Table 3. *Measurement of basal and secretin-stimulated bile flow, bicarbonate concentration and secretion in normal and rats that (immediately after BDI) underwent castration surgery followed by the administration of vehicle or testosterone*

	Bile Flow		Bicarbonate Concentration		Bicarbonate Secretion	
Treatment	Basal, μl · min^{-1} · kg body wt^{-1}	Secretin, μl · min^{-1} · kg body wt^{-1}	Basal, meq/l	Secretin, meq/l	Basal, μl · min^{-1} · kg body wt^{-1}	Secretin, μl · min^{-1} · kg body wt^{-1}
Normal rats (n = 4)	81.7 ± 6.3	83.0 ± 5.8	29.4 ± 0.9	26.8 ± 0.3	2.4 ± 0.2	2.2 ± 0.1
Normal rats + castration (n = 4)	70.2 ± 4.3	71.1 ± 8.9	30.7 ± 3.7	27.5 ± 6.1	2.3 ± 0.2	1.2 ± 0.3
BDI rats (n = 4)	126.8 ± 15.0^{a}	187.8 ± 21.9^{b}	37.1 ± 3.4^{a}	45.5 ± 2.6^{b}	4.9 ± 0.2^{a}	8.6 ± 1.1^{b}
BDI rats + castration + vehicle (n = 4)	114.1 ± 24.7	133.0 ± 15.7^{ns}	26.8 ± 11.2	35.1 ± 14.9^{ns}	3.1 ± 1.8	4.9 ± 2.2^{ns}
BDI rats + castration + testosterone (n = 4)	109.1 ± 13.3^{c}	146.3 ± 13.9^{c}	32.5 ± 0.8	41.1 ± 1.1^{c}	3.6 ± 0.4	6.1 ± 0.6^{c}

Values are means ± SE; *n*, no. of rats. BDI, bile duct incannulated.When steady spontaneous bile flow was reached [60-70 min from the infusion of Krebs-Ringer-Henseleit (KRH)], rats were infused for 30 min with secretin followed by a final infusion of KRH for 30 min. After the rats were surgically prepared for bile flow experiments, bile was collected every 10 min in preweighed tubes and used for determining bicarbonate concentration. $^{a}P < 0.05$ vs. corresponding basal value of bile flow, bicarbonate concentration, or bicarbonate secretion of normal rats without castration. $^{b}P < 0.05$ vs. corresponding basal value of bile flow, bicarbonate concentration. or bicarbonate secretion of BDI rats. ^{ns}Vs. corresponding basal value of bile flow, bicarbonate concentration, or bicarbonate secretion of BDI rats without castration. $^{c}P < 0.05$ vs. corresponding basal value of bile flow, bicarbonate concentration, or bicarbonate secretion of BDI castrated rats treated with testosterone for 1 wk. Differences between groups were analyzed by the Student's unpaired *t*-test when two groups were analyzed and ANOVA when more than two groups were analyzed.

was increased IBDM compared with their corresponding normal rats (Table 2) (3). Also, testosterone increased IBDM in normal (data not shown) and BDL female and male rats (Table 2) compared with rats treated with vehicle. Consistent with the concept that testosterone is a trophic factor for cholangiocyte growth, castration (which reduces serum testosterone levels) (31) decreased IBDM in both normal (data not shown) and BDL rats compared with rats without castration (Table 2). We next demonstrated that administration of an antitestosterone antibody (which reduces the circulating levels of testosterone) decreased IBDM compared with control BDL rats (Table 2), and administration of testosterone partly prevented castration-induced loss of IBDM (Table 2). In BDL castrated rats and BDL rats treated with antitestosterone antibody, there was an increase in apoptosis compared with BDL rats (data not shown).

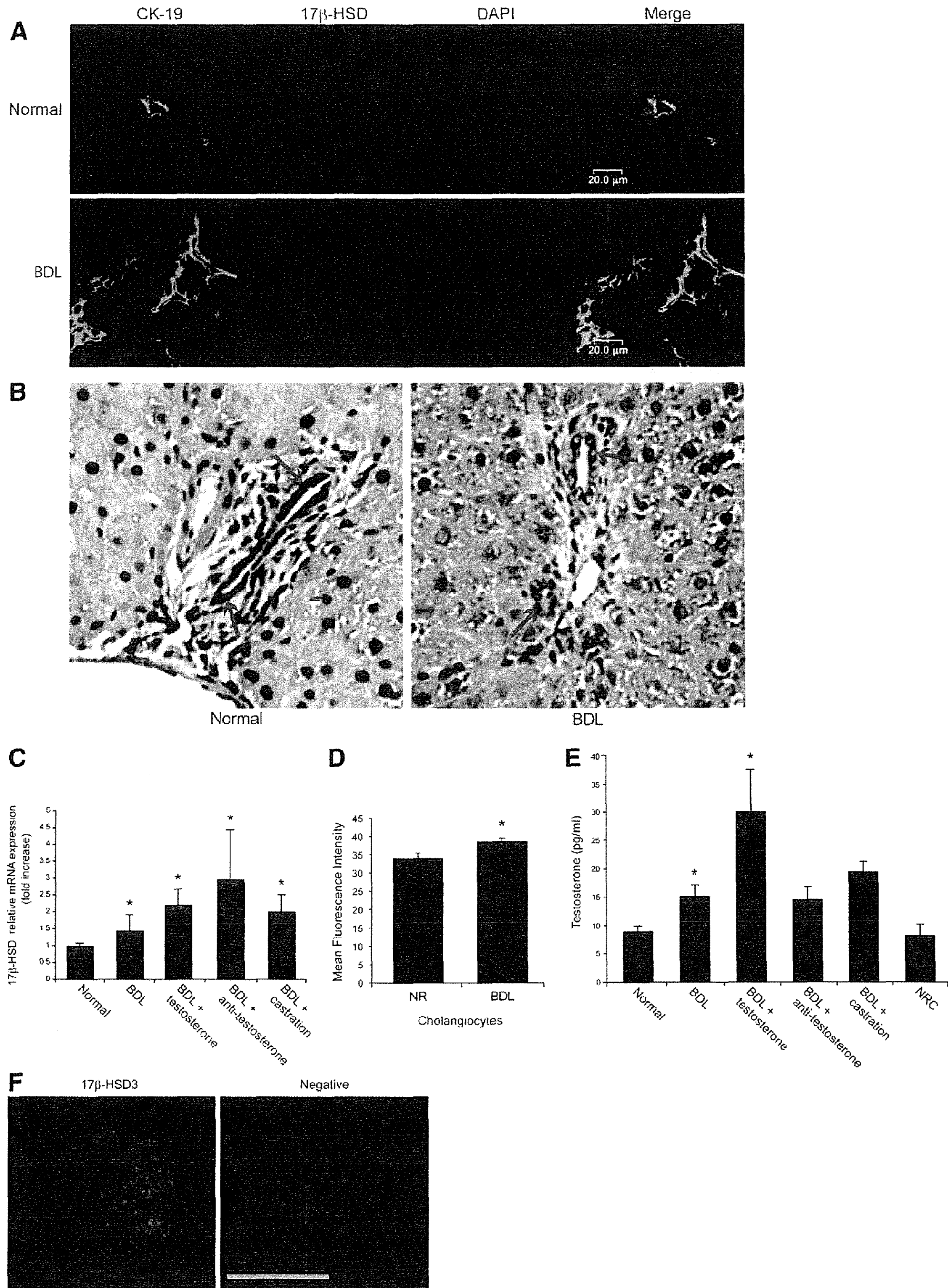
Castration inhibits secretin-stimulated cAMP levels and bile secretion. Secretin increased cAMP levels of cholangiocytes from normal but not normal castrated rats (Fig. 3). Basal cAMP levels of cholangiocytes from BDL rats were higher than cAMP levels of normal cholangiocytes (Fig. 3). As expected (3, 35), secretin did not induce changes in bile and bicarbonate secretion in normal rats with or without castration (Table 3). In agreement with previous studies (2, 3), in BDL rats (without castration), secretin increased cAMP levels in purified cholangiocytes (Fig. 3) and bicarbonate-rich choleresis in bile fistula rats (Table 2). After castration to BDL rats, the stimulatory effects of secretin on cAMP levels in purified cholangiocytes (Fig. 3) and bile and bicarbonate secretion in bile fistula rats (Table 2) were ablated. Chronic administration (1 wk) of testosterone to BDL castrated rats restored the functional secretory activity of cholangiocytes, since secretin was able to stimulate bile and bicarbonate secretion (Table 3) in these rats.

Expression of 17β-HSD3 in liver sections and cholangiocytes: Evaluation of testosterone secretion in NRICC and determination of the effect of pharmacological inhibition and molecular silencing of 17β-HSD3 on NRICC growth. By immunofluorescence and immunohistochemistry in liver sections, 17β-HSD3 was expressed by intrahepatic bile ducts from normal and BDL male rats (Fig. 4, *A* and *B*). The immunoreactivity was higher in bile ducts from male BDL rats compared with their corresponding normal rats. mRNA (by real-time PCR) and protein (by FACS) for 17β-HSD3 was expressed by normal male cholangiocytes and increased following BDL (Fig. 4, *C* and *D*). In purified cholangiocytes from male BDL rats with castration or receiving antitestosterone antibody, the expression of 17β-HSD3 mRNA was similar to or higher than that of BDL cholangiocytes (Fig. 4*C*), which is likely due to a compensatory mechanism by cholangiocytes in response to decreased testosterone serum levels after castration or the administration of antitestosterone antibody to BDL rats. We have also demonstrated that normal cholangiocytes and NRICC secrete testosterone in the supernatant, and the levels of testosterone increased in the supernatant of BDL cholangiocytes compared with normal cholangiocyte supernatant (Fig. 4*E*). In purified cholangiocytes from BDL rats with castration or receiving antitestosterone antibody, the secretion of testosterone was similar to that of BDL cholangiocytes (Fig. 4*E*). By

Fig. 4. *A*: by immunofluorescence, 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3) was expressed by intrahepatic bile ducts of liver sections from normal and BDL rats with and without castration. Colocalization with CK-19 (green staining) of bile ducts expressing the AR (red staining) is also visible. Bar = 50 μm. *B*: by immunohistochemistry, cholangiocytes from normal and BDL rats express 17β-HSD3. Original magnification, ×40. The immunoreactivity was higher in bile ducts from male BDL rats (++) compared with their corresponding normal rats (+). *C* and D: the message and protein for 17β-HSD3 was expressed by normal male cholangiocytes and increased following BDL. Data are means ± SE of 3 real-time PCR and FACS experiments. *C*: in purified cholangiocytes from BDL rats with castration or receiving antitestosterone antibody, the expression of 17β-HSD3 mRNA was similar to that of BDL cholangiocytes. Data are means ± SE of 3 real-time PCR experiments. *$P < 0.05$ vs. all of the other groups. *D*: the protein expression levels of 17β-HSD3 were determined by FACS analysis. *$P < 0.05$ vs. normal rat. *E*: normal cholangiocytes and NRICC secrete testosterone in the supernatant. The levels of testosterone increased in the supernatant of BDL cholangiocytes compared with normal cholangiocyte supernatant. In purified cholangiocytes from BDL rats with castration or receiving antitestosterone antibody, the secretion of testosterone was similar to that of BDL cholangiocytes. Data are means ± SE of 8 evaluations. *$P < 0.05$ vs. all of the other groups. *F*: by immunofluorescence, NRICC express the protein for 17β-HSD3. Bar = 50 μm.

immunofluorescence, NRICC express the protein for 17β-HSD3 (Fig. 4*F*).

Similar to what is shown in vivo (Table 2), testosterone (10^{-11} to 10^{-5} M) in vitro increased the proliferation (by MTS) of NRICC compared with the corresponding basal value (Fig. 5*A*). Also, testosterone (100 nM) increased the mRNA expression of 17β-HSD3 (the key enzyme regulating testosterone synthesis) (20) in NRICC compared with the



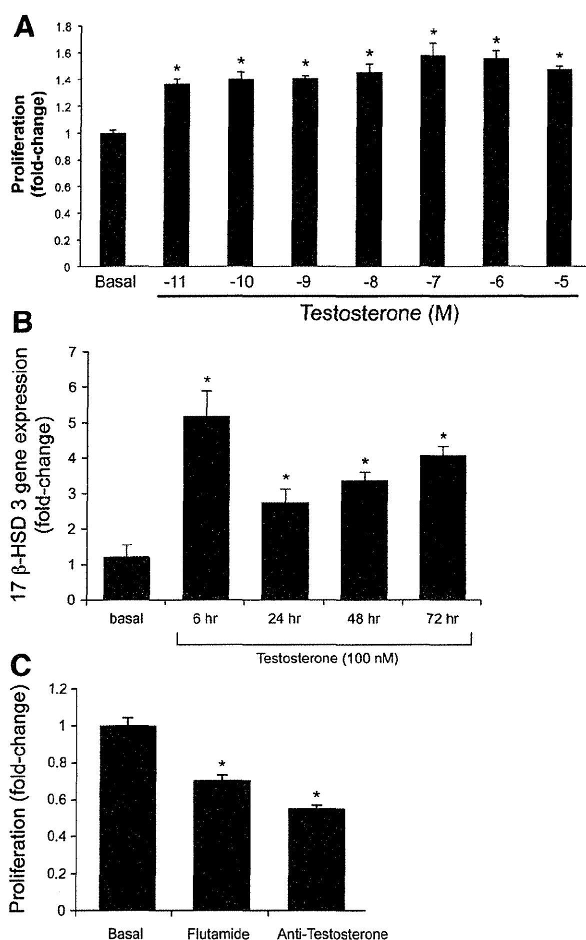


Fig. 5. *A*: measurement of NRICC proliferation following treatment with 0.2% BSA or testosterone. Testosterone (10^{-11} to 10^{-5} M) in vitro increased the proliferation (by MTS) of NRICC compared with the corresponding basal value. Data are means ± SE of 6 experiments. $*P < 0.05$ vs. the corresponding basal value. *B*: testosterone in vitro increased the mRNA expression of 17β-HSD3 (the key enzyme regulating testosterone synthesis) in NRICC compared with the corresponding basal value. Data are means ± SE of 6 experiments. $*P < 0.05$ vs. the corresponding basal value. *C*: measurement of cell growth (by MTS assays) in NRICC stimulated with BSA (basal), flutamide (a specific antagonist of AR), or antitestosterone antibody for 48 h. When NRICC were incubated with flutamide or antitestosterone antibody, there was a decrease in cell growth compared with NRICC treated with BSA. Data are means ± SE of 6 experiments. $*P < 0.05$ vs. the corresponding basal value.

corresponding basal value (Fig. 5*B*). Consistent with the concept that testosterone (synthesized and secreted by freshly isolated cholangiocytes and NRICC) regulates the growth of these cells by an autocrine mechanism, we have shown that, when NRICC were incubated with flutamide (a specific antagonist of AR) (28) or antitestosterone antibody for 48 h, there was a decrease in cell growth compared with NRICC treated with 0.2% BSA (basal) (Fig. 5*C*). Furthermore, knock down of 17β-HSD3 (80% decrease by real-time PCR) (Fig. 6*A*) in NRICC decreased the basal proliferative activity of NRICC (Fig. 6*B*), supporting the concept that testosterone is an autocrine factor sustaining biliary growth.

DISCUSSION

The present study demonstrates the paracrine and autocrine role of testosterone in the stimulation of cholangiocyte growth in normal and cholestatic states. We first demonstrated that intrahepatic bile ducts and hepatocytes (in liver sections) and freshly isolated cholangiocytes and hepatocytes from normal and BDL rats, and male NRICC express testosterone receptors. We have also shown that: *1*) testosterone serum levels were lower in female and male BDL compared with normal rats and *2*) castration and administration of neutralizing antitestosterone antibody significantly decreased testosterone serum levels in normal and BDL male rats. The administration of testosterone increased testosterone serum levels in both normal and BDL male rats and partly prevented castration-induced reduction of testosterone serum levels. We have also demonstrated that: *1*) testosterone increased IBDM of both normal and BDL female and male rats; *2*) castration and administration of antitestosterone antibody decreased IBDM in BDL male rats; and *3*) administration of testosterone partly prevented castration-induced loss of IBDM in BDL male rats. At the functional level, in BDL castrated rats, there was ablation of secretin-stimulated cAMP levels and bicarbonate-rich choleresis, two functional indexes of biliary growth (2, 3, 22, 36). Similar to in vivo, in in vitro studies, we have demonstrated that testosterone increased the proliferation of male NRICC. We have also

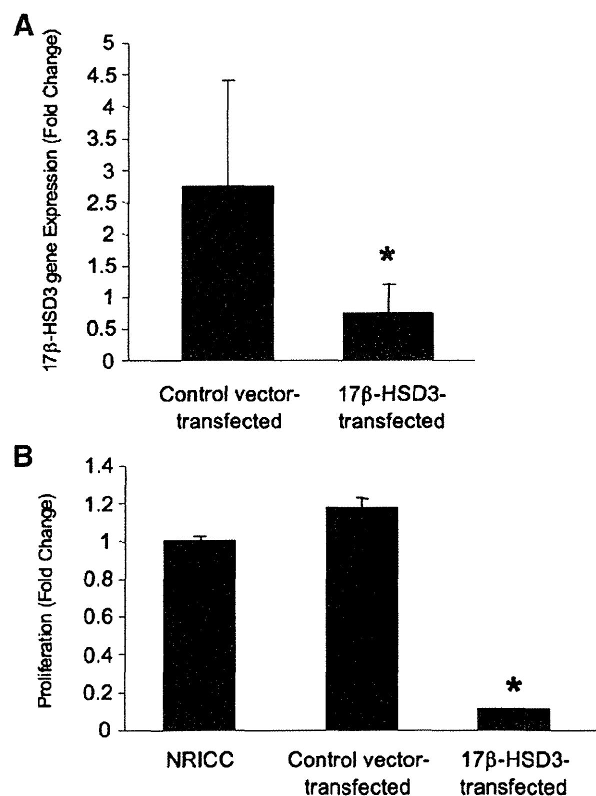


Fig. 6. *A* and *B*: effect of knock down of 17β-HSD3 on the basal proliferative activity of NRICC by MTS assays. Knock down of 17β-HSD3 (~80%) in NRICC (*A*) decreased the proliferative activity of NRICC (*B*), supporting the concept that testosterone is an autocrine factor sustaining biliary growth. Data are means ± SE of 6 experiments. $*P < 0.05$ vs. the corresponding basal value.

shown that: *1*) intrahepatic cholangiocytes and NRICC express 17β-HSD3 (the key enzyme regulating testosterone synthesis) (20) and secrete testosterone; *2*) the biliary expression of 17β-HSD3 and secretion of testosterone increases after BDL and in NRICC after in vitro treatment with testosterone; and *3*) stable transfection of 17β-HSD3 in NRICC decreases the proliferative activity of these cells. The finding that testosterone stimulates biliary proliferation by an autocrine mechanism may be an important compensatory mechanism for sustaining biliary growth and ductal secretory activity in ductopenic pathologies of the biliary epithelium.

Cholangiopathies, which specifically target cholangiocytes, are characterized by dysregulation of the balance between biliary growth/damage (5). Thus, studies aimed to understand the intracellular mechanisms of this phenomenon are necessary. In this context, the cholestatic BDL rodent model has been used widely to pinpoint the mechanisms of biliary growth/apoptosis (2, 3, 6, 18, 19, 22, 34, 49). A number of studies have demonstrated that *1*) gastrointestinal and sex hormones, bioagenic amines, and neuropeptides regulate the growth of bile ducts and *2*) in the course of chronic cholestasis, cholangiocytes acquire neuroendocrine phenotypes regulating biliary functions by both autocrine and paracrine pathways (2, 3, 5–8, 18, 19, 22, 34, 49). Our study provides the first evidence that ARs are expressed by cholangiocytes and testosterone stimulates biliary growth and secretion during cholestasis. We propose that testosterone is important in sustaining biliary proliferation and ductal secretory activity in pathological conditions associated with functional damage of the biliary epithelium.

We first demonstrated in male rats the expression of functional testosterone receptors in bile ducts, cholangiocytes, hepatocytes, and NRICC. Supporting our findings, a number of studies have shown the presence of functional ARs in liver cells, including hepatocytes and bile ducts from PBC patients (26, 29, 38). Also, the AR mRNA has been detected in human liver biopsy samples, fetal liver, and HepG2 cells (48). In the next sets of experiments, we demonstrated that enhanced testosterone serum levels correlate with increased IBDM in male rats and decreased testosterone serum levels are associated with reduction of the number of intrahepatic bile ducts in male rats. We first validated our models, demonstrating that testosterone serum levels decreased in cholestatic BDL male rats (compared with normal rats) and in castrated rats and increased in rats chronically treated with testosterone. The decrease in testosterone serum levels is likely due to hypotrophy of seminal vesicles as observed in rats during puberty and BDL-induced cholestasis (33, 54, 57). Testicular atrophy has been observed in cirrhotic patients (52), whereas lower serum testosterone levels have been shown in patients with PBC (14). Contrasting results exist regarding serum testosterone levels in other liver diseases. For example, while a study has demonstrated lowered serum testosterone levels in cirrhotic rats (53), other studies have shown elevated serum testosterone may promote the development of hepatocellular carcinoma in cirrhosis (50). The decrease in serum testosterone levels observed in our animals is supported by a number of studies in rodents (31, 55) in which the decrease is prevented by the chronic administration of testosterone (55). Similar to our finding, other studies have demonstrated that chronic administration of testosterone increased testosterone serum levels (9). A number of studies have shown that sex hormones, including estrogens, prolactin, follicle-stimulating hormone, and progesterone, sustain biliary proliferation by both paracrine and autocrine mechanisms (6, 7, 23, 39, 49). However, no information exists regarding the role of testosterone in modulating the balance between cholangiocyte growth/loss. The fact that testosterone increases biliary hyperplasia and prevents the loss of biliary growth and function (following castration) supports the concept that androgens can be important for ameliorating the cholestatic conditions associated with testicular hypotrophy and ductopenic conditions associated with decreased testosterone levels as occurs in PBC (14). These findings and the fact that the administration of neutralizing antitestosterone antibody reduces testosterone serum levels and biliary hyperplasia introduce the concept that the administration of testosterone receptor antagonists or antitestosterone antibodies may be new therapeutic approaches for decreasing the aberrant growth of cholangiocytes. At the functional level, we demonstrated that the decrease in testosterone serum levels was associated with ablation of secretin-stimulated cAMP synthesis in cholangiocytes and bicarbonate-rich choleresis in bile fistula male rats, two functional parameters that were restored by the chronic administration of testosterone. Indeed, enhanced secretin receptor expression and secretin-stimulated ductal secretory activity is associated with enhanced biliary hyperplasia (2, 3, 22), whereas reduced secretory capacity in response to secretin is an index of functional cholangiocyte damage (34, 36).

We next performed experiments in male rats aimed at demonstrating that: *1*) purified cholangiocytes and NRICC express 17β-HSD3 (the key enzyme regulating testosterone synthesis) (20) and secrete testosterone and *2*) testosterone stimulates in vitro the growth of NRICC by directly interacting with ARs on cholangiocytes by an autocrine mechanism stimulating the expression of 17β-HSD3. The higher expression of 17β-HSD3 and the enhanced secretion of testosterone by purified cholangiocytes from BDL rats and BDL rats treated in vivo with testosterone (that proliferate at higher rates) support the concept that testosterone (secreted by cholangiocytes) is a trophic autocrine hormone for sustaining biliary hyperplasia. The concept that testosterone stimulates biliary growth by an autocrine fashion is also supported by the fact that testosterone increases the mRNA expression of 17β-HSD3 in vitro in NRICC and flutamide (a specific antagonist of AR) and antitestosterone antibody inhibit the growth of NRICC in vitro. Conclusive evidence that testosterone is an important autocrine trophic factor sustaining biliary growth is also supported by the finding that, in conditions of lowered serum testosterone levels (after castration and administration of neutralizing antitestosterone antibody to BDL rats), there is enhanced testosterone secretion likely by a compensatory mechanism. The possible role of testosterone as a key autocrine trophic was also supported by knock down of 17β-HSD3, which caused a marked decreased in NRICC proliferation. In support of our findings, a number of studies demonstrate the presence of 17β-HSD3 in the liver (41) and show that this enzyme isoform modulates the stimulatory effects on the mitosis of a number of cells, including cholangiocytes. This idea is supported by recent studies showing that specific inhibitors of 17β-HSD3 have been shown to inhibit hormone-dependent prostate cancer and benign prostate hyperplasia (12). The novel concept that cholangiocytes are secretory cells synthesizing a number of factors (including

testosterone) regulating the homeostasis of the biliary epithelium is supported by a number of studies. For example, cholangiocytes synthesize a number of neuroendocrine factors, such progesterone, prolactin, vascular endothelial growth factor, and nerve growth factor, that stimulate biliary growth (19, 21, 23, 49). Also, serotonin inhibits biliary hyperplasia in BDL rats, since cholangiocytes secrete serotonin, the blockage of which enhances cholangiocyte proliferation in the course of cholestasis (42).

In summary, we have demonstrated the presence of functional ARs on cholangiocytes and that testosterone stimulates biliary growth by an autocrine mechanism by increasing the expression of 17β-HSD3 in cholangiocytes. We have also demonstrated that testosterone prevents the decrease in biliary hyperplasia typical of BDL after castration and the administration of neutralizing antitestosterone antibody. We propose that drug targeting of 17β-HSD3 may be important to regulate the balance between biliary proliferation/loss and ductal secretory activity in cholangiopathies.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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Melatonin inhibits cholangiocyte hyperplasia in cholestatic rats by interaction with MT1 but not MT2 melatonin receptors

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Renzi A, Glaser S, DeMorrow S, Mancinelli R, Meng F, Franchitto A, Venter J, White M, Francis H, Han Y, Alvaro D, Gaudio E, Carpino G, Ueno Y, Onori P, Alpini G. Melatonin inhibits cholangiocyte hyperplasia in cholestatic rats by interaction with MT1 but not MT2 melatonin receptors. *Am J Physiol Gastrointest Liver Physiol* 301: G634–G643, 2011. First published July 21, 2011; doi:10.1152/ajpgi.00206.2011.—In bile duct-ligated (BDL) rats, large cholangiocytes proliferate by activation of cAMP-dependent signaling. Melatonin, which is secreted from pineal gland as well as extrapineal tissues, regulates cell mitosis by interacting with melatonin receptors (MT1 and MT2) modulating cAMP and clock genes. In the liver, melatonin suppresses oxidative damage and ameliorates fibrosis. No information exists regarding the role of melatonin in the regulation of biliary hyperplasia. We evaluated the mechanisms of action by which melatonin regulates the growth of cholangiocytes. In normal and BDL rats, we determined the hepatic distribution of MT1, MT2, and the clock genes, CLOCK, BMAL1, CRY1, and PER1. Normal and BDL (immediately after BDL) rats were treated in vivo with melatonin before evaluating *1*) serum levels of melatonin, bilirubin, and transaminases; *2*) intrahepatic bile duct mass (IBDM) in liver sections; and *3*) the expression of MT1 and MT2, clock genes, and PKA phosphorylation. In vitro, large cholangiocytes were stimulated with melatonin in the absence/presence of luzindole (MT1/MT2 antagonist) and 4-phenyl-2-propionamidotetralin (MT2 antagonist) before evaluating cell proliferation, cAMP levels, and PKA phosphorylation. Cholangiocytes express MT1 and MT2, CLOCK, BMAL1, CRY1, and PER1 that were all upregulated following BDL. Administration of melatonin to BDL rats decreased IBDM, serum bilirubin and transaminases levels, the expression of all clock genes, cAMP levels, and PKA phosphorylation in cholangiocytes. In vitro, melatonin decreased the proliferation, cAMP levels, and PKA phosphorylation, decreases that were blocked by luzindole. Melatonin may be important in the management of biliary hyperplasia in human cholangiopathies.

cAMP; cholestasis; mitosis; PKA; secretin

CHOLANGIOCYTES ARE THE TARGET CELLS in human cholangiopathies, including primary biliary cirrhosis and primary sclerosing cholangitis (5), and animal models of cholestasis such as bile duct ligation (BDL) and acute administration of carbon tetrachloride (CCl_4) (4, 40). These pathologies are characterized by cholangiocyte hyperplasia/damage (5) that is restricted to bile ducts of certain sizes (3, 40). In BDL rats only large cholangiocytes (lining large ducts) (2, 6, 23) proliferate (leading to enhanced large intrahepatic bile ductal mass, IBDM) (3, 40) by the activation of cAMP→PKA signaling (3, 24, 40). Studies (4, 24, 40, 42) have demonstrated the key role of secretin and its receptor (SR, only expressed by cholangiocytes in rodent liver) (2, 8, 24) in the regulation and functional evaluation of biliary hyperplasia/damage. Following BDL the enhanced biliary hyperplasia is associated with increased SR expression and secretin-stimulated cAMP levels and bile secretion (3, 4, 7, 8, 40, 42). Conversely, during damage of cholangiocytes, there is reduced SR expression and functional response to secretin (40, 41). A number of neuroendocrine factors and neuropeptides such as secretin, sex hormones, vascular endothelial growth factor, α-calcitonin gene-related peptide, serotonin, cholinergic and adrenergic receptor agonists, and biogenic amines have been shown to exert inhibitory and/or stimulatory effects on biliary growth in normal and cholestatic conditions (3, 5, 9, 20–22, 24, 39, 42).

Melatonin is an indole formed enzymatically from L-tryptophan by the activity of the enzymes serotonin N-acetyltransferase (AANAT), and hydroxyindole-0-methyltransferase (32, 37) and is produced predominantly by the pineal gland (53). Extrapineal sites (e.g., the gastrointestinal tract) of melatonin production have been demonstrated (13). Melatonin exerts its effects by interacting with G protein-coupled membrane receptors, such as melatonin 1A receptor (MT1), MT2, and MT3 (found only in nonmammals) (37, 55), modulating intracellular messengers such as cAMP, a key molecule regulating large cholangiocyte functions (40), and $[Ca^{2+}]_i$, an important signaling molecule regulating the function of small cholangiocytes (21). Indeed, melatonin-activation of G protein-coupled receptors inhibits cAMP levels in a number of cells including rat pancreatic β-cells (50).

Melatonin receptors are distributed in the central nervous system (46) as well as in peripheral tissues including small intestine and hepatocytes (45, 51). In the liver, melatonin suppresses oxidative damage, attenuates proliferation, and stimulates apoptosis of hepatocytes in rats subjected to partial hepatectomy (35). Melatonin improves liver fibrosis in rats with BDL (62) and ameliorates BDL-induced systemic oxidative stress in cholestatic rats (19). No information exists re-

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garding the role and mechanisms of action by which melatonin modulates biliary hyperplasia in cholestatic rats. Melatonin regulates cell mitosis by modulation of the circadian rhythm, which is under the control of a core set of clock genes: Period 1, 2, and 3 (PER1–3); Cryptochrome 1 and 2 (CRY1 and CRY2); CLOCK; and BMAL1 and BMAL2 (33). For example, melatonin exerts antiproliferative effects in breast cancer cells by resynchronization of deregulated core clock circuitry (27). The aims of the study were to demonstrate in the BDL rats that *1*) melatonin decreases the proliferation of large cholangiocytes and liver damage and *2*) melatonin inhibition of large biliary hyperplasia is associated with downregulation of cAMP-dependent phosphorylation of PKA and modulation of clock genes.

METHODS AND MATERIALS

Materials. Reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): *1*) mouse monoclonal antibody against rat proliferating cell nuclear antigen (PCNA); *2*) MT1, an affinity-purified goat polyclonal antibody raised against a peptide mapping near the COOH terminus of MT1 of rat origin; *3*) MT2, an affinity-purified goat polyclonal antibody raised against a peptide mapping within an internal region of MT2 of mouse origin; and *4*) the rabbit polyclonal antibodies to the CLOCK transcription factor, the goat polyclonal antibody to BMAL1 transcription factor, CRY1, and PER1 proteins. The mouse anti-cytokeratin-19 (CK-19) antibody was purchased from Caltag Laboratories (Burlingame, CA). The phospho-PKA catalytic subunit antibody was purchased from Cell Signaling (Boston, MA). The RNeasy Mini Kit to purify RNA was purchased from Qiagen (Valencia, CA). The radioimmunoassay (RIA) kits for the determination of cAMP levels were purchased from GE Healthcare (Arlington Heights, IL). 4-Phenyl-2-propionamidotetralin (4-P-PDOT, a specific MT2 antagonist, >300-fold selective for the MT2 vs. the MT1 subtype) (61) and the MT1/MT2 antagonist, luzindole (18), were purchased from Tocris Bioscience, Ellisville, MO.

Animal models. Male 344 Fischer rats (150–175 g) were purchased from Charles River (Wilmington, MA) and kept in a temperature-controlled environment (22°C) with 12-h:12-h light/dark cycles. Animals were fed ad libitum and had free access to food and drinking water. The studies were performed in normal rats and in rats that, immediately after BDL (4) or bile duct incannulation (BDI, for bile collection) (4), had ad libitum access to water or water containing melatonin (20 mg/l corresponding to a melatonin intake of 2 mg/g body wt per day) (10) for 1 wk (Table 1). Melatonin (20 mg) was dissolved in 2.5 ml of ethanol and then diluted at 1 l with water. Control animals received water containing the same amount of ethanol. This estimated intake of melatonin is based on the fact that rats drink ~15 ml of water per day mostly throughout the night (57) when melatonin secretion from the pineal gland is higher (37). The facts that *1*) circulating melatonin is mostly metabolized (4–6 h in rats) (56) and excreted with the bile to small bowel and returns to the liver through enterohepatic circulation (with a minimal quantity excreted through the urine) (37, 65) and that *2*) melatonin serum levels increased in our model (see Table 1) support the validity of our route of melatonin administration. The administration of melatonin by drinking water has been previously used in rodents (10). Before each surgical procedure, animals were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). All animal experiments were performed in accordance with a protocol approved by the Scott and White and Texas A&M HSC IACUC Committee. All animals were used for the harvest of tissues and purification of liver cells at 8:00 AM. In all animals, we measured wet liver weight, body weight, and wet liver weight-to-body weight ratio, an index of cell growth (4).

Table 1. *Liver and body weight, liver-to-body weight ratio, and serum levels of melatonin, transaminases, and bilirubin in selected animal groups*

Groups	Liver Weight, g	Body Weight, g	Liver-to-Body Weight Ratio	Melatonin serum, pg/ml	SGPT, U/l	SGOT, U/l	Total Bilirubin, mg/l
Normal rats + tap water	7.98 ± 0.7 (*n* = 5)	190.4 ± 3.2 (*n* = 5)	4.2 ± 0.4 (*n* = 5)	45.5 ± 12.7 (*n* = 7)	83.2 ± 14.8 (*n* = 7)	194.5 ± 42.0 (*n* = 6)	<0.1 (*n* = 8)
Normal rats + tap water containing melatonin	9.4 ± 0.4 (*n* = 5)	206.6 ± 3.9 (*n* = 5)	4.5 ± 0.2 (*n* = 5)	140.2 ± 12.7 (*n* = 7)	69.4 ± 45.9 (*n* = 7)	143.0 ± 36.0 (*n* = 6)	<0.1 (*n* = 8)
BDL rats + tap water	8.3 ± 0.2 (*n* = 15)	145.6 ± 3.9 (*n* = 15)	5.7 ± 0.1 (*n* = 15)	117.6 ± 38.5 (*n* = 7)	390.7 ± 107.0 (*n* = 6)	1415.0 ± 325.8 (*n* = 6)	12.4 ± 1.5 (*n* = 8)
BDL rats + tap water containing melatonin	6.7 ± 0.1 (*n* = 15)	150.1 ± 7.9 (*n* = 15)	4.5 ± 0.1 (*n* = 15)	164.7 ± 66.8 (*n* = 7)	210.8 ± 45.9 (*n* = 6)	585.0 ± 162.4 (*n* = 6)	7.3 ± 1.9 (*n* = 8)

Values are means ± SE. BDL, bile duct ligation; SGOT, serum glutamic oxaloacetic transaminases; SGPT, serum glutamate pyruvate transaminases.

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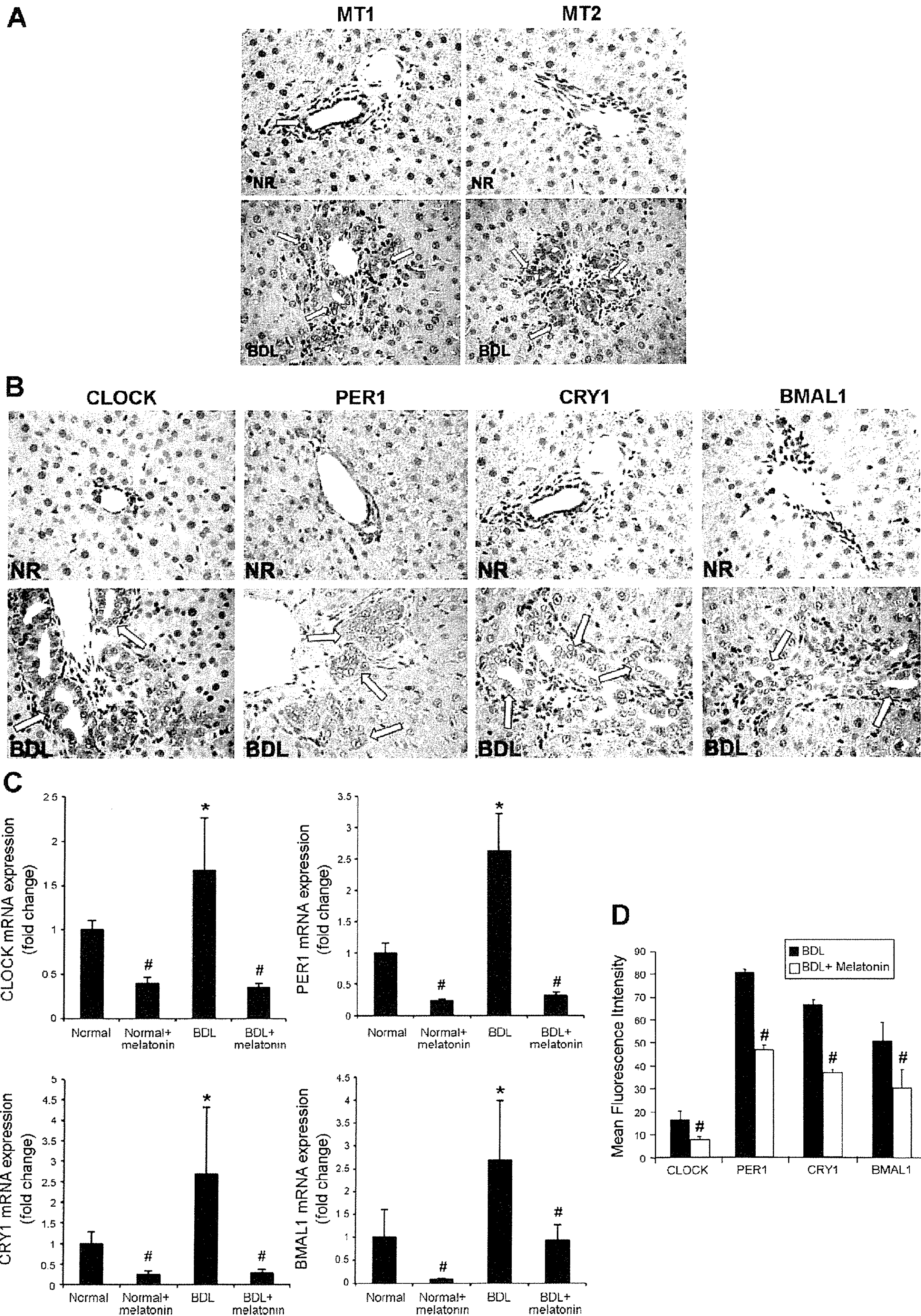

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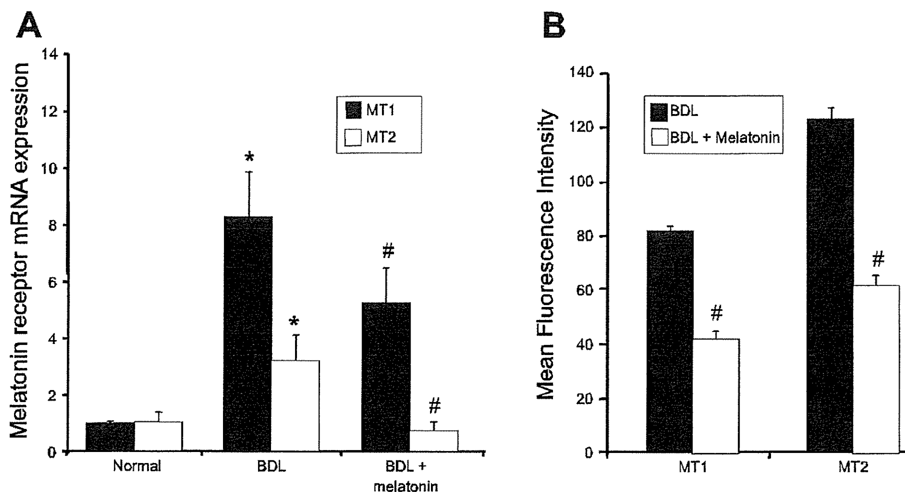

Fig. 2. *A*: by real-time PCR, the mRNA expression of MT1 and MT2 increased in BDL compared with normal cholangiocytes but decreased in purified cholangiocytes from BDL rats treated with melatonin in vivo compared with cholangiocytes from control BDL rats. Data are means ± SE of 3 evaluations. *$P < 0.05$ vs. the values of normal cholangiocytes. #$P < 0.05$ vs. the values of BDL cholangiocytes from rats treated with regular tap water. *B*: by FACS analysis the protein expression of MT1 and MT2 decreased in cholangiocytes from BDL rats treated with melatonin in vivo compared with cholangiocytes from BDL rats treated with vehicle. Data are means ± SE of 3 evaluations. #$P < 0.05$ vs. the values of BDL cholangiocytes from rats treated with regular tap water.

Expression of MT1 and MT2, CLOCK, BMAL1, CRY1, and PER1 in liver sections and purified cholangiocytes. We evaluated the expression of MT1 and MT2 and CLOCK, BMAL1, CRY1, and PER1 in liver sections (4–5 μm thick) from normal and BDL rats by immunohistochemistry (42) and in total RNA (0.5 μg) by real-time PCR (21) from purified cholangiocytes.

Immunohistochemical observations were taken in a coded fashion by BX-51 light microscopy (Olympus, Tokyo, Japan) with a Videocam (Spot Insight; Diagnostic Instrument, Sterling Heights, MI) and analyzed with an Image Analysis System (Delta Sistemi, Rome, Italy). For all immunoreactions, negative controls (with normal serum from the same species substituted for the primary antibody) were included. To evaluate the expression of the messages for MT1 and MT2, CLOCK, BMAL1, CRY1, and PER1, we used the RT^2 Real-Time assay from SABiosciences (Frederick, MD) (21). A $\Delta\Delta C_T$ analysis was performed (21) using normal cholangiocytes as control. Data were expressed as relative mRNA levels ± SE of the selected gene-to-GAPDH ratio. The primers for melatonin receptors 1A and 1B, CLOCK, BMAL1, CRY1, and PER1 (SABiosciences) were designed according to the NCBI GenBank Accession numbers: XM_341441 (MT1), NM_053330 (MT2), NM_021856 (CLOCK gene), NM_024362 (BMAL1 gene), NM_198750 (CRY1), and NM_001034125 (PER1).

We evaluated by fluorescence-activated cell sorting (FACS) analysis (49) the expression of MT1 and MT2, CLOCK, BMAL1, CRY1, and PER1 in purified large cholangiocytes. FACS analysis was performed using a C6 flow cytometer and analyzed by CFlow Software (Accuri Cytometers, Ann Arbor, MI) (49). The expression of the selected protein was identified and gated on FL1-A/Count plots. The relative quantity of the selected protein (mean selected protein fluorescence) was expressed as mean FL1-A (samples)/mean FL-1A (secondary antibodies only).

Isolated and immortalized large cholangiocytes. The isolation of cholangiocytes started each morning at 8:00 AM. Pure (100% by γ-glutamyltransferase histochemistry) (58) cholangiocytes were isolated by immunoaffinity separation (7) using a monoclonal antibody (from Dr. R. Faris, Brown University, Providence, RI). The rationale for performing these studies in large cholangiocytes is based on the fact that, following BDL, only these cells undergo mitosis (3, 23, 40). The in vitro studies were performed in immortalized large cholangiocytes (from large bile ducts) (63) displaying phenotypes similar to that of freshly isolated cholangiocytes (3, 21, 24, 63).

Evaluation of serum levels of melatonin, transaminases, and bilirubin and cholangiocyte proliferation and apoptosis. The serum levels of the transaminases, glutamate pyruvate transaminases, and glutamic oxaloacetic transaminase and total bilirubin were evaluated using a Dimension RxL Max Integrated Chemistry system (Dade Behring, Deerfield, IL) by the Chemistry Department, Scott & White. Serum levels of melatonin were measured by commercially available ELISA kits (Genway, San Diego, CA).

We evaluated in liver sections (4–5 μm thick) *1)* the percentage of cholangiocyte proliferation by semiquantitative immunolocalization for PCNA (41), *2)* IBDM of cholangiocytes (41), and *3)* the percentage of cholangiocyte apoptosis by semiquantitative terminal deoxynucleotidyltransferase biotin-dUTP nick-end labeling (TUNEL) kit (Apoptag; Chemicon International, Temecula, CA) (24). We evaluated by hematoxylin and eosin of sections whether melatonin administration induces the damage of kidney, heart, stomach, spleen, and small and large intestine. Sections were evaluated in a blinded fashion by a BX-51 light microscope (Olympus, Tokyo, Japan).

Measurement of PCNA expression and phosphorylation of PKA. We evaluated by immunoblots (21) PCNA protein expression (21) and the phosphorylation of PKA in protein (10 μg) from spleen (positive) and large cholangiocytes from melatonin- or vehicle-treated BDL rats. The intensity of the bands was determined by scanning video densitometry using the phospho-imager Storm 860 and the ImageQuant TL software version 2003.02 (GE Healthcare).

Measurement of secretin-stimulated cAMP levels and bile and bicarbonate secretion. We evaluated the effect of secretin on cAMP levels in large cholangiocytes and bile and bicarbonate secretion in

Fig. 1. *A*: by immunohistochemistry in liver sections, bile ducts (yellow arrow) from normal rats (NR) were weakly positive for melatonin 1A receptor (MT1) and did not stain for MT2; bile ducts (yellow arrows) from bile duct-ligated (BDL) rats showed immunoreactivity for both MT1 and MT2. Original magnification ×20. *B*: expression of CLOCK and BMAL1 was virtually absent in normal bile ducts; immunoreactivity was observed in BDL bile ducts (yellow arrows). Normal bile ducts stained positively for PER1 and CRY1, whose expression increased in bile ducts from BDL rats. Original magnification ×20. *C*: by real-time PCR, the mRNA expression of PER1, BMAL1, CRY1, and CLOCK increased in BDL compared with normal cholangiocytes but decreased in cholangiocytes from normal and BDL rats treated with melatonin in vivo compared with control cholangiocytes. *D*: by fluorescence-activated cell sorting (FACS) analysis, the protein expression of PER1, BMAL1, CRY1, and CLOCK decreased in cholangiocytes from BDL rats treated with melatonin in vivo compared with cholangiocytes from BDL rats treated with vehicle. Data are means ± SE of 3 evaluations. *$P < 0.05$ vs. the values of normal cholangiocytes. #$P < 0.05$ vs. the values of normal and BDL cholangiocytes from rats treated with regular tap water.

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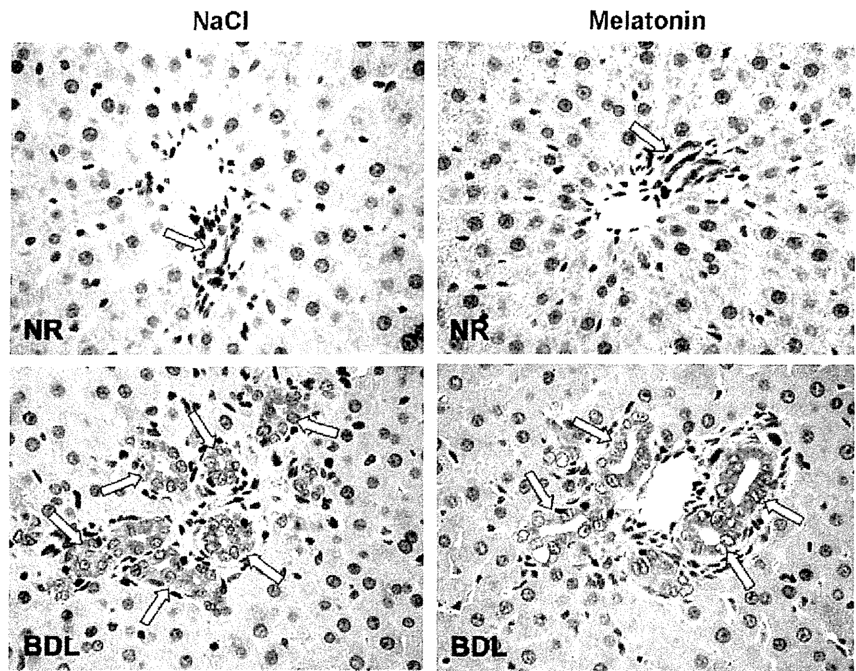

Fig. 3. Effect of melatonin on large intrahepatic bile duct mass (IBDM) of BDL rats, immunoreactivity for CK19. The administration of melatonin to BDL rats decreased IBDM compared with their corresponding controls rats (for semiquantitative data see Table 2) (yellow arrows: bile ducts) Original magnification, ×20.

bile fistula rats. Following isolation, cholangiocytes (1×10^5 cells) were incubated for 1 h at 37°C (31) before stimulation with 0.2% bovine serum albumin or secretin (100 nM) for 5 min at room temperature before evaluation of cAMP levels by RIA (3, 40). After anesthesia, rats were surgically prepared for bile collection (4). When 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) (39, 40) for 30 min followed by intravenous infusion of KRH for 30 min. Bicarbonate levels in bile were determined by a COBAS Mira Plus automated clinical chemistry analyzer (Bohemia, NY) (47).

In vitro effect of melatonin on the proliferation of large cholangiocytes. We evaluated by immunofluorescence (21) and immunoblots (20) the expression of MT1 and MT2 receptors in immortalized large cholangiocytes. Images were visualized using an Olympus IX-71 confocal microscope. For all immunoreactions, negative controls were included. The intensity of the bands was determined by scanning video densitometry (see above).

We evaluated by RIA kits (3, 34, 40) cAMP levels in cholangiocytes treated with vehicle (basal) or melatonin (10^{-11} M for 5 min) in the absence/presence of preincubation with 4-P-PDOT or luzindole (both 10 μM) (18, 61). The rationale for using this dose (10^{-11} M) for melatonin is based on the finding that serum levels of melatonin in rodents and humans are on the picomolar to nanomolar ranges (12, 66). After trypsinization, cholangiocytes were treated at 37°C for 48 h with vehicle (DMSO diluted with 1× PBS), melatonin (10^{-11} M) in the absence/presence of preincubation with 4-P-PDOT or luzindole (10 μM) (18, 61), 4-P-PDOT or luzindole alone (10 μM) before evaluating by immunoblots (21) the expression of PCNA and PKA phosphorylation. Melatonin was first dissolved in DMSO and then diluted with 1× PBS at the stock solution of 2.5×10^{-2} M before being diluted to the desired working solution with 1× PBS.

Statistical analysis. All data are expressed as means ± SE. Differences between groups were analyzed by Student's unpaired *t*-test when two groups were analyzed and ANOVA when more than two groups were analyzed, followed by an appropriate post hoc test.

Table 2. *Percentage of PCNA- and TUNEL-positive cholangiocytes and IBDM*

Groups	PCNA-Positive Cholangiocytes, %	IBDM, %	Cholangiocytes Positive by TUNEL, %
Normal rats + tap water	7.919 ± 0.338	0.28 ± 0.03	Negative
Normal rats + tap water containing melatonin	6.740 ± 0.303	0.24 ± 0.03	Negative
BDL rats + tap water	47.436 ± 1.227	3.657 ± 0.302	5.514 ± 0.142
BDL rats + tap water containing melatonin	41.501 ± 0.973*	2.036 ± 0.100*	7.146 ± 0.163*

Data are mean ± SE. *$P < 0.01$ vs. the corresponding values of BDL rats. IBDM, intrahepatic bile duct mass; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling.

RESULTS

Cholangiocytes express MT1 and MT2, CLOCK, BMAL1, CRY1, and PER1. By immunohistochemistry in liver sections, normal bile ducts were weakly positive for MT1 (but not MT2) but showed strong immunoreactivity for MT1 and MT2 following BDL (Fig. 1*A*). The expression of CLOCK and BMAL1 was absent in normal bile ducts; however, immunoreactivity was observed in BDL bile ducts (Fig. 1*B*). Normal bile ducts stained positively for PER1 and CRY1, whose expression slightly increased in bile ducts from BDL rats (Fig. 1*B*). By real-time PCR and FACS analysis (Fig. 1, *C* and *D*), the expression of PER1, BMAL1, CRY1, and CLOCK increased in BDL compared with normal cholangiocytes but decreased in cholangiocytes from normal and BDL rats treated in vivo with melatonin compared with control cholangiocytes. The mRNA expression of MT1 and MT2 increased in BDL compared with normal cholangiocytes (Fig. 2*A*). By real-time PCR and FACS analysis, the expression of MT1 and MT2 decreased in cholangiocytes from BDL rats treated with melatonin in vivo compared with cholangiocytes from control BDL (Fig. 2, *A* and *B*).

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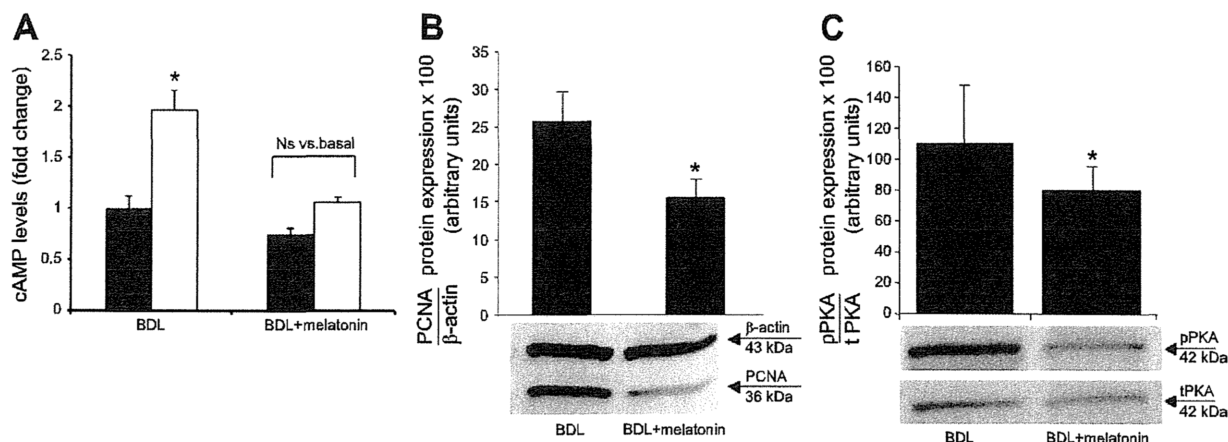

Fig. 4. *A*: effect of secretin on cAMP levels in large cholangiocytes from BDL rats treated with regular tap water or melatonin in drinking water for 1 wk. Secretin increased the intracellular cAMP levels of large cholangiocytes from BDL rats. Secretin did not enhance the intracellular levels of cAMP of large cholangiocytes from melatonin-treated rats. Data are means ± SE of 6 evaluations from cumulative preparations of cholangiocytes. $*P < 0.05$ vs. the corresponding basal values of large cholangiocytes from BDL controls. *B–C*: effect of melatonin on the expression of proliferating cell nuclear antigen (PCNA) (*B*) and the phosphorylation of PKA (*C*) in purified large cholangiocytes. By immunoblots, there was decreased PCNA expression and PKA phosphorylation in large cholangiocytes from melatonin-treated BDL rats compared with large cholangiocytes from BDL controls. Data are means ± SE of 4 blots from cumulative preparations of cholangiocytes. $*P < 0.05$ vs. the corresponding values of large cholangiocytes from BDL controls.

Evaluation of serum levels of melatonin and transaminases, and bilirubin levels, cholangiocyte proliferation, and apoptosis. There was a 15–20% decrease of body weight in BDL compared with normal rats (Table 1). No difference was observed in body weight between normal and BDL rats treated with melatonin compared with controls (Table 1). There was a decrease in liver-to-body weight ratio in BDL rats treated with melatonin compared with BDL controls (Table 1). The serum levels of melatonin of normal rats were similar to that of previous studies and increased following BDL (64) and after the administration of melatonin to normal and BDL rats (Table 1). The serum levels of transaminases increased in BDL rats compared with normal rats and were decreased in both normal and BDL rats by the administration of melatonin (Table 1). The administration of melatonin to BDL rats decreased large BDM (Fig. 3 and Table 2) and the percentage of PCNA-positive cholangiocytes (Table 2) compared with control rats. Melatonin inhibition of biliary hyperplasia in BDL rats was associated with enhanced cholangiocyte apoptosis (Table 2). Melatonin had no effects in normal rats (Table 2). No morphological changes of kidney, heart, stomach, spleen, and small and large intestine were observed in rats treated with melatonin (not shown).

Effect of secretin on cAMP levels in cholangiocytes and bile and bicarbonate secretion in bile fistula rats. In BDL rats treated with melatonin, basal levels of cAMP and basal bile and bicarbonate secretion were lower than those of the corresponding values of BDL control rats (Fig. 4*A* and Table 3). As expected (7), secretin increased cAMP levels of large cholangiocytes from BDL rats but did not enhance the levels of cAMP of large cholangiocytes from BDL rats treated with melatonin for 1 wk (Fig. 4*A*). Secretin increased bile and bicarbonate secretion of BDL controls but not of melatonin-treated BDL rats (Table 3).

Effect of melatonin on the expression of PCNA and phosphorylation of PKA in large cholangiocytes. There was decreased PCNA expression in large cholangiocytes from BDL rats treated with melatonin compared with cholangiocytes from BDL control rats (Fig. 4*B*). We found decreased phosphorylation of PKA in large cholangiocytes from melatonin-treated BDL rats compared with large cholangiocytes from BDL controls (Fig. 4*C*).

Effect of melatonin on the proliferation of large cholangiocytes. By immunofluorescence and immunoblots, large cholangiocytes express both MT1 and MT2 (Fig. 5, *left* and *right*). Melatonin decreased cAMP levels, a decrease that was prevented by luzindole (a MT1/MT2 antagonist) (18) but not 4-P-PDOT (a specific MT2 antagonist) (61) (Fig. 6*A*), demonstrating that melatonin inhibitory effects on cholangiocyte growth are mediated by MT1. Melatonin decreased gene and protein expression for PCNA and the phosphorylation of PKA, decreases that were prevented by luzindole but not 4-P-PDOT (Fig. 6, *B–D*).

Table 3. *Measurement of basal and secretin-stimulated bile flow and bicarbonate secretion in rats*

	Bile Flow		Bicarbonate Secretion	
Treatment	Basal, $\mu l \cdot min^{-1} \cdot kg$ body wt^{-1}	Secretin, $\mu l \cdot min^{-1} \cdot kg$ body wt^{-1}	Basal, $\mu l \cdot min^{-1} \cdot kg$ body wt^{-1}	Secretin, $\mu l \cdot min^{-1} \cdot kg$ body wt^{-1}
BDI rats + tap water (n = 4)	124.6 ± 11.9	226.7 ± 28.2†	4.0 ± 0.3	11.0 ± 1.4†
BDI rats + tap water containing melatonin (n = 4)	90.1 ± 11.9*	94.3 ± 11.6‡	2.5 ± 0.4*	3.4 ± 0.4‡

Values are means ± SE. Differences between groups were analyzed by the Student's unpaired t-test when 2 groups were analyzed and ANOVA when more than 2 groups were analyzed. $*P < 0.05$ vs. basal values of bile flow, bicarbonate secretion of bile duct incannulation (BDI) treated with vehicle for 1 wk. $†P < 0.05$ vs. corresponding basal value of bile flow or bicarbonate secretion of BDI control rats. ‡Nonsignificant vs. corresponding basal value of bile flow, bicarbonate concentration, or bicarbonate secretion of BDI rats that had ad libitum access to regular tap water for 1 wk.

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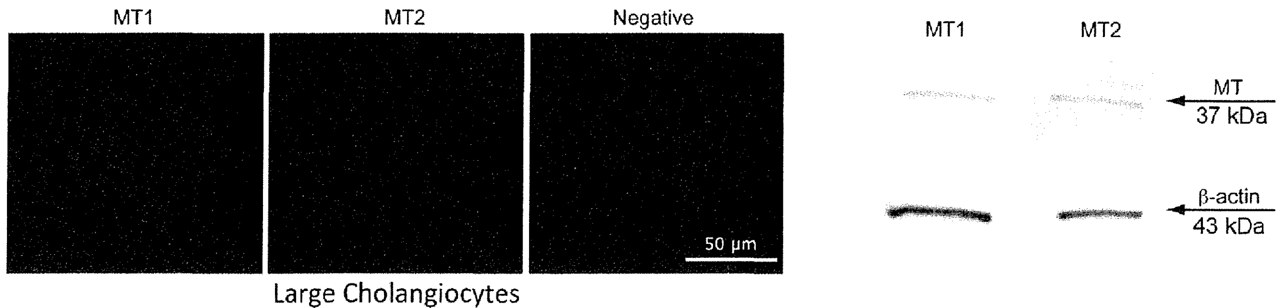

Fig. 5. Evaluation of MT1 and MT2 expression by immunofluorescence in cell smears (*left*) and immunoblots in protein of large cholangiocytes (*right*). We demonstrated that large cholangiocytes express both MT1 and MT2. Bar = 50 μm. Specific receptor immunoreactivity is depicted in red, whereas cells were counterstained with DAPI (blue).

Neither luzindole nor 4-P-PDOT alters cholangiocyte proliferation (not shown). By FACS analysis, in vitro melatonin decreased the expression of PER1, BMAL1, CRY1, and CLOCK (Fig. 7*A*) and MT1 and MT2 (Fig. 7*B*) compared with their corresponding basal value.

DISCUSSION

Our study demonstrated *1*) that freshly isolated and lines of large cholangiocytes express MT1 and MT2, CLOCK, BMAL1, CRY1, and PER1 and *2*) that in vivo administration of melatonin to BDL rats reduces the serum levels of transaminases and bilirubin and inhibits cholangiocyte proliferation and IBDM typical of BDL (4). The antiproliferative effects of melatonin on biliary growth were associated with decreased basal cAMP levels and spontaneous bile and bicarbonate secretion as well as with loss of responsiveness to secretin and enhanced biliary apoptosis. The biliary expression of MT1 and MT2 and clock genes increased in BDL compared with normal cholangiocytes and decreased in cholangiocytes from normal and BDL rats treated with melatonin. There was decreased phosphorylation of PKA in cholangiocytes. In vitro melatonin decreased proliferation, cAMP levels, and PKA phosphorylation in large cholangiocytes, decreases that were mediated by MT1 receptors.

As it mimics typical features of human cholangiopathies (5), the BDL model is commonly used for evaluating the mechanisms of biliary growth/damage (3, 4, 24). Cholangiopathies share common pathological characteristics such as the damage of cholangiocytes and the proliferation of residual ducts (as a mechanism of compensatory repair to maintain the homeostasis of the biliary tree) (9), but they evolve toward ductopenia that represents the terminal stage of these diseases (9).

We provided the first evidence for the presence of functional melatonin receptors in the biliary epithelium. A recent study

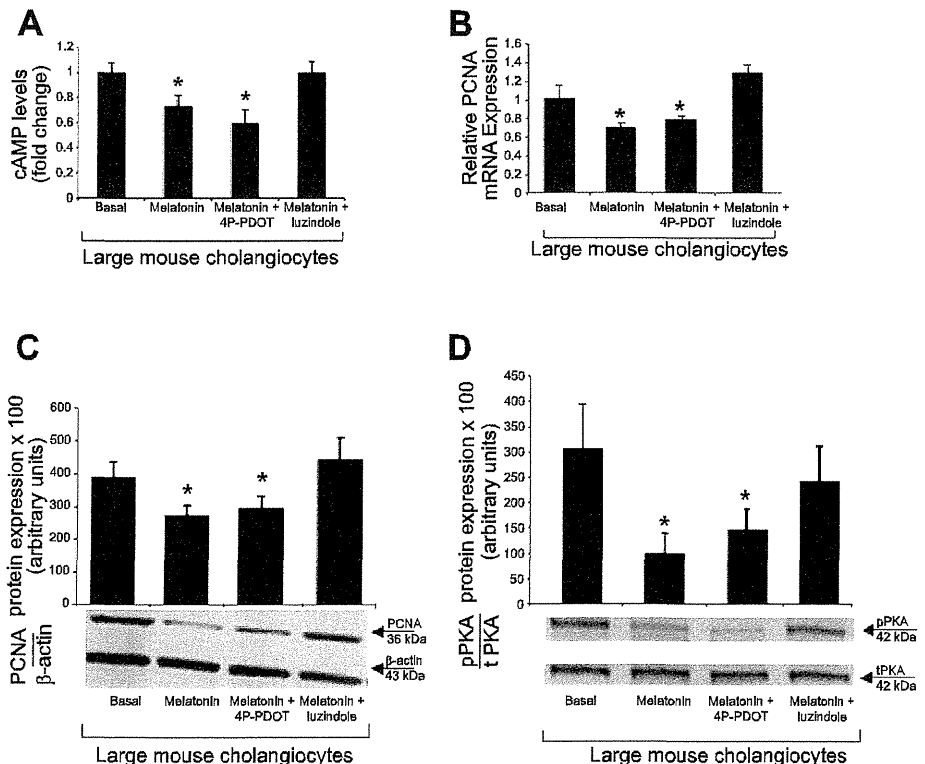

Fig. 6. Effect of melatonin on cAMP levels (*A*), expression of mRNA PCNA (*B*), PCNA protein expression (*C*), and PKA phosphorylation (*D*) of large cholangiocyte lines. Melatonin decreased cAMP levels, a decrease that was prevented by luzindole but not 4-phenyl-2-propionamidotetralin (4-P-PDOT). Melatonin decreased PCNA protein expression and the phosphorylation of PKA, decreases that were prevented by luzindole but not 4-P-PDOT. Data are means ± SE of 6 evaluations from cumulative preparations of cholangiocytes. *$P < 0.05$ vs. the corresponding basal values of large cholangiocyte lines.

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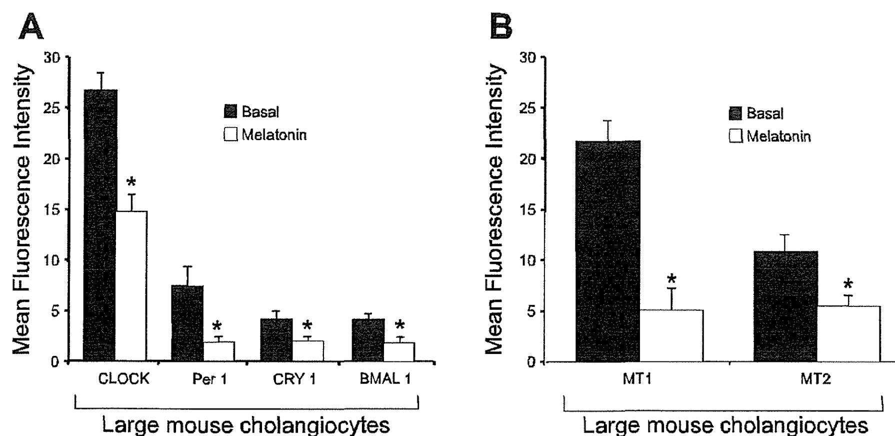

Fig. 7. *A*: by FACS analysis the protein expression of PER1, BMAL1, CRY1, and CLOCK decreased in large mouse cholangiocytes treated with melatonin compared with basal large mouse cholangiocyte cell lines. Data are means ± SE of 3 evaluations. $*P < 0.05$ vs. the corresponding basal values of large cholangiocyte lines. *B*: by FACS analysis the protein expression of MT1 and MT2 decreased in large mouse cholangiocyte lines after melatonin treatment compared with basal large mouse cholangiocyte lines. Data are means ± SE of 3 evaluations. $*P < 0.05$ vs. the corresponding basal values of large cholangiocyte lines.

showing that melatonin attenuates the damage caused by scolicidal agents on bile ducts alluded to the presence of melatonin receptors on cholangiocytes (60). MT1s are expressed by human gallbladder epithelia (11). The reason why these inhibitory melatonin receptors are upregulated in proliferating BDL cholangiocytes may be due to a compensatory mechanism. The decrease in the expression of MT1 and MT2 by melatonin is likely due to desensitization of these receptors as suggested by other studies (36). Another explanation may be due to the increased expression of AANAT (the enzyme regulating melatonin secretion by cholangiocytes, G. Alpini, unpublished observations), an increase that may lead to decreased expression of melatonin receptors. Studies are being undertaken in our laboratory to demonstrate the presence and role of this autocrine loop (AANAT→MT1/MT2) in the autocrine regulation of biliary growth.

The validity of our model was supported by the fact that, following chronic administration of melatonin, the serum levels of this hormone increased compared with BDL control rats. The serum levels of melatonin in normal rats were similar to that of previous studies (30) and, in agreement with previous findings (e.g., in liver cirrhosis) (14), increased following BDL. The finding that administration of melatonin to normal and BDL rats increases its circulating levels is supported by studies in rats (52) and humans (17). A number of studies support the inhibitory effect of melatonin on cell mitosis. For example, melatonin inhibits the hyperplastic growth of gastric mucosal in rats (1). The finding that the in vivo administration of melatonin decreases the serum levels of transaminases and bilirubin (observed in BDL rats) is supported by previous studies (48). This finding suggests that melatonin protects the biliary epithelium from cholestatic injury as supported by the fact that melatonin ameliorates oxidative stress in cholestatic rats (19).

We demonstrated that melatonin inhibition of biliary hyperplasia is associated with downregulation of basal and secretin-stimulated cAMP levels and bile secretion and phosphorylation of PKA, regulators of large cholangiocyte proliferation (3, 40–42). Indeed, activation of cAMP-dependent signaling has been shown to stimulate large cholangiocyte hyperplasia (22, 24). Conversely, downregulation of the cAMP-dependent transduction pathway inhibits BDL-induced biliary hyperplasia (20, 40).

We performed in vitro experiments in large cholangiocytes aimed to demonstrate that melatonin exerts its effects by direct interaction with specific melatonin receptors (MT1) by downregulating cAMP signaling and selected clock genes. The concept that MT1 (but not MT2) is the predominant receptor modulating the inhibitory effects of melatonin on biliary hyperplasia is supported by studies in other cells (43, 50). These findings raise the potential important concept that drug targeting of MT1 may be important in the management of cholangiopathies.

Recent studies have demonstrated the role of circadian rhythm (26) and the key circadian hormone, melatonin, in the pathogenesis of disease states and carcinogenesis (54, 59). Several studies have implicated melatonin in the pathogenesis of liver disease and damage. Melatonin synthesis, release, and resulting circadian rhythms are dysregulated in a number of liver diseases. Abnormal melatonin circadian rhythms are found in patients with hepatic cirrhosis and correlated with the severity of liver insufficiency (64). This melatonin arrhythmia is corrected after liver transplantation (15). In addition, melatonin has been shown to protect against liver damage by attenuating oxidative stress and apoptosis in animal models of hepatic cirrhosis and fibrosis (16, 28, 29). Melatonin regulates the expression of circadian genes, which include Per1 and Per2, Cry1 and Cry2, BMAL1, and CLOCK. Circadian genes are linked with downregulation of cell proliferation and dysregulation of cell-cycle control during carcinogenesis (38). Mice lacking the circadian genes Per1 and Per2 and Cry1 and Cry2 are deficient in cell-cycle regulation, and Per2 mutant mice are cancer prone (38). Studies have shown that clock genes regulate cell mitosis and that melatonin regulates cell proliferation by changes in clock gene expression (27). For example, the circadian clock component BMAL1 is a critical regulator of p21WAF1/CIP1 expression and hepatocyte proliferation (25). The loss of CLOCK activity in $Cry1^{-/-}$/$Cry2^{-/-}$ double mutant mice results in delayed liver regeneration (44). Supporting these studies, we have demonstrated that melatonin inhibition of biliary hyperplasia is associated with downregulation of PER1, CLOCK, BMAL1, and CRY1 expression. These previous findings suggest that circadian genes play a key role in the mechanisms regulating liver cell proliferation, and our current findings suggest that

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clock genes may play an important role in liver pathogenesis. Future studies are necessary to fully elucidate the individual roles of each circadian gene in the proliferative signaling cascade by which melatonin regulates biliary proliferation. Studies aimed to evaluate the expression and function of clock genes in cholangiocytes in vivo over at least one full 24-h cycle are undergoing because melatonin secretion from the pineal gland (37) and the biliary epithelium (G. Alpini unpublished observations) is higher during the night. Our findings have important clinical implications because melatonin (an over-the-counter drug used for curing sleep disorders) may be an important therapeutic tool for managing the cholangiocyte hyperplasia in biliary disorders.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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Activation of Alpha$_1$-Adrenergic Receptors Stimulate the Growth of Small Mouse Cholangiocytes Via Calcium-Dependent Activation of Nuclear Factor of Activated T Cells 2 and Specificity Protein 1

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Small cholangiocytes proliferate via activation of calcium (Ca^{2+})-dependent signaling in response to pathological conditions that trigger the damage of large cyclic adenosine monophosphate–dependent cholangiocytes. Although our previous studies suggest that small cholangiocyte proliferation is regulated by the activation of Ca^{2+}-dependent signaling, the intracellular mechanisms regulating small cholangiocyte proliferation are undefined. Therefore, we sought to address the role and mechanisms of action by which phenylephrine, an α_1-adrenergic agonist stimulating intracellular D-*myo*-inositol-1,4,5-triphosphate (IP_3)/Ca^{2+} levels, regulates small cholangiocyte proliferation. Small and large bile ducts and cholangiocytes expressed all AR receptor subtypes. Small (but not large) cholangiocytes respond to phenylephrine with increased proliferation via the activation of IP_3/Ca^{2+}-dependent signaling. Phenylephrine stimulated the production of intracellular IP_3. The Ca^{2+}-dependent transcription factors, nuclear factor of activated T cells 2 (NFAT2) and NFAT4, were predominantly expressed by small bile ducts and small cholangiocytes. Phenylephrine stimulated the Ca^{2+}-dependent DNA-binding activities of NFAT2, NFAT4, and Sp1 (but not Sp3) and the nuclear translocation of NFAT2 and NFAT4 in small cholangiocytes. To determine the relative roles of NFAT2, NFAT4, or Sp1, we knocked down the expression of these transcription factors with small hairpin RNA. We observed an inhibition of phenylephrine-induced proliferation in small cholangiocytes lacking the expression of NFAT2 or Sp1. Phenylephrine stimulated small cholangiocyte proliferation is regulated by Ca^{2+}-dependent activation of NFAT2 and Sp1. *Conclusion*: Selective stimulation of Ca^{2+}-dependent small cholangiocyte proliferation may be key to promote the repopulation of the biliary epithelium when large bile ducts are damaged during cholestasis or by toxins. (HEPATOLOGY 2011;53:628-639)

In human and experimental cholangiopathies, the proliferation/loss of bile ducts is restricted to specific-sized bile ducts.[1-3] The secretory and proliferative capacity of large cholangiocytes depends on the activation of adenosine 3′,5′-monophosphate (cAMP)-dependent mechanisms.[2-4] Large (but not small)

Abbreviations: AR, adrenergic receptor; [Ca^{2+}]$_i$, intracellular calcium; BAPTA/AM, 1,2-bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester; BMY 7378 dihydrochloride, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride; BRL 37344, (±)-(R,R*)-[4-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]acetic acid; BSA, bovine serum albumin; CAI, calcineurin autoinhibitory peptide; cAMP, 3′-5′-cyclic adenosine monophosphate; CaMK, calmodulin-dependent protein kinase; CK-19, cytokeratin-19; clen, clenbuterol; $\Delta\Delta C_T$, delta delta of the threshold cycle; dobut, dobutamine; EMSA, electrophoretic mobility shift assay; IBDM, intrahepatic bile duct mass; IP_3, D-*myo*-inositol-1,4,5-triphosphate; MiA, mithramycin A; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; NFAT, nuclear factor of activated T cells; PCR, polymerase chain reaction; phenyl, phenylephrine; Rec 15/2615 dihydrochloride, 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-[[2-methoxy-6-(1-methylethyl)phenoxy]acetyl]piperazine dihydrochloride; RS-17053, N-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-alpha, alpha-dimethyl-1H-indole-3-ethanamine hydrochloride; Sp1/3, specificity protein 1/3.*

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cholangiocytes are more susceptible to toxins (e.g., carbon tetrachloride [CCl_4]) that induce the loss of proliferative and secretory activity.[3] Immortalized small cholangiocyte lines[5] proliferate via D-myo-inositol 1,4,5-triphosphate (IP_3)/Ca^{2+}/calmodulin-dependent protein kinase (CaMK) I–dependent mechanisms regulated via activation of H1 histamine receptor subtypes.[6] Ca^{2+}-dependent small cholangiocyte proliferation may be a key compensatory mechanism for maintaining homeostasis and overall bile duct function in pathological ductopenic conditions associated with damage of large ducts.[3,7]

We have demonstrated that: (1) cholangiocytes express adrenergic receptors (ARs) including $\alpha_{1A/1C}$, α_{1B}, α_{2A}, α_{2B}, α_{2C}, β_1, and β_2 subtypes; and (2) administration of agonists for these receptors regulate large cholangiocyte function by modulation of cAMP-dependent signaling.[8-10] For example, activation of $\alpha_{1A/1C}$, α_{1B} AR (by phenylephrine) stimulates secretin-stimulated cholangiocyte choleresis of bile duct–ligated rats via Ca^{2+}-dependent stimulation of cAMP signaling.[10] The expression of α_1-AR receptors, which are G-protein–coupled receptors signaling via Ca^{2+},[11] in small and large cholangiocytes and the possible effects of their stimulation on proliferation has not been explored. In particular, activation of Ca^{2+}-dependent signaling in small cholangiocytes by AR agonists, such as phenylephrine, that are known to trigger intracellular Ca^{2+} signaling,[10] has not been studied.

Nuclear factor of activated T cells (NFAT) is a ubiquitous transcription factor initially described in T-lymphocytes. Five NFAT family members have been described: NFAT1 (also known as NFATp or NFATc2), NFAT2 (NFATc or NFATc1), NFAT3, NFAT4 (NFATx or NFATc3), and NFAT5.[12] NFAT1, NFAT2, NFAT3, and NFAT4 are regulated by calcium/calcineurin signaling,[13] whereas activation of NFAT5 is calcineurin independent.[14] In nonstimulated cells, NFAT proteins are located in the cytoplasm in a hyper-phosphorylated state. Following increases in $[Ca^{2+}]_i$, the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, calcineurin, directly dephosphorylates NFAT, which induces rapid nuclear import providing a direct link between $[Ca^{2+}]_i$ signaling and gene expression.[13] In the nucleus, NFAT proteins bind to target promoter elements alone or in combination with other nuclear elements such as Sp1/Sp3 to regulate gene transcription. Nevertheless, the potential role of Ca^{2+}/calcineurin dependent activation of NFAT in the regulation of small cholangiocyte proliferation has not been addressed.

Materials and Methods

Materials. Reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. The mouse monoclonal antibodies for NFAT1 and NFAT2 were purchased from Novus Biologicals, Inc. (Littleton, CO). The rabbit polyclonal antibody for NFAT3 and the mouse monoclonal antibody for NFAT4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The radioimmunoassay kits for the determination of intracellular cAMP (cAMP [^{125}I] Biotrak Assay System) and IP_3 (IP_3 [^{3}H] Biotrak Assay System) levels were purchased from GE Healthcare (Piscataway, NJ).

Immortalized and Freshly Isolated Small and Large Cholangiocytes. Small and large cholangiocytes from normal mice (BALB/c) were immortalized by the introduction of the simian virus-40 large T antigen gene[5] and cultured as described.[6] These cell lines display morphological, phenotypic and functional features similar to freshly isolated small and large mouse cholangiocytes.[5,6,15] Freshly isolated small and large mouse cholangiocytes were purified as described.[2,6,15-17]

Animal Models. Male C57/Bl6N mice (20-25 g) were purchased from Charles River (Wilmington, MA). Animals received humane care according to the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by

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the National Institutes of Health. Normal mice were treated with saline or phenylephrine (10 mg/kg body weight, daily intraperitoneal injections)[18] for 1 week in the absence/presence of: (1) 11R-VIVIT, a cell-permeable NFAT inhibitor peptide, 10 mg/kg, daily intraperitoneal injections; or (2) mithramycin A (MiA), an Sp1 inhibitor, 0.5 mg/kg, intraperitoneal injections twice weekly)[19,20] before evaluating intrahepatic bile duct mass (IBDM, by immunohistochemistry for cytokeratin-19 [CK-19])[21] of small and large bile ducts. Stained sections were analyzed for each group using a BX-51 light microscopy (Olympus, Tokyo, Japan).

Expression of Adrenergic Receptors. The expression of α_{1A}, α_{1B}, α_{1D}-AR was evaluated by: (1) immunohistochemistry in liver sections and (2) immunofluorescence and fluorescence-activated cell sorting (FACS) analysis in immortalized small and large cholangiocytes. We evaluated the presence of the message for α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, and β_3, AR by real-time polymerase chain reaction (PCR) in freshly isolated and immortalized small and large cholangiocytes. Immunohistochemistry was performed in paraffin-embedded liver sections (4-5 μm thickness).[6] Light microscopy photographs of 10 non-overlapping fields liver sections were evaluated for receptor expression. Immunofluorescence in cell smears was performed as described.[6] Negative controls were performed by usage of a pre-immune serum instead of the primary antibody. FACS analysis was performed as described[22] using a C6 flow cytometer (Accuri, Inc., Ann Arbor, MI) and analyzed by CFlow Software. At least 20,000 events in the light-scatter (side scatter/forward scatter) were acquired. AR receptors were identified and gated on FL1-A/Count plots. The relative quantity of AR (mean AR fluorescence) was expressed as mean FL1-A (samples)/mean FL-1A (secondary antibodies only). For real-time PCR, a $\Delta\Delta C_T$ (delta delta of the threshold cycle) analysis was performed.[23] The primers for α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3 AR subtypes were designed by SABiosciences (Frederick, MD) according to the sequences listed in the National Center for Biotechnology Information. Data were expressed as fold-change of the ratio of relative messenger RNA levels $\pm$ standard error of the mean of AR to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Expression of NFAT Isoforms. The expression of the different NFAT isoforms (NFAT1, NFAT2, NFAT3, and NFAT4) was evaluated by: (1) immunohistochemistry in normal liver sections[6]; and (2) immunofluorescence in immortalized small and large cholangiocytes.[6] In liver sections, when 0%-5% of bile ducts were positive for NFAT isoforms, we assigned a negative score; a +/− score was assigned when 6%-10% of bile ducts were positive; a + score was assigned when 11%-30% of bile ducts were positive; and a ++ score was assigned with 31%-60% of bile ducts positive.[15]

Effect of Adrenergic Receptor Agonists on the Proliferation of Immortalized Small and Large Cholangiocytes. The effect of phenylephrine on small cholangiocyte proliferation was evaluated at varying dosages (10^{-11} to 10^{-3} M) and times (24-72 hours) by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] proliferation assay.[6] The effects (24 hours at 37°C) of α_1 (phenylephrine, 10 μM),[10] α_2 (UK14,304, 50 μM),[8] β_1 (dobutamine, 10 μM),[9,10] β_2 (clenbuterol, 10 μM)[9] or β_3 (BRL 37344, 10 μM)[24] AR agonists on the proliferation of small and large cholangiocytes were evaluated by MTS proliferation assays.[6] The concentration of 10 μM for phenylephrine was chosen based on the fact that: (1) at this concentration (10 μM) phenylephrine stimulates cholangiocyte secretory activity[10]; and (2) the doses (10^{-11} to 10^{-5} M) used for phenylephrine induced a similar increase in small cholangiocyte proliferation (Fig. 3A). Because phenylephrine was the only α_1-AR agonist to increase small cholangiocyte proliferation (see results section), in separate sets of experiments we evaluated by MTS assay[6] the effect of phenylephrine on small cholangiocyte proliferation in the absence or presence of: (1) BAPTA/AM (intracellular Ca^{2+} chelator, 5 μM)[6]; (2) CAI (calcineurin autoinhibitory peptide, 50 μM)[4]; (3) 11R-VIVIT (1 nM)[19]; or (4) MiA (50 nM).[25] To demonstrate that the effects of phenylephrine on cholangiocyte proliferation are due to selective interaction with α-1 AR, we evaluated the effect of phenylephrine (10 μM for 24 hours) on the proliferation of small cholangiocytes in the absence or presence of: (1) RS-17053 (selective α_{1A}-AR antagonist, 1 nM)[26]; (2) Rec 15/2615 dihydrochloride (selective α_{1B}-AR antagonist, 10 nM)[27]; or (3) BMY 7378 dihydrochloride (selective α_{1D}-AR antagonist, 1 nM).[28]

Effect of Phenylephrine on Intracellular cAMP and IP3 Levels. Immortalized small and large cholangiocytes were stimulated at room temperature for 5 minutes with 0.2% bovine serum albumin (BSA; basal) or phenylephrine (10 μM in 0.2% BSA).[10] Intracellular cAMP and IP_3 levels were measured by commercially available kits according to the instructions provided by the vendor.

Effect of Phenylephrine on the Nuclear Translocation and DNA-Binding Activity of NFAT2, NFAT4, Sp1, and Sp3 of Immortalized Small Cholangiocytes. Experiments were performed to evaluate the effect of phenylephrine on: (1) the nuclear

translocation of NFAT2 and NFAT4, the isoforms expressed by immortalized small cholangiocytes by immunofluorescence; and (2) NFAT2, Sp1, and Sp3 DNA-binding activity by enzyme-linked immunosorbent assay (ELISA)[29] and electrophoretic mobility shift assay (EMSA)[30] in immortalized small cholangiocytes. Nuclear translocation of NFAT2 and NFAT4 was evaluated by immunofluorescence[6] in small cholangiocytes treated with 0.2% BSA or phenylephrine (10 μM in 0.2% BSA) for 1 hour at 37°C in the presence/absence of pretreatment for 30 minutes with benoxathian (nonsubtype selective α_1-AR antagonist, 50 μM),[31] BAPTA/AM or CAI. NFAT2 (a kit is not available for NFAT4), Sp1, and Sp3 DNA-binding activity was measured by a commercially available ELISA-based kit that detects transcription factor activation (TransAM transcription factor assay kit; Active Motif, Carlsbad, CA). Immortalized small cholangiocytes were stimulated with 0.2% BSA (basal) or phenylephrine (10 μM in 0.2% BSA) for 1 hour at 37°C in the presence/absence of BAPTA/AM, or CAI or MiA. Nuclear extracts were analyzed transcription for factor activation according to the manufacturer's protocol (Active Motif, Carlsbad, CA). The relative DNA-binding of NFAT2/4 and Sp1was assessed by EMSA in immortalized small cholangiocytes treated with phenylephrine (10 μM) for 0-minute, 30-minute, and 60-minute timepoints at 37°C as described.[30] Double-stranded oligonucleotides containing either the consensus binding motif for NFAT (Santa Cruz Biotechnology), Sp1 (Promega, Madison, WI) or Oct (Promega) were end-labeled with [^{32}P]deoxyadenosine triphosphate using T4 polynucleotide kinase for 10 minutes at room temperature. The NFAT consensus sequence binds both NFAT2 and NFAT4 isoforms.[32] In parallel, to prove specificity of the relevant DNA-binding activities, cold competition assays were performed by adding 50-fold excess of unlabeled consensus sequences for NFAT, a mutant NFAT sequence that differs from the native sequence by three base pairs (Santa Cruz Biotechnology), Oct or Sp1 prior to the addition of the labeled sequence.

Knockdown of NFAT2, NFAT4, and Sp1 Expression in Immortalized Small Cholangiocytes. Immortalized small cholangiocyte cell lines lacking NFAT2, NFAT4 and Sp1 expression were established using SureSilencing short hairpin RNA (shRNA; SuperArray, Frederick, MD) plasmids for mouse NFAT2, NFAT4 and Sp1, containing neomycin (for NFAT2) and puromycin (for NFAT4 and Sp1) resistance for the selection of stably transfected cells, according to the instructions provided by the vendor.[33] Approximately 70% knockdown of NFAT2, NFAT4, and Sp1 messenger RNA expression was achieved in immortalized small cholangiocytes (Supporting Information Fig. 1A). Immunofluorescence and DNA-binding activity for NFAT2, NFAT4, and Sp1 by EMSA were used to validate the knockdown of protein expression in small cholangiocytes (Supporting Information Fig. 1B). There was no inadvertent knockdown of NFAT2, NFAT4, and Sp1 in each case. The small cholangiocyte cell line, mock-transfected clone (Neo-Control shRNA or Puro-Control shRNA), the NFAT2 knockdown clone, NFAT4 knockdown clone, and the Sp1 knockdown clone were stimulated with 0.2% BSA (basal) or phenylephrine (10 mM in 0.2% BSA) for 24 hours before evaluation of proliferation by MTS assays.[6]

Results

Cholangiocytes Express α1-AR Subtypes. In normal liver sections, we demonstrated that α_{1A}, α_{1B}, α_{1D}-AR are expressed by small (yellow arrow) and large (red arrow) bile ducts (Fig. 1A). Immortalized small and large cholangiocytes were positive for α_{1A}, α_{1B}, α_{1D}-AR expression (Fig. 1B). By real-time PCR, freshly isolated and immortalized small and large cholangiocytes express the messages for α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, and β_3 AR (Supporting Information Fig. 2A). By FACS, we demonstrated that immortalized small and large cholangiocytes express the protein for α_{1A}, α_{1B}, α_{1D}-AR (Supporting Information Fig. 2B).

Small Bile Ducts and Cholangiocytes Express NFAT2 and NFAT4. By immunohistochemistry, small bile ducts in liver sections express the NFAT2 and NFAT4 isoforms (Fig. 2A). Large bile ducts in liver sections expressed lower levels of NFAT2 and NFAT4 (Fig. 2A) as determined by semiquantitative immunohistochemical analysis (Supporting Information Table 1). By immunofluorescence, we demonstrated that NFAT2 and NFAT4 were predominantly expressed by immortalized small cholangiocytes and that NFAT3 was expressed by large cholangiocytes (Fig. 2B). NFAT1 was not expressed by small or large bile ducts or immortalized small and large cholangiocytes (Fig. 2A,B).

Phenylephrine Stimulates In Vivo and In Vitro the Proliferation of Small but not Large Cholangiocytes. Chronic *in vivo* administration of phenylephrine to normal mice induces a significant increase in IBDM of small cholangiocytes, increase that was blocked by 11R-VIVIT and mithramycin A (Fig. 3). The *in vitro* doses (10^{-11} to 10^{-5} M) used for phenylephrine induced a similar increase in the proliferation of immortalized small