 1) Goα, resulting in adenyl cyclase activation (37) and cAMP formation and 2) Goα that inhibits cAMP formation. On the basis of this background, we aim to demonstrate that SP and its receptor, NK-1R, regulate the proliferation of cAMP-dependent large cholangiocytes in the cholestatic BDL mouse model.

**MATERIALS AND METHODS**

**Materials.** Reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. The nuclear dye 4,6-diamidino-2-phenylindole (DAPI) was obtained from Molecular Probes, Eugene, OR. The NK-1R antibody against the rat COOH-terminal (393–407) peptide was purchased from Enzo Life Sciences International (Plymouth Meeting, PA). SP was purchased from Phoenix Pharmaceuticals (Burlingame, CA). The antibody against proliferating cell nuclear antigen (PCNA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-cytokeratin-19 (CK-19) antibody (clone RCK105) was purchased from Caltag Laboratories (Burlingame, CA). The cAMP-dependent phospho-PKA catalytic subunit (Thr197) antibody (Cell Signaling, Boston, MA) detects endogenous levels of PKA catalytic subunit (α, β, and γ) only when phosphorylated at Thr197. The cAMP-dependent PKA catalytic subunit-α antibody (Cell Signaling) detects endogenous levels of total PKA catalytic subunit-α. The antibodies for the rabbit anti-ERK1 (which detects p44 and p42) and goat anti-pERK (which detects phosphorylated p44 and p42) were purchased from Santa Cruz Biotechnology RIA kits for the determination of cAMP and IP3 levels were purchased from GE Healthcare (Arlington Heights, IL).

**Animal models.** The majority of the studies were performed in wild-type (WT, +/+ ) and NK-1R knockout (NK-1R −/− ) normal (sham-operated) and 1-wk BDL mice (Table 1); since we did not see

Table 1. Evaluation of NK-1R expression, liver and body weight, liver-to-body weight ratio in the selected groups of animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>NK-1R Expression</th>
<th>Liver Weight, g</th>
<th>Body Weight, g</th>
<th>Liver/body weight × 100, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT normal mice (n = 84)</td>
<td>(−/+ )</td>
<td>1.98 ± 0.05</td>
<td>26.3 ± 0.5</td>
<td>7.58 ± 0.16</td>
</tr>
<tr>
<td>NK-1R −/− normal mice</td>
<td>(−/− )</td>
<td>1.80 ± 0.05</td>
<td>28.3 ± 0.7</td>
<td>6.25 ± 0.18*</td>
</tr>
<tr>
<td>WT BDL mice (n = 41)</td>
<td>(−/+ + )</td>
<td>1.94 ± 0.06</td>
<td>21.92 ± 0.34</td>
<td>8.84 ± 0.21†</td>
</tr>
<tr>
<td>NK-1R −/− BDL mice</td>
<td>(−/− )</td>
<td>2.07 ± 0.09</td>
<td>25.9 ± 0.74†</td>
<td>7.96 ± 0.27‡</td>
</tr>
</tbody>
</table>

Data are means ± SE. ND, not detectable; NK-1R, neurokinin-1 receptor. *, †P < 0.05 vs. the corresponding value of normal wild-type (WT) mice. ‡P < 0.05 vs. the corresponding value of WT bile duct-ligated (BDL) mice.

**Fig. 1.** A: expression of NK-1 receptor (NK-1R) was low in bile ducts from normal wild-type (WT; red arrow) mice but increased in bile ducts from bile duct-ligated (BDL) WT mice (yellow arrows; see Table 1). NK-1R was absent in intrahepatic bile ducts from normal and BDL NK-1R knockout (NK-1R −/− ) mice. Original magnification ×40. B: protein expression of NK-1R increased in large cholangiocytes from BDL WT mice compared with large cholangiocytes from normal WT mice. Data are means ± SE of 6 immunoblots derived from protein obtained from cumulative preparations of cholangiocytes. *P < 0.05 vs. the corresponding value of BDL large cholangiocytes. C: specific immunoreactivity for NK-1R in representative fields of large cholangiocytes is shown in red; cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; blue). Bar size = 25 μm.
any difference in biliary growth between normal and sham-operated mice (not shown), we used normal mice in our studies. Some of the experiments were also performed in normal and 1-wk BDL heterozygous mice (derived from the same breeding) to evaluate hepatocyte apoptosis and steatosis, lobular necrosis, the degree of inflammation and intrahepatic bile duct mass, and biliary apoptosis in vivo. BDL was performed as described (22, 23, 35). The NK-1R

expression was evaluated by semiquantitative immunohistochemistry in

was performed as described (22, 23, 35). The NK-1R

apoptosis and steatosis, lobular necrosis, the degree of inflammation

and BDL WT mice. The intensity of the bands was determined by

1

measured

mouse model lacking the NK-1R gene was generated as described

by the Chemistry Department, Scott & White.

We evaluated J) lobular necrosis and the degree of inflammation by

hematocytein eosin (H&E) staining; 2) the percentage of apoptotic

hepatocytes by terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) kit (33) (Apoptag; Chemicon International); and J) the number of large cholangiocytes (lining large bile ducts, > 15 μm diameter) (2) positive for PCNA (15) and CK-19 (39) or by TUNEL kit (33) in liver sections (4–5 μm thick) for each slide 6 nonoverlapping fields were evaluated) from the selected groups of animals. The degree of hepatocyte steatosis was evaluated in frozen liver sections (4–5 μm thick) by both H&E staining and the oil red O staining kit (32) (IHC World, Woodstock, MD). All sections were examined in a coded fashion by two board certified pathologists by a BX-51 light microscope (Olympus, Tokyo, Japan) equipped with a camera. We also evaluated the damage of a number of tissues/organs in the experimental groups listed in Table 1.

Evaluation of gene expression for collagen 1α and α-smooth muscle actin in total liver samples. We measured the expression of the messages for collagen 1α and α-smooth muscle actin (α-SMA) in RNA (0.5 μg) in total liver tissue by the RT² Real-Time assay from SABiosciences (Frederick, MD) (16). A △△Ct (delta delta of the threshold cycle) analysis was performed by using RNA from normal WT mice as the control sample. Data were expressed as relative mRNA levels ± SE of the selected gene-to-GAPDH ratio. The pri-

mers for collagen 1α and α-SMA (purchased from SABiosciences) were designed according to the NCBI GenBank Accession numbers: NM_007742 (for collagen 1α) (18) and NM_007392 (for α-SMA) (34).

Measurement of PCNA protein expression and phosphorylation of PKA in purified large cholangiocytes. In protein (10 μg) from whole cell lysate from spleen (positive control) and purified large cholangiocytes from WT and NK-1R+/− BDL mice, we evaluated by immunoblotting (16, 21) the phosphorylation of PKA in purified large cholangiocytes by PCNA protein expression (measured as ratio to β-actin protein expression) (16), and the phosphorylation of cAMP-dependent PKA (expressed as ratio to protein expression of the corresponding total protein), a molecule playing an important role in cAMP-dependent regulation of large cholangiocyte proliferation (8, 15, 17). The intensity of the bands was determined by scanning video densitometry using the phospho-imager

<table>
<thead>
<tr>
<th>Groups</th>
<th>Alanine Aminotransferase, units/l</th>
<th>Aspartate Aminotransferase, units/l</th>
<th>Total Bilirubin, mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT normal mice</td>
<td>20.2 ± 2.4 (n = 8)</td>
<td>55.2 ± 3.9 (n = 8)</td>
<td>&lt;0.1 (n = 8)</td>
</tr>
<tr>
<td>NK-1R+/− normal mice</td>
<td>123.8 ± 20.7 (n = 11)</td>
<td>229.3 ± 23.2 (n = 12)</td>
<td>&lt;0.1 (n = 16)</td>
</tr>
<tr>
<td>WT BDL mice</td>
<td>657.1 ± 119.1 (n = 6)</td>
<td>1,134.6 ± 22.1 (n = 5)</td>
<td>13.58 ± 1.74 (n = 7)</td>
</tr>
<tr>
<td>NK-1R+/− BDL mice</td>
<td>389 ± 351 (n = 6)</td>
<td>708.6 ± 50.6 (n = 5)</td>
<td>9.22 ± 1.1 (n = 7)</td>
</tr>
</tbody>
</table>

Data are means ± SE of 7 evaluations. *P < 0.05 vs. the corresponding value of WT normal mice. 1P < 0.05 vs. the corresponding value of WT mice with BDL for 7 days.

Table 2. Evaluation of serum levels of transaminases and bilirubin

Table 3. Evaluation of the percentage of hepatocytes positive by TUNEL

<table>
<thead>
<tr>
<th>Groups</th>
<th>Percentage of Hepatocytes positive by TUNEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT normal mice</td>
<td>4.2 ± 0.44</td>
</tr>
<tr>
<td>NK-1R+/− normal mice</td>
<td>4.9 ± 0.48*</td>
</tr>
<tr>
<td>WT 7 BDL mice</td>
<td>31.8 ± 2.3*</td>
</tr>
<tr>
<td>NK-1R+/− BDL mice</td>
<td>34.0 ± 2.6</td>
</tr>
</tbody>
</table>

Data are means ± SE. TUNEL, terminal deoxynucleotidyltransferase biotin-dUTP nick-end labeling. *P < 0.05 vs. the corresponding value of normal WT mice.
In vitro effect of SP on cAMP and IP₃ levels, proliferation, and phosphorylation of PKA and ERK1/2 of immortalized large cholangiocytes. For the measurement of cAMP or IP₃ levels, large immortalized cholangiocytes were treated at room temperature for 5 (cAMP) (3, 15) or 10 (IP₃) (16) min with 0.2% bovine serum albumin (BSA) or SP (10⁻⁹ M) before evaluation of the levels of these two molecules by RIA (2, 3, 15, 16). In other experiments, large cholangiocyte lines were treated at 37°C for 24, 48, and 72 h with 0.2% BSA or SP (10⁻⁹ M) for 1, 2, 3, 5, 7, 10, 20, 30, 60, and 90 min, 1, 2, and 6 h) before evaluation of ERK1/2 phosphorylation by immunoblots (19).

RESULTS

Expression of NK-1R in liver sections and isolated and immortalized large cholangiocytes. By semiquantitative immunohistochemistry, the expression of NK-1R was low in bile ducts from WT (red arrow) normal mice but increased in bile ducts from WT BDL mice (Fig. 1A; yellow arrows and Table 1). NK-1R was absent in bile ducts from normal and BDL NK-1R⁻/⁻ mice (Fig. 1A and Table 1). These findings were confirmed by immunoblotting: the expression of NK-1R increased in large cholangiocytes from BDL WT mice compared with large cholangiocytes from normal WT mice (Fig. 1B); NK-1R was also expressed by small bile ducts in liver sections (not shown). By immunofluorescence, specific immunoreactivity for NK-1R in representative fields of large murine cholangiocyte lines (44) is shown in red; cell nuclei were stained with DAPI (blue) (Figs. 1C).

Evaluation of serum levels of transaminases and bilirubin, lobular necrosis, inflammation, hepatocyte apoptosis and steatosis, and cholangiocyte proliferation and apoptosis. Surprisingly, body weight was significantly lower in WT BDL mice compared with NK-1R⁻/⁻ BDL mice (Table 1). In both normal and BDL NK-1R⁻/⁻ mice there was a significant decrease in liver-to-body weight ratio (an index of liver growth including cholangiocytes) (4) compared with the corresponding WT mice.
(Table 1). In agreement with previous studies in rodents (4), the serum levels of transaminases (alanine aminotransferase and aspartate aminotransferase) and total bilirubin were higher in WT BDL mice compared with normal WT mice and decreased in NK-1R-/− BDL mice compared with WT BDL mice (Table 2). No difference in the serum levels of total bilirubin was observed between WT normal mice and NK-1R-/− normal mice (Table 2). Surprisingly, we observed a significant increase in the serum levels of transaminases in normal NK-1R-/− mice compared with WT normal mice (Table 2).

In liver sections from normal NK-1R-/− mice, we observe higher hepatocyte apoptosis (Table 3), some necrotic areas (yellow arrow, Fig. 2A) and inflammatory infiltrates (yellow arrow, Fig. 2B) compared with normal WT mice. There was a decrease in necrotic areas in NK-1R-/− BDL mice compared with BDL WT mice (see yellow arrows) (Fig. 2A). No marked difference in inflammatory infiltrate was observed between WT and NK-1R-/− BDL mice (see yellow arrow) (Fig. 2B). In NK-1R-/− normal mice, centrolobar liver parenchyma shows round-shaped areas (Fig. 2C) and red scattered spots by oil red O staining evocative of steatosis (Fig. 2C), whereas WT (Fig. 2C) and heterozygous (not shown) normal mice samples display normal liver morphology. All BDL liver sections do not present round-shaped areas evocative of steatosis (not shown). No significant differences in these parameters were seen between WT and NK-1R-/− normal mice (not shown). These histomorphological changes (Fig. 2, A–C and Table 3) likely explain the significant increase in the serum levels of transaminases observed in normal NK-1R-/− mice compared with WT normal mice. The number of PCNA-positive cholangiocytes was low and similar in both WT (0.4 ± 0.2) and NK-1R-/− (0.2 ± 0.2) normal mice. In NK-1R-/− BDL mice, there was a decrease in the number of PCNA-positive large cholangiocytes (17.4 ± 1.3) compared with WT BDL mice (12.0 ± 1.3). The number of CK-19-positive large cholangiocytes was similar among WT, heterozygous, and NK-1R-/− normal mice and increased following BDL in both WT and heterozygous BDL mice (Fig. 3 and Table 4). In NK-1R-/− BDL mice, there was a decrease in the number of CK-19-positive large cholangiocytes compared with the degree of cholangiocyte apoptosis observed in the corresponding WT BDL mice (Table 4). No difference in cholangiocyte apoptosis was observed between WT and heterozygous normal mice and NK-1R-/− normal mice (Table 4). No significant gross postmortem or pathological changes were detected in the body cavities, integumentary, alimentary, respiratory, circulatory, nervous, urogenital, hematopoietic, and musculoskeletal systems of normal WT mice and NK-1R-/− normal mice (not shown).

mRNA expression for collagen 1α and α-SMA. No changes in the mRNA and protein expression of collagen 1α were observed in total liver samples from WT and NK-1R-/− normal mice (Fig. 4A). The expression of α-SMA decreased in total liver samples from normal NK-1R-/− mice compared with normal WT mice (Fig. 4B). The expression of collagen 1α and α-SMA increased in total liver samples from BDL WT mice compared with normal WT mice (Fig. 4, A and B). There was a decrease expression of collagen 1α and α-SMA mRNA expression in total liver samples from BDL NK-1R-/− mice compared with total liver samples from BDL WT mice (Fig. 4, A and B).

Measurement of PCNA protein expression, and phosphorylation of cAMP-dependent PKA in isolated large cholangiocytes. There was a decrease in PCNA expression in large cholangiocytes from NK-1R-/− BDL mice compared with large cholangiocytes from WT BDL mice (Fig. 5A). In large cholangiocytes from NK-1R-/− BDL mice there was a decrease in the phosphorylation of cAMP-dependent PKA compared with large cholangiocytes from WT BDL mice (Fig. 5B).

Table 4. Evaluation of the number of CK-19 or TUNEL-positive large cholangiocytes

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of CK-19-Positive Cholangiocytes</th>
<th>Number of Cholangiocytes Positive by TUNEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT normal mice</td>
<td>17.2 ± 1.0</td>
<td>ND</td>
</tr>
<tr>
<td>Heterozygous normal mice</td>
<td>17.3 ± 1.1</td>
<td>ND</td>
</tr>
<tr>
<td>NK-1R-/− normal mice</td>
<td>17.1 ± 1.8</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>WT 7 BDL mice</td>
<td>78.8 ± 4.3*</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Heterozygous BDL mice</td>
<td>65.1 ± 2.0*</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>NK-1R-/− BDL mice</td>
<td>53.0 ± 2.6†</td>
<td>6.6 ± 0.4†</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05 vs. the corresponding value of normal WT mice. †P < 0.05 vs. the corresponding value of WT mice with BDL for 7 days.
In vitro effect of SP on cAMP and IP₃ levels, cholangiocyte proliferation, and phosphorylation of PKA and ERK1/2 of large cholangiocytes. A marked increase in cAMP levels was observed with forskolin (an activator of adenylyl cyclase) (17), whereas secretin and SP induced a modest yet significant increase in cAMP levels in large cholangiocytes (Fig. 6A). The increase in cAMP levels observed with secretin in immortalized large cholangiocytes was similar to that observed in our previous studies (17). SP did not increase IP₃ levels of large cholangiocytes (Fig. 6B). By MTS assays, SP induced a similar and sustained (both at 48 and 72 h) increases in the proliferation of large cholangiocytes compared with controls (Fig. 7, A and B); no increase was seen at 24 h of treatment with SP (not shown). SP stimulation of large cholangiocyte growth was blocked by preincubation with spantide and H89 at 48 h and partly at 72 h (Fig. 7C and D); BAPTA/AM did not block substance stimulation of large cholangiocyte proliferation (Fig. 7C and D). Short-term treatment with SP increased PCNA protein expression and phosphorylation of PKA (but not ERK1/2, not shown) compared with controls (Fig. 8, A and B).

**DISCUSSION**

Previous studies have demonstrated that 1) circulating levels of the sensory neuropeptides CGRP, SP, and adrenomedullin are elevated in humans, and rodent models of cirrhosis and biliary hyperplasia (11, 23, 29, 40); and 2) SP serum levels are elevated in cholestatic patients and BDL rats (42). Our study provides the first evidence regarding the role of the SP→NK-1R axis in sustaining the proliferation of large cholangiocytes by activation of cAMP signaling. We found an increase in the serum levels of transaminases in normal NK-1R⁻/⁻ mice compared with WT normal mice. The serum levels of transaminases and total bilirubin were decreased in NK-1R⁻/⁻ BDL mice compared with WT BDL mice. We demonstrated the presence of NK-1R in large cholangiocytes that was higher in BDL compared with normal rats. Knockdown of the NK-1R gene in BDL mice induces a decrease (~40%) in the number of large cholangiocytes (associated with enhanced biliary apoptosis) compared with BDL WT mice. There was decreased PCNA protein expression and phosphorylation of PKA in large cholangiocytes from NK-1R⁻/⁻ BDL mice.
compared with controls. The expression of collagen 1α and α-SMA increased in total liver samples from BDL WT mice compared with normal WT mice and decreased in BDL NK-1R−/− mice compared with total liver samples from BDL WT mice. In vitro, SP increased cAMP levels, enhanced the phosphorylation of PKA but not ERK1/2, and induced a sustained increase in the proliferation of large cholangiocytes. Pharmacological targeting of NK-1R may be important in the inhibition of biliary proliferation in cholestatic liver disorders.

In support for the presence of NK-1R in liver, previous studies have demonstrated the presence of NK-1R in hepatocytes (9, 10). Although both small (not shown) and large cholangiocytes express NK-1R, we evaluated the role of the SP→NK-1R axis on the regulation of large cholangiocyte growth since large, but not small, cholangiocytes proliferate in response to BDL (3, 15, 31). Previous studies have emphasized the importance of cAMP/PKA/ERK1/2 signaling in the regulation of large biliary functions (8). For example, the stimulation of adenyl cyclase by forskolin stimulates large cholangiocyte proliferation (17). Maintenance of cholangiocyte cAMP levels by administration of forskolin prevents the functional damage of bile ducts induced by vagotomy (30). Since both small cholangiocytes (whose function is regulated by IP3/Ca2+)/Ca2+ signaling (16, 20) express NK-1R and SP exerts its cellular function by the activation of both cAMP and IP3/Ca2+ signaling (26, 36), studies aimed to evaluate the role of SR in small cholangiocyte functions are necessary. Also, further experiments aimed to evaluate the effects of SP on the phosphorylation of other MAPK isoforms such as JNK and p38 in large cholangiocytes are underway and part of another project.

In our NK-1R−/− BDL model, the extent of the reduction (~40%) of biliary mass is consistent with the concept that cholangiocyte proliferation is coordinately modulated by a number of stimulatory/inhibitory neuroendocrine factors (7). A similar reduction in biliary was observed in α-CGRP BDL−/− mice (23) since other sensory neuropeptides such as β-CGRP...

Fig. 6. In vitro effect of forskolin (10−4 M), secretin (100 nM) and substance P (10 μM) on cAMP (A) and substance P (10 μM) on d-myso-inositol 1,4,5-trisphosphate (IP3) levels (B) of large cholangiocytes. A: a massive increase in cAMP levels was observed with forskolin, whereas secretin and substance P induced a modest albeit significant increase in cAMP levels in large cholangiocytes. B: substance P did not increase IP3 levels of large cholangiocytes. Substance P increased cAMP (A) but not IP3 (B) levels of large cholangiocytes. Data are means ± SE of 6 values obtained from cumulative preparations of cholangiocytes. *P < 0.05 vs. the corresponding basal value of large cholangiocytes treated with 0.2% BSA (basal).

Fig. 7. A and B: by MTS assays, substance P induced a similar (at all the doses used) and sustained (24 and 72 h) increase in the proliferation of large cholangiocytes compared with BSA-treated cells. Data are means ± SE of 6 experiments. *P < 0.05 vs. the corresponding basal value of large cholangiocytes. C and D: effect of 0.2% BSA (basal) or substance P (10−9 M) for 48 h in the absence or presence of preincubation with spantide (specific NK-1R inhibitor), H89 (PKA inhibitor), or BAPTA/AM (intracellular Ca2+ chelator). Substance P stimulation of large cholangiocyte growth was blocked by preincubation with spantide and H89 but not BAPTA/AM. Data are means ± SE of 6 experiments. *P < 0.05 vs. the corresponding basal value of large cholangiocytes.
stimulate cholangiocyte proliferation during cholestasis. Also, knockout of the secretin receptor gene induces a similar decrease in biliary mass in mice with BDL (22). The reduction of the serum levels of transaminases and bilirubin observed in NK-1R−/− BDL mice further supports the concept that blockage of the NK-1R induced signaling is important in the reduction of liver damage and biliary hyperplasia. The increase in hepatocyte apoptosis and steatosis likely explains the significant increase in the serum levels of transaminases observed in normal NK-1R−/− mice compared with WT normal mice. This finding also suggests that SP signaling may play a role in hepatic metabolism and that lack of the NK-1R may trigger hepatocyte steatosis (41) that was we speculate was resolved during cholestasis induced by BDL. Compared with WT animals, in NK-1R−/− mice a significant reduction in the mRNA expression of collagen 1α and α-SMA after BDL. These data reflect the reduction of the expansion of the biliary tree in NK-1R−/− mice, with a consequent reduction of biliary fibrogenesis, which is mostly likely due to reduced hepatic stellate cell activity (46). In addition to alterations in hepatic stellate cell activation, we cannot rule out that an ancillary part of those differences may be accounted to reduced collagen deposition by hepatocytes, by cholangiocytes, and, in particular, by inflammatory cells, since SP is a one of the mediators of neurogenic inflammation and NK1-R antagonist have been shown to protect mice from cytokine, CD95 and TNF-α mediated liver injury (9, 10).

The biological and pathophysiological significance of our findings is supported by a number of studies. For example, the neurokinin-1 receptor antagonists CP-96,345 and L-733,060 protect mice from cytokine-mediated liver injury, most likely by inhibiting SP effects (10). NK-1R antagonists have been shown to protect mice from CD95- and TNF-α-mediated liver damage (9). As a direct outgrowth of the present study, since a number of neuroendocrine factors regulate biliary functions by autocrine mechanisms, we propose to evaluate the possible autocrine role of SP in the growth and damage of the biliary epithelium.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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