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α-1 Adrenergic Receptor Agonists Modulate Ductal Secretion of BDL Rats via Ca^{2+}- and PKC-Dependent Stimulation of cAMP

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Acetylcholine potentiates secretin-stimulated ductal secretion by Ca^{2+}-calcineurin–mediated modulation of adenylyl cyclase. D2 dopaminergic receptor agonists inhibit secretin-stimulated ductal secretion via activation of protein kinase C (PKC)-γ. No information exists regarding the effect of adrenergic receptor agonists on ductal secretion in a model of cholestasis induced by bile duct ligation (BDL). We evaluated the expression of α-1A/1C, -1β and β-1 adrenergic receptors in liver sections and cholangiocytes from normal and BDL rats. We evaluated the effects of the α-1 and β-1 adrenergic receptor agonists (phenylephrine and dobutamine, respectively) on bile and bicarbonate secretion and cholangiocyte IP_3 and Ca^{2+} levels in normal and BDL rats. We measured the effect of phenylephrine on lumen expansion in intrahepatic bile duct units (IBDUs) and cyclic adenosine monophosphate (cAMP) levels in cholangiocytes from BDL rats in the absence or presence of BAPTA/AM and Gö6976 (a PKC-α inhibitor). We evaluated if the effects of phenylephrine on ductal secretion were associated with translocation of PKC isoforms leading to increased protein kinase A activity. α-1 and β-1 adrenergic receptors were present mostly in the basolateral domain of cholangiocytes and, following BDL, their expression increased. Phenylephrine, but not dobutamine, increased secretin-stimulated choleresis in BDL rats. Phenylephrine did not alter basal but increased secretin-stimulated IBDU lumen expansion and cAMP levels, which were blocked by BAPTA/AM and Gö6976. Phenylephrine increased IP_3 and Ca^{2+} levels and activated PKC-α and PKC-β-II. In conclusion, coordinated regulation of ductal secretion by secretin (through cAMP) and adrenergic receptor agonist activation (through Ca^{2+}/PKC) induces maximal ductal bicarbonate secretion in liver diseases. (*Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2004;40:1116–1127.))

Secretin stimulates ductal HCO_3^- secretion[1–5] by interacting with receptors expressed only by rat cholangiocytes.[6] This interaction induces an increase in intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) levels,[4,5,7] which leads to the opening of cystic fibrosis transmembrane conductance regulator channels,[8] followed by activation of the apically located Cl^-/HCO_3^- exchanger[9] with secretion of HCO_3^- into bile.[1]

Acetylcholine increases intracellular Ca^{2-} ($[Ca^{2+}]_i$) levels and oscillations in rat cholangiocytes due to influx of extracellular Ca^{2+} and mobilization of thapsigargin-sensitive $[Ca^{2+}]_i$ stores.[10] It also increases secretin-stimulated ductal HCO_3^- secretion via Ca^{2-}-calcineurin mediated modulation of adenylyl cyclase.[9] The D2 dopaminergic receptor agonist quinelorane inhibits secretin-stimulated ductal secretion via activation of the Ca^{2+}-dependent protein kinase C (PKC)-γ.[11] Adrenergic nerve stimulation causes a decrease in bile flow in the isolated perfused rat liver via interaction with α-1 adrenergic receptors.[12] In isolated perfused rat liver, adrenaline induces

Abbreviations: PKC, protein kinase C; BDL, bile duct ligation; IBDU, intrahepatic bile duct unit; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; BDI, bile duct incannulation; BSA, bovine serum albumin; KRH, Krebs Ringer Hemelett.

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a complex response of bile secretion including rapid, reversible stimulation, reversible inhibition, and prolonged stimulation via interaction with α-1 adrenergic receptors.[13] Adrenergic receptor agonists modulate the functions of target epithelial cells by acting on α-1 and/or β-1 adrenergic receptors, which in turn activate D-myo-inositol 1,4,5-triphosphate (IP_3)–, $[Ca^{2+}]_i$-, or cAMP-dependent intracellular pathways.[14–16] No information exists regarding the role and mechanism of action through which adrenergic receptor agonists regulate ductal secretion in a rat model of cholestasis induced by extrahepatic bile duct ligation (BDL).[1]

We addressed the following questions: (1) Are functional α-1 and β-1 adrenergic receptors expressed by cholangiocytes from normal and BDL rats? (2) Do α-1 and β-1 adrenergic receptor agonists regulate basal and secretin-stimulated ductal secretion in the BDL rat model via the Ca^{2+}-dependent PKC pathway? (3) Are adrenergic effects on ductal bile secretion of BDL rats associated with activation of Ca^{2+}-dependent (α, β-I, β-II and γ) and/or Ca^{2+}-independent novel (ε and θ) and atypical (η and ζ) PKC isoforms? and (4) Is membrane translocation of PKC-α and PKC-β-II (by phenylephrine) associated with upregulation of protein kinase A (PKA) activity leading to enhanced secretin-stimulated ductal secretion of BDL rats?

Experimental Procedures

Materials

Reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified. Porcine secretin was purchased from Peninsula Laboratories (Belmont, CA). The antibodies (mouse monoclonal immunoglobulin G1) reacting with the rat α-1A/-1C, -1B, or β-1 adrenergic receptors were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The classical α-1A and the cloned α-1C are the same adrenergic receptors.[17] Radioimmunoassay kits for the determination of cAMP and IP_3 levels were purchased from Amersham (Arlington Heights, IL). The antibodies (immunoglobulin G) against the selected PKC isoforms were purchased from Santa Cruz Biotechnology, Inc.

Animal Model

Male Fischer 344 rats (150-175 g) were purchased from Charles River Laboratories (Wilmington, MA). Studies were performed in normal rats and rats with BDL (for isolation of cholangiocytes[5] or intrahepatic bile duct units [IBDUs][18]) or bile duct incannulation (BDI, for bile collection)[1] for 2 weeks. Before experiments, animals were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg body weight). Study protocols were performed in compliance with the institution guidelines.

Purification of Cholangiocytes and IBDUs

Cholangiocytes (98%-99% positive for γ-GT)[19] were obtained via immunoaffinity bead purification.[5,20] Cell viability (≈97%) was determined via trypan blue exclusion. Large secretin-responsive IBDUs (size range of 50-100 μm with a mean diameter of 70 μm)[21] from BDL rats were isolated as described by the authors.[21]

Expression of α-1A/1C -1B, and β-1 Adrenergic Receptors

Liver sections (n = 3; 5-μm thick) from normal and BDL rats were incubated with or without the mouse monoclonal antibodies against α-1A/1C, -1B, or β-1 adrenergic receptors (1:50 dilution). The reactive sites were detected using a DAKO LSAB kit (DAKO, Carpinteria, CA) according to the manufacturer's instructions. Sections not incubated with a primary antibody served as negative controls. To determine the specificity of the antibodies for the respective adrenergic receptor isoforms, each antibody (α-1A/1C, -1B, or β-1) was incubated for 2 hours at room temperature with the peptides corresponding to the different isoforms (*i.e.*, α-1A/-1C antibody was incubated with the α-1B corresponding blocking peptide, sc-1476 P, and β-1 was incubated with the corresponding blocking peptide, sc-1477 P) before incubation with liver sections. Following counterstaining, slides were examined with a microscope (BX40, Olympus Optical Co., Tokyo, Japan). The expression of the α-1A/1C, -1B, and β-1 adrenergic receptors was evaluated via immunoblotting in whole lysate from rat brain (10 μg, positive), bovine serum albumin (BSA) (10 μg, negative), or cholangiocytes (10 μg) from normal and BDL rats. The amount of protein loaded was normalized by immunoblots for β-actin.[22] The intensity of the bands was determined via scanning video densitometry using the ChemiImager 4000 low light imaging system (Alpha Innotech Corp., San Leandro, CA). We evaluated (via immunoblotting) the protein expression for α-1A/1C, -1B, or β-1 adrenergic receptors in basolateral or apical cholangiocyte membranes[23] from BDL rats. The amount of cholangiocyte apical and basolateral membrane protein was determined using a Pierce BSA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). To demonstrate the purity of the membrane preparations, we measured the activity of specific markers for the basolateral (Na^+, K^+-ATPase) and apical (alkaline phosphatase) domain of cholangiocyte membranes.[23,24]

Effect of Adrenergic Agonists on Ductal Secretion

Bile Flow. The animals used in the *in vivo* studies of biliary physiology were not suffering or hypoxic. Follow-

ing anesthesia, rats were surgically prepared for bile collection.[1] When steady-state bile flow was reached (60-70 minutes from the infusion of Krebs Ringer Henseleit [KRH]), normal or BDI rats were subsequently infused with phenylephrine (10 μmol/L) for 30 minutes; KRH for 60 minutes; phenylephrine (10 μmol/L) + secretin (100 nmol/L) for 30 minutes; and KRH for 60 minutes followed by secretin (100 nmol/L) for 30 minutes and a final infusion of KRH for 30 minutes. We evaluated the effect of benoxathian (10 μmol/L), an α-1 adrenergic receptor antagonist,[25] on basal and phenylephrine stimulation of secretin-induced bile and bicarbonate secretion of BDI rats. Following infusion of KRH for 60 to 70 minutes, BDI rats were subsequently infused with phenylephrine (10 μmol/L) for 30 minutes; KRH for 60 minutes; phenylephrine (10 μmol/L) + secretin (100 nmol/L) for 30 minutes in the presence of benoxathian (10 μmol/L)[25]; and KRH for 60 minutes followed by secretin (100 nmol/L) for 30 minutes and a final infusion of KRH for 30 minutes. In other experiments, normal or BDI rats were subsequently infused with KRH for 60 to 70 minutes; dobutamine (10 μmol/L), a selective β-1 adrenergic receptor agonist, for 30 minutes; KRH for 60 minutes; dobutamine (10 μmol/L) + secretin (100 nmol/L) for 30 minutes; and KRH for 60 minutes followed by secretin (100 nmol/L) for 30 minutes and a final infusion of KRH for 30 minutes. Biliary bicarbonate concentration (measured as total CO_2) was determined by an ABL™ 520 Blood Gas System (Radiometer Medical A/S, Copenhagen, Denmark).

IBDU Secretion. We evaluated the effects of phenylephrine on basal and secretin-stimulated fluid secretion in large IBDUs from BDL rats.[21] Ductal secretion was evaluated from the changes in the area of IBDU lumen space, as described by the authors,[18] after basolateral perfusion with: (1) 0.2% BSA (basal) for 10 minutes; (2) secretin (100 nmol/L) with 0.2% BSA for 10 minutes; or (3) phenylephrine or dobutamine (10 μmol/L) for 10 minutes in the absence or presence of secretin (100 nmol/L) with 0.2% BSA for 10 minutes. Because phenylephrine increased the stimulatory effect of secretin on IBDU lumen space, we evaluated the effect of benoxathian (10 μmol/L); BAPTA/AM (50 μmol/L), an intracellular Ca^{2+} chelator[7,9]); and Gö6976 (1 μmol/L), a Ca^{2+}-dependent PKC-α inhibitor,[26] in the presence of phenylephrine (10 μmol/L) + secretin (100 nmol/L) with 0.2% BSA. We did not evaluate the effect of BAPTA/AM or Gö6976 on IBDU lumen space, because we have previously shown that neither BAPTA/AM nor PKC inhibitors alter secretin-stimulated fluid secretion in IBDU.[5,9]

Is Phenylephrine Stimulation of Secretin-Induced Ductal Secretion of BDL Rats Associated With Increased Secretin-Stimulated cAMP Levels?

Following incubation at 37°C for 1 hour,[4] cholangiocytes (1×10^5) from BDL rats were incubated at room temperature[4,5,7] with: (1) 0.2% BSA (basal) for 5 minutes; (2) secretin (100 nmol/L) for 5 minutes with 0.2% BSA; (3) phenylephrine or dobutamine (10 μmol/L for 5 minutes) in the absence or presence of secretin (100 nmol/L for 5 minutes) with 0.2% BSA; or (4) BAPTA/AM (50 μmol/L) for 10 minutes and Gö6976 (1 μmol/L) for 10 minutes in the presence of phenylephrine (10 μmol/L for 10 minutes) + secretin (100 nmol/L for 5 minutes) with 0.2% BSA. Cholangiocyte cAMP levels were measured via Radioimmunoassay.[4,5,7,21]

Are Phenylephrine Effects on Secretin-stimulated Ductal Secretion of BDL Rats Associated With Changes in IP_3/Ca^{2+} Levels and Activation of PKC Isoforms?

Before IP_3 and Ca^{2+} measurements, cholangiocytes were incubated for 1 hour at 37°C.[4] For IP_3 measurements, cholangiocytes were stimulated for 10 minutes at 22°C with 0.2% BSA or phenylephrine (10 μmol/L) with 0.2% BSA. Intracellular IP_3 levels were assessed using the IP_3 [^{3}H] kit (Amersham). Calcium fluorescence measurements in cholangiocytes were performed using fluo-3 AM (Molecular Probes, Eugene, OR) and a Fluoroskan Ascent FL (ThermoLabsystems, Helsinki, Finland) microplate reader equipped with three injectors.[11,27] Cholangiocytes (4×10^4 per well) were loaded for 1 hour at room temperature with 5 μmol/L of fluo-3 AM in Tyrode's salt solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.2 mmol/L NaH_2PO_4, 12 mmol/L $NaHCO_3$, and 5.5 mmol/L glucose) with 0.1% Pluronic F-127 (Molecular Probes). After washes with Tyrode's salt solution, the loaded cells were added to a 96-well black microplate. The baseline fluorescence was measured 50 times at 2-second intervals. Tyrode's salt solution alone or phenylephrine (10 μmol/L) dissolved in buffer was injected sequentially into separate wells, and the fluorescence intensity was measured at 538 nm for 3 minutes at 1-second intervals. The excitation wavelength was 485 nm. $[Ca^{2+}]_i$ concentration was calculated as follows: $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$. F_{max} refers to fluorescence intensity measured after permeabilization of the cells with 1% NP-40. Then, 0.1 mol/L EGTA was added to chelate Ca^{2+} and minimum fluorescence intensity (F_{min}) was obtained.

We evaluated if the stimulatory effects of phenylephrine on secretin-stimulated ductal secretion are as-

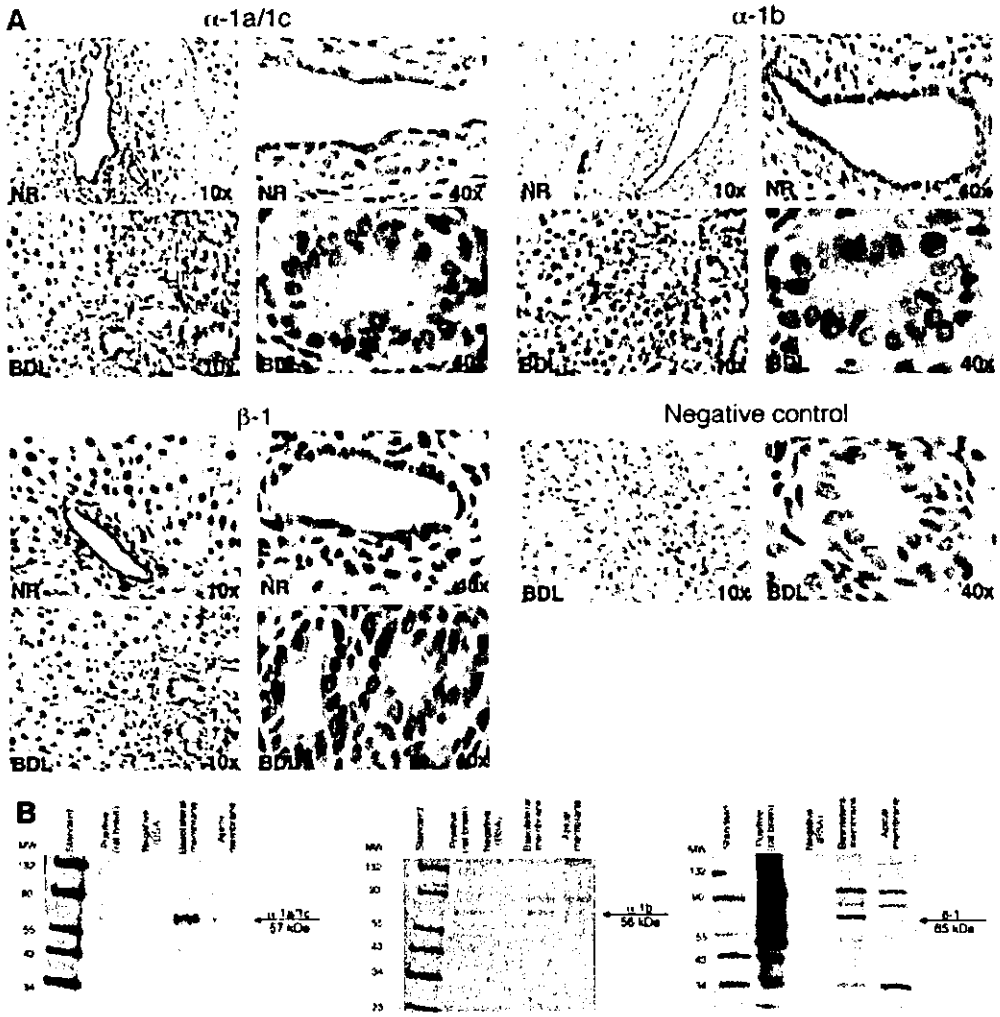

Fig. 1. (A) Low- and high-power micrographs of liver sections show a specific reaction in bile ducts from normal and BDL rats after incubation with α-1A/-1C, -1B, and β-1 adrenergic receptor antibodies. Hepatocytes also show a positive staining. The negative control is also shown. (B) Distribution of α-1A/-1C, -1B, and β-1 adrenergic receptors in apical and basolateral membranes from pure cholangiocytes in BDL rats. A band for the α-1A/-1C, -1B, and β-1 adrenergic receptor was mostly present in the basolateral domain of cholangiocytes from BDL rats; the expression of α-1A/-1C, -1B, and β-1 adrenergic receptors was very low in the apical membranes of cholangiocytes from BDL rats. NR, normal; BDL, bile duct ligation; BSA, bovine serum albumin; MW, molecular weight.

sociated with increased protein expression of Ca^{2+}-dependent (α, -βI, -βII, and -γ) and/or Ca^{2+}-independent novel (δ, ϵ, and θ) and atypical (η and ζ) PKC isoforms. Cholangiocytes from BDL rats were stimulated for 90 minutes[7,28] at 37°C with (1) 0.2% BSA (basal) or (2) phenylephrine (10 μmol/L) with 0.2% BSA and analyzed for protein expression for the selected PKC isoform in cytosolic or membrane fractions[29] via immunoblotting. To determine if phenylephrine induces membrane translocation of PKC-α or PKC-β-II, cholangiocytes from BDL rats were treated *in vitro* at 37°C for 90 minutes with (1) 0.2% BSA or (2) phenylephrine (10 μmol/L) with 0.2% BSA. To overcome methodological doubts, we performed studies to demonstrate that the mobile PKC isoforms (α, β-II, ϵ, and θ) are translocated to membranes by phorbol esters in cholangiocytes treated *in vitro* with 0.2% BSA or phorbol 12-myristate 13-acetate (1 μmol/L), a PKC activator,[30] for 90 minutes at room temperature, whereas nonmobile isoforms (η and ζ) and β-actin are not translocated to membranes. The intensity of the bands was determined via scanning video densitometry using the ChemiImager 4000 low light imaging system.

Table 1. Characterization of the Basolateral and Apical BDL Cholangiocyte Membranes by Measurement of the Activity of Specific Markers for the Apical (*i.e.*, Alkaline Phosphatase) and Basolateral (*i.e.*, Na^+, K^+-ATPase) Domains of Cholangiocyte Membranes

Markers	Relative Specific Activity (Apical Membranes)	Relative Specific Activity (Basolateral Membranes)
Alkaline phosphatase (apical marker)	4.31 ± 0.51	0.60 ± 0.02
Na^+K^+-ATPase (basolateral marker)	0.123 ± 0.07	7.10 ± 2.00

NOTE. Relative specific activity = specific activity of membrane fraction/specific activity of homogenate. Similar to previous studies,[23,24] the apical cholangiocyte membranes were enriched in alkaline phosphatase, whereas the basolateral cholangiocyte membranes were enriched in Na^+, K^+-ATPase when compared with homogenate. Data are the mean ± SEM of 4 experiments.

Is Phenylephrine-Induced Increase in PKC-α or PKC-β-II Associated With Upregulation of PKA Activity in Cholangiocytes?

We evaluated cholangiocytes to determine if activation of PKC-α or PKC-β-II (by phenylephrine) causes stimulation of secretin-stimulated PKA activity, which regulates ductal secretion.[3] PKA assay was performed using a PepTag Assay Protein Kinase Kit (Promega, Madison, WI) for PKA according to the manufacturer's instructions.[21] Cholangiocytes (5×10^6) from BDL rats were stimulated at 37°C with: (1) 0.2% BSA for 30 minutes; (2) secretin (30 minutes at 100 nmol/L) with 0.2% BSA; (3) phenylephrine (30 minutes at 10 μmol/L) with 0.2% BSA; (4) phenylephrine (30 minutes at 10 μmol/L) before stimulation with secretin (30 minutes at 100 nmol/L) with 0.2% BSA; or (5) BAPTA/AM (20 minutes at 50 μmol/L or Gö6976 (20 minutes at 1 μmol/L) before treatment with phenylephrine (30 minutes at 10 μmol/L) followed by secretin (30 minutes at 100 nmol/L) stimulation. Phosphorylated peptide bands were quantitated via scanning densitometry using the ChemiImager 4000 low light imaging system.

Statistical Analysis

All data are expressed as the mean ± SEM. The differences between groups were analyzed using Student *t* test when two groups were being analyzed or ANOVA if more than two groups were being analyzed.

Results

Cholangiocytes Express α-1A/-1C, α-1B and β-1 Adrenergic Receptors. Low- (×10) and high- (×40) power micrographs of liver sections show positive staining for α-1A/-1C, -1B, and β-1 adrenergic receptor in bile ducts from normal and BDL rats; hepatocytes show positive staining (Fig. 1A). Through immunoblotting, α-1A/-1C, -1B, and β-1 adrenergic receptors were expressed by normal cholangiocytes and up-regulated following BDL (Supplementary Fig. 1). These receptors were present mostly in the basolateral membranes of cholangiocytes (Fig. 1B). The expression of adrenergic receptors was very low in the apical membranes of BDL cholangiocytes (Fig. 1B). Parallel to previous studies,[23,24] the apical cholangiocyte membranes were enriched in alkaline phosphatase, whereas the basolateral cholangiocyte membranes were enriched in Na^+, K^+-ATPase when compared with homogenate (Table 1).

Phenylephrine Increases Secretin-Stimulated Bicarbonate-Rich Choleresis. Secretin (infused intravenously) did not increase bile and bicarbonate secretion of normal rats (Table 2), but enhanced bile and bicarbonate

Table 2. Effects of Phenylephrine and Dobutamine on Basal and Secretin-Stimulated Bile Flow, Bicarbonate Concentration, and Secretion in Normal Rats

Treatment	Bile Flow (μL/min/kg Body Weight)	Bicarbonate Concentration (mEq/L)	Bicarbonate Secretion (μEq/min/kg Body Weight)
Basal before phenylephrine	72.5 ± 8.9 (n = 8)	32.9 ± 0.9 (n = 8)	2.4 ± 0.3 (n = 8)
Phenylephrine	75.4 ± 9.6 (n = 8)	34.2 ± 0.8 (n = 8)	2.5 ± 0.3 (n = 8)
Basal before secretin	81.5 ± 4.8 (n = 6)	29.5 ± 0.5 (n = 6)	2.4 ± 0.1 (n = 6)
Secretin	83.1 ± 5.1 (n = 6)	26.8 ± 0.2 (n = 6)	2.2 ± 0.1 (n = 6)
Basal before phenylephrine + secretin	83.7 ± 2.8 (n = 10)	35.0 ± 0.8 (n = 10)	2.9 ± 0.1 (n = 10)
Phenylephrine + secretin	89.1 ± 2.8 (n = 10)	37.7 ± 1.4 (n = 10)	3.3 ± 0.1 (n = 10)
Basal before dobutamine	71.6 ± 3.4 (n = 7)	35.2 ± 0.9 (n = 7)	2.5 ± 0.1 (n = 7)
Dobutamine	74.9 ± 3.3 (n = 7)	33.8 ± 1.0 (n = 7)	2.5 ± 0.1 (n = 7)
Basal before secretin	77.9 ± 3.9 (n = 6)	27.9 ± 0.7 (n = 6)	2.2 ± 0.1 (n = 6)
Secretin	80.2 ± 4.4 (n = 6)	26.3 ± 0.4 (n = 6)	2.1 ± 0.1 (n = 6)
Basal before dobutamine + secretin	71.8 ± 3.4 (n = 5)	34.6 ± 1.1 (n = 5)	2.4 ± 0.1 (n = 5)
Dobutamine + secretin	78.4 ± 3.2 (n = 5)	35.5 ± 0.4 (n = 5)	2.7 ± 0.1 (n = 5)

NOTE. Data are the mean ± SE of 3 to 5 experiments. Statistical analysis was performed by both unpaired Student's *t* and ANOVA test.

Table 3. Effects of Phenylephrine and Dobutamine on Basal and Secretin-Stimulated Bile Flow, Bicarbonate Concentration, and Secretin in 2 Week BDI Rats

Treatment	Bile Flow (μL/min/Kg Body Weight)	Bicarbonate Concentration (mEq/L)	Bicarbonate Secretion (μEq/min/Kg Body Weight)
Basal before phenylephrine	141.6 ± 18.0 (n = 18)	37.5 ± 2.0 (n = 18)	5.2 ± 0.5 (n = 18)
Phenylephrine	148.9 ± 15.6 (n = 18)	39.7 ± 1.5 (n = 18)	5.7 ± 0.5 (n = 18)
Basal before secretin	138.8 ± 13.6 (n = 25)	36.00 ± 2.2 (n = 25)	4.8 ± 0.5 (n = 25)
Secretin	188.1 ± 15.6* (n = 25)	43.5 ± 1.6* (n = 25)	8.1 ± 0.6* (n = 25)
Basal before phenylephrine + secretin	125.4 ± 9.3 (n = 22)	39.2 ± 1.1 (n = 22)	4.9 ± 0.3 (n = 22)
Phenylephrine + secretin	200.0 ± 16.7*† (n = 22)	57.8 ± 3.0*† (n = 22)	12.0 ± 1.6*† (n = 22)
Basal before benoxathian + phenylephrine + secretin	124.6 ± 9.5 (n = 12)	39.8 ± 2.8 (n = 12)	4.8 ± 0.6 (n = 12)
Benoxathian + phenylephrine + secretin	179.2 ± 10.8* (n = 12)	57.0 ± 2.8* (n = 12)	10.3 ± 0.8* (n = 12)
Basal before benoxathian	97.7 ± 5.5 (n = 12)	40.5 ± 1.5 (n = 12)	3.9 ± 0.3 (n = 12)
Benoxathian	98.9 ± 4.6 (n = 12)	38.7 ± 1.7 (n = 12)	3.8 ± 0.3 (n = 12)
Basal before dobutamine	123.5 ± 10.5 (n = 18)	41.7 ± 2.1 (n = 18)	5.0 ± 0.4 (n = 18)
Dobutamine	127.4 ± 10.8 (n = 18)	42.8 ± 3.2 (n = 18)	5.2 ± 0.4 (n = 18)
Basal before secretin	127.0 ± 13.2 (n = 12)	36.0 ± 1.9 (n = 12)	4.4 ± 0.4 (n = 12)
Secretin	177.4 ± 16.3* (n = 12)	51.0 ± 3.3* (n = 12)	8.6 ± 0.6* (n = 12)
Basal before dobutamine + secretin	118.0 ± 11.8 (n = 13)	36.2 ± 4.0 (n = 13)	4.2 ± 0.6 (n = 13)
Dobutamine + secretin	165.8 ± 17.7* (n = 13)	49.7 ± 4.5* (n = 13)	8.1 ± 0.9* (n = 13)

NOTE. Data are the mean ± SEM. The differences between groups were analyzed by Student *t* test when two groups were being analyzed or ANOVA if more than two groups were being analyzed.

*$P < .05$ versus corresponding basal value of bile flow, bicarbonate concentration, or secretion, respectively.

†$P < .05$ versus corresponding value of secretin-stimulated bile flow, concentration, or secretion, respectively.

secretion of BDL rats (Table 3). Phenylephrine or dobutamine did not alter basal or secretin-stimulated bile flow or bicarbonate secretion of normal rats (Table 2). In BDL rats, phenylephrine increased the stimulatory effects of secretin on bile and bicarbonate secretion (Table 3). The stimulatory effect of phenylephrine on secretin-stimulated bile and bicarbonate secretion of BDL rats was blocked by benoxathian (Table 3). Benoxathian did not alter basal bile flow of BDL rats (Table 3). Dobutamine did not alter basal or secretin-stimulated bile flow, and bicarbonate concentration and secretion of BDL rats (Table 3). A shortcoming of these *in vivo* studies of biliary physiology is that the effect of phenylephrine on secretin-stimulated choleresis of BDL rats may be influenced by the *in vivo* vascular effects of phenylephrine.[31] However, in support of the concept that the effects of phenylephrine are due to a direct interaction with cholangiocytes rather than vascular effects,[31] we demonstrated that phenylephrine increases secretin-stimulated ductal lumen expansion in IBDUs from BDL rats.

Phenylephrine Increases the Stimulatory Effect of Secretin on IBDU Lumen Space. Secretin increased lumen space of isolated IBDUs from BDL rats (Fig. 2). Neither phenylephrine nor dobutamine alone altered IBDU lumen space (Fig. 2). Phenylephrine (but not dobutamine) increased the stimulatory effect of secretin in IBDU lumen space (Fig. 2). Benoxathian blocked the stimulatory effect of phenylephrine on secretin-induced increases on IBDU lumen space (Fig. 2). Phenylephrine stimulation of secretin-induced increase in IBDU lumen space was blocked by benoxathian, BAPTA/AM, and Gö6976 (Fig. 2). Benoxathian did not alter lumen space in IBDUs from BDL rats (Fig. 2).

Phenylephrine Increases Secretin-Stimulated cAMP Levels of Cholangiocytes from BDL Rats. Parallel to previous studies,[4,5,7,21] secretin increased cAMP levels of

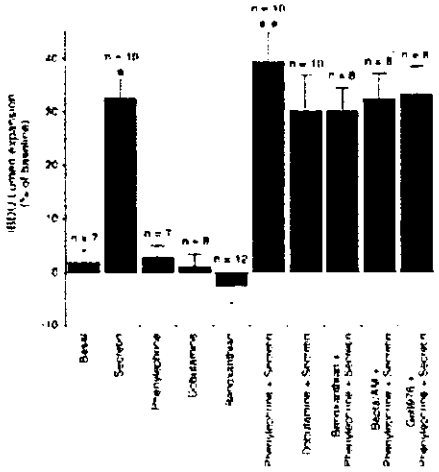

Fig. 2. Effect of phenylephrine and dobutamine on basal and secretin-induced increases in IBDU lumen space in BDL rats. Neither phenylephrine nor dobutamine altered IBDU lumen space, but phenylephrine (but not dobutamine) increased the stimulatory effect of secretin on IBDU lumen space. The α1-adrenergic receptor antagonist benoxathian, BAPTA/AM, and Gö6976 blocked the stimulatory effect of phenylephrine on secretin-induced increases on IBDU lumen space. Benoxathian did not alter lumen space in IBDU from BDL rats. Data are expressed as the mean ± SEM. *$P < .05$ versus the corresponding basal value. **$P < .05$ versus secretin-induced increases in IBDU lumen space. IBDU, intrahepatic bile duct unit.

cholangiocytes from BDL rats (Fig. 3). Phenylephrine did not increase basal cAMP levels of cholangiocytes from BDL rats (Fig. 3). Phenylephrine increased secretin-stimulated cAMP levels of cholangiocytes from BDL rats (Fig. 3). Dobutamine did not alter basal or secretin-stimulated cAMP levels of cholangiocytes from BDL rats (results not shown). Phenylephrine-induced increase in secretin-stimulated cAMP levels was blocked by BAPTA/AM and Gö6976 (Fig. 3).

Phenylephrine Induces Membrane Translocation of PKC-α and PKC-β-II. Phenylephrine increased intracellular IP_3 and $[Ca^{2+}]_i$ levels more in normal than BDL cholangiocytes (Table 4). The lowered effect of phenylephrine on IP_3 and $[Ca^{2+}]_i$ of cholangiocytes from BDL rats (compared with normal cholangiocytes) may be due to the loss of inositol 1,4,5-triphosphate receptors in cholangiocytes following BDL.[32] In support of this concept, studies have shown[33] that attenuation of phenylephrine effects on $[Ca^{2+}]_i$ in cholestatic hepatocytes of BDL rats is due to the lowered expression of receptor-operated Ca^{2+} channels.

The protein for PKC-α, -βI, -βII, -γ, δ, ϵ, θ, η, and ζ was expressed by cholangiocytes from BDL rats (Fig. 4A). Phenylephrine did not increase protein expression for PKC-β-I, -γ, -δ, -ϵ, -θ, -η, and -ζ but increased PKC-α and -β-II protein expression (Fig. 4A). Phenylephrine decreases the expression of the Ca^{2+}-independent PKC-ϵ, -θ, and -η (Fig. 4A). The effect of adrenergic receptor agonists on the expression of PKC-ϵ and θ is cell specific. Although adrenergic receptor agonists activate PKC-ϵ in adult rat ventricular myocytes,[34,35] these agonists downregulate PKC-θ in rat soleus muscle.[36]

In cholangiocytes treated with BSA, the majority of PKC-α and -β-II was found in the cytosolic fraction (Supplementary Fig. 2); however, upon the addition of phenylephrine, PKC-α and -β-II protein expression decreases in the cytosolic fraction (Supplementary Fig. 2). Following phenylephrine treatment, loss of PKC-α and -β-II from the cytosolic fraction was associated with increased protein expression of these PKC isoforms in the membrane fraction of cholangiocytes (Supplementary Fig. 2). The mobile PKC isoforms (α, β-II, ϵ, and θ) were translocated to cholangiocyte membranes (with corresponding loss of PKC protein expression in the cytosol fraction) by phorbol 12-myristate 13-acetate, whereas nonmobile isoforms (η and ζ) and β-actin were not translocated to cholangiocyte membranes (Fig. 4B).

Phenylephrine-Induced Increase in PKC-α and PKC-β-II Membrane Translocation Is Associated With Upregulation of PKA Activity in Cholangiocytes. We evaluated if a phenylephrine-induced increase in PKC-α and PKC-β-II expression causes enhancement of

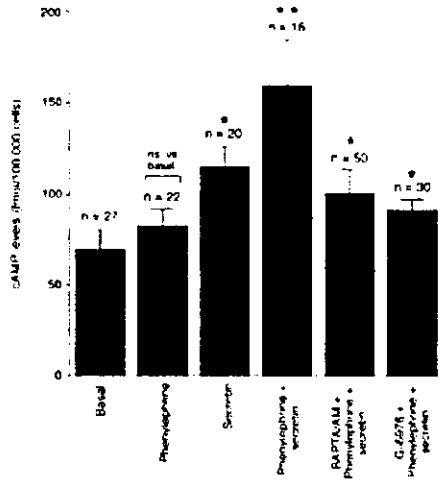

Fig. 3. Effect of phenylephrine on basal and secretin-stimulated cAMP levels of cholangiocytes from BDL rats. Phenylephrine did not increase basal cAMP levels of cholangiocytes from BDL rats. Phenylephrine increased secretin-stimulated cAMP levels of cholangiocytes from BDL rats. Phenylephrine-induced increase in secretin-stimulated cAMP levels was blocked by BAPTA/AM and Gö6976. Data are expressed as the mean ± SEM. *$P < .05$ versus the corresponding basal value. **$P < .05$ versus secretin-stimulated cAMP levels. cAMP, cyclic adenosine monophosphate; ns, not significant.

Table 4. Effect of Phenylephrine on IP_3 and $[Ca^{2+}]i$ Levels in Purified Cholangiocytes From Normal and BDL Rats

	IP_3 Levels (pmol/1 × 10^6 Cells)		$[Ca^{2+}]i$ Levels (nmol/L)	
	Basal	Phenylephrine	Basal	Phenylephrine
Normal cholangiocytes	0.11 ± 0.01 (n = 8)	0.37 ± 0.01*† (n = 8)	203.44 ± 1.18 (n = 10)	249.8 ± 2.48*† (n = 10)
BDL cholangiocytes	0.12 ± 0.01 (n = 8)	0.21 ± 0.01* (n = 8)	204.72 ± 0.13 (n = 10)	234.24 ± 0.33* (n = 10)

NOTE. Before IP_3 and Ca^{2+} measurements, isolated cholangiocytes from normal and BDL rats were incubated for 1 hour at 37°C. For IP_3 measurements, cholangiocytes were stimulated for 10 minutes at 22°C with 0.2% BSA or phenylephrine (10 μmol/L) with 0.2% BSA. Intracellular IP_3 levels were assessed with the IP_3 [^{3}H] kit (Amersham). Calcium fluorescence measurements in cholangiocytes from BDL rats were performed using fluo-3 AM (Molecular Probes) and a Fluoroskan Ascent FL (ThermoLabsystems) microplate reader equipped with three injectors. Phenylephrine increased intracellular IP_3 and $[Ca^{2+}]i$ levels more in normal cholangiocytes than cholangiocytes from BDL rats. Data are the mean ± SEM.

*$P < .05$ versus the corresponding basal value.

†$P < .05$ indicates that phenylephrine-induced increases in intracellular IP_3 and $[Ca^{2+}]i$ levels are higher in normal cholangiocytes than cholangiocytes from BDL rats.

secretin-stimulated PKA activity and found that phenylephrine alone did not alter PKA activity (Fig. 5). Secretin increased PKA activity and phenylephrine enhanced secretin-induced increases in PKA activity in cholangiocytes (Fig. 5). The stimulatory effect of phenylephrine on secretin-stimulated PKA activity was ablated by benoxathian, BAPTA/AM and Gö6976 (Fig. 5).

Discussion

This study demonstrated that: (1) α-1A/-1C, -1B, and β-1 adrenergic receptors are expressed by normal cholangiocytes and up-regulated following BDL; (2) adrenergic receptors are mostly present in the basolateral domain of cholangiocytes; (3) phenylephrine increases IP_3 and $[Ca^{2+}]_i$ levels more in normal than BDL cholangiocytes; (4) phenylephrine (but not dobutamine) increases secretin-stimulated ductal secretion in BDL rats, an increase that was blocked by benoxathian, BAPTA/AM, and Gö6976; and (5) the stimulatory effect of phenylephrine on secretin-induced ductal secretion was associated with enhanced secretin-stimulated cAMP levels and PKA activity and membrane translocation of PKC-α and -β-II. Upregulation of adrenergic receptors in cholangiocytes (following BDL) may be important in the regulation of ductal secretion in liver diseases.

We first showed that: (1) α-1A/-1C, -1B, and β-1 adrenergic receptors are present in the basolateral domain of cholangiocytes; and (2) the expression of these receptors is low in normal cholangiocytes and upregulated following BDL. Although the adrenergic receptors are preferentially distributed in the basolateral membrane of cholangiocytes, the internalization due to different stimuli (*e.g.*, stress due to handling before anesthesia, which is a powerful stressor) can explain the cytoplasmic staining observed by immunohistochemistry in liver sections. In support of this, studies have shown[37] that the majority of β-1 adrenergic receptors is localized on the membrane of peripheral cytoplasmic vesicles as a result of the internalization of surface receptors before killing. The internalization of adrenergic receptors is due to a dramatic rise in plasma catecholamines, which is induced by stress.[38] In newborn rat liver, β-adrenoreceptor internalization occurs a few minutes after delivery as a consequence of a surge in plasma catecholamines.[39] Next, we evaluated the effect of phenylephrine on basal and secretin-stimulated choleresis in normal and BDL rats and IBDUs from BDL rats. Phenylephrine did not affect basal ductal secretion but potentiated—by approximately 40%—secretin-stimulated choleresis and secretin-induced IBDU expansion in BDL rats. Secretin-stimulated choleresis is not present *in vivo* (when infused through a jugular vein) in normal rats (where cholangiocytes represent less than 2%-3% of total liver cells), but it becomes evident in BDL rats in which cholangiocyte mass increases to up to 40% of the total liver mass.[1] The potentiation of secretin-stimulated ductal secretion in BDL rats by phenylephrine was due to a specific effect on the secretory activity of bile ducts as demonstrated in IBDUs from BDL rats, where phenylephrine increased secretin-induced expansion of lumen space, which was blocked by benoxathian. These findings demonstrated a direct interaction of phenylephrine with α-1 adrenergic receptors on cholangiocytes and allowed us to exclude that the potentiation of secretin-stimulated choleresis observed *in vivo* was a consequence of a systemic vascular effect of phenylephrine.[31]

We focused on the mechanisms involved in the potentiation of secretin-stimulated ductal secretion by phenylephrine. Similar to what is shown in other cells,[40] the activation of cholangiocyte α-1 adrenergic receptors by phenylephrine increased $[Ca^{2+}]_i$ and IP_3 levels. The potentiation of secretin-stimulated fluid secretion in IBDUs was abolished by BAPTA/AM and Gö6976, suggesting that mobilization of IP_3-sensitive Ca^{2+} stores is required for adrenergic potentiation of

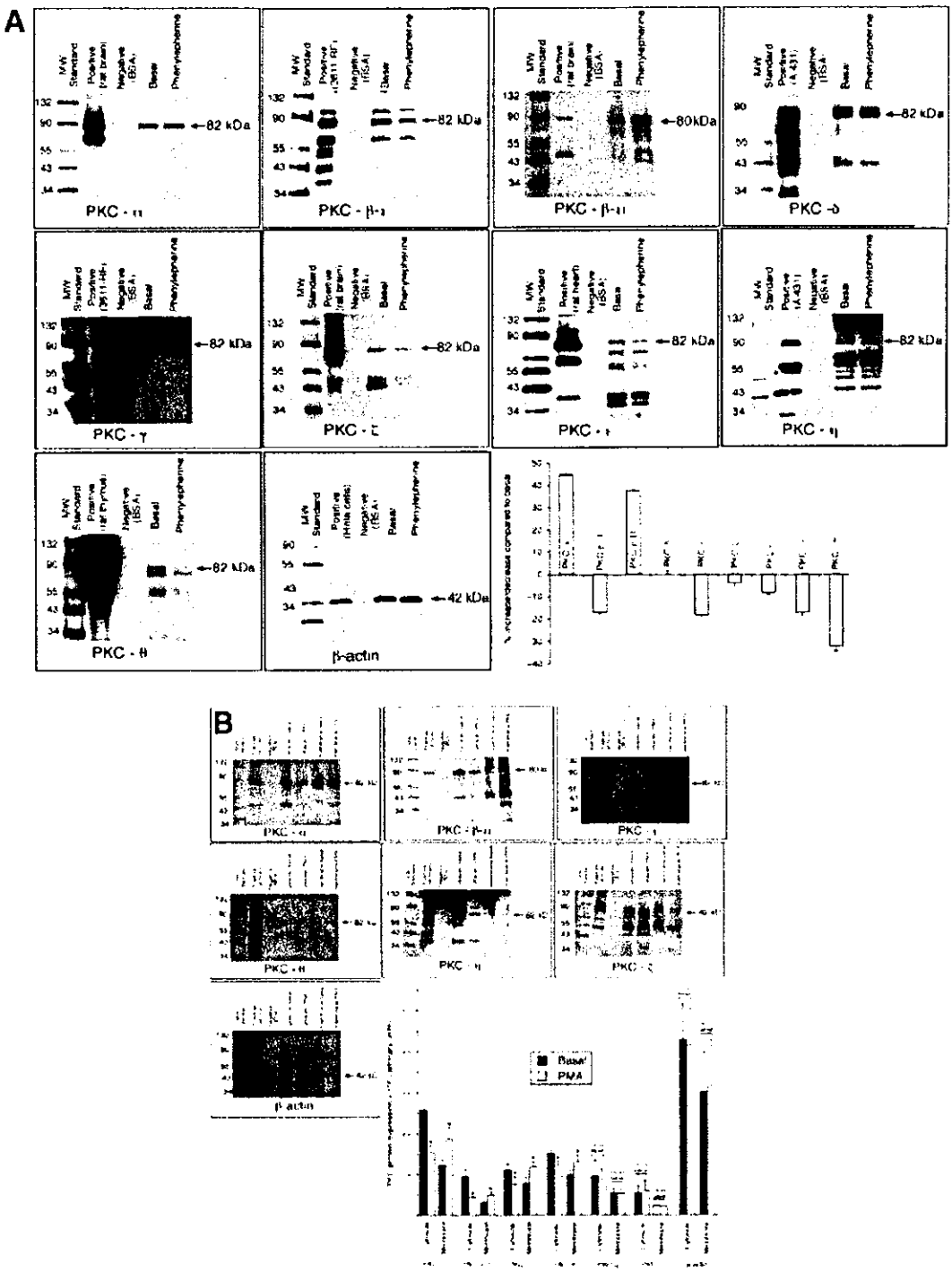

Fig. 4. (A) Effect of phenylephrine on protein expression for the Ca^{2+}-dependent PKC-α, PKC-β-I, PKC-β-II and PKC-γ, and the Ca^{2+}-independent novel (δ, ϵ, and θ) and atypical (η and ζ) isoforms in cholangiocytes from BDL rats treated *in vitro* for 90 minutes with 0.2% BSA (basal) or phenylephrine (100 μmol/L in 0.2% BSA). Phenylephrine increased protein expression of PKC-α and PKC-β-II in purified cholangiocytes. $^*P < .05$ versus basal values. Data are expressed as the mean ± SEM. A-431, human epidermoid carcinoma; HeLa, human epithelioid carcinoma; 3611-RF, rat-Raf1-transformed fibroblasts. (B) The mobile PKC isoforms (α, β-II, ϵ, and θ) were translocated to cholangiocyte membranes by phorbol 12-myristate 13-acetate, whereas nonmobile isoforms (η and ζ) and β-actin were not translocated to cholangiocyte membranes. $^*P < .05$ vs. corresponding basal values. $P < .05$ vs. corresponding value of the cytosolic fraction. Data are expressed as the mean ± SEM of 6 experiments. BSA, bovine serum albumin; PKC, protein kinase C; ns, not significant.

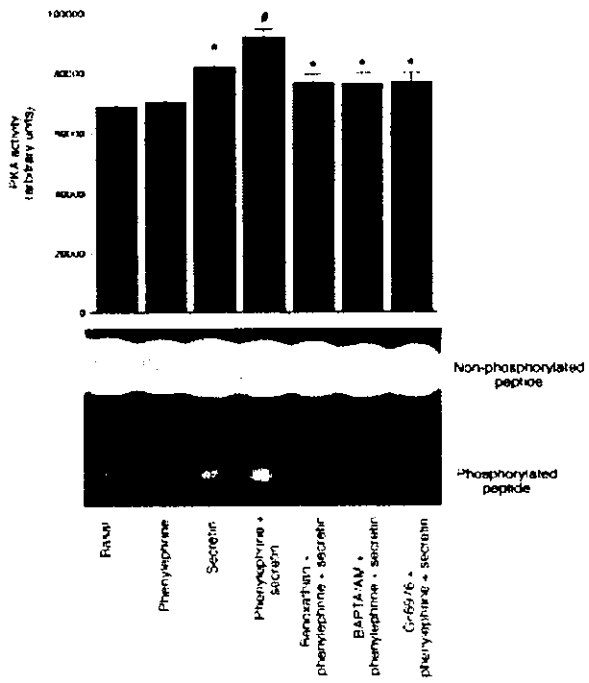

Fig. 5. Measurement of PKA activity in purified cholangiocytes (5×10^6) from BDL rats treated at 37°C with: (1) 0.2% BSA (basal) for 30 minutes; (2) secretin (30 minutes at 100 nmol/L) with 0.2% BSA; (3) phenylephrine (30 minutes at 10 μmol/L) with 0.2% BSA; (4) phenylephrine (30 minutes at 10 μmol/L) before stimulation with secretin (30 minutes at 100 nmol/L) with 0.2% BSA; or (5) benoxathian, BAPTA/AM, or Gö6976 (20 minutes each) before treatment with phenylephrine (30 minutes at 10 μmol/L) followed by secretin (30 minutes at 100 nmol/L) stimulation with 0.2% BSA. Phenylephrine did not alter PKA activity. Our data showed that secretin increased PKA activity and that phenylephrine enhanced secretin-induced increase in PKA activity in cholangiocytes. The stimulatory effect of phenylephrine on secretin-stimulated PKA activity was ablated by benoxathian, BAPTA/AM, and Gö6976. PKA assay was performed according to the manufacturer's instructions using PepTag Assay Protein Kinase Kits (Promega). Phosphorylated peptide bands were quantitated via scanning densitometry using the Chemilmager 4000 low light imaging system (Alpha Innotech Corp.). Data are expressed as the mean ± SEM of 11 experiments. $^*P < .05$ vs. its corresponding basal value. $^\#P < .05$ vs. PKA activity of cholangiocytes treated *in vitro* with: (1) secretin, (2) benoxathian + phenylephrine + secretin, (3) BAPTA/AM + phenylephrine + secretin, or (4) Gö6976 + phenylephrine + secretin. PKA, protein kinase A.

secretin-stimulated ductal secretion. In support of this, in other cells including salivary glands, adrenergic agonists induce Cl^- secretion by increasing $[Ca^{2+}]_i$ release from IP_3-sensitive stores.[41] Similar to the previous study by Shibao et al. showing impaired acetylcholine-induced Ca^{2+} and IP_3 signaling in cholangiocytes and reduced acetylcholine-dependent bicarbonate secretion in BDL rats that appears to be principally related to loss of IP_3 receptors,[32] our study showed blunted IP_3 and Ca^{2+} responses to phenylephrine in BDL cholangiocytes compared with normal cholangiocytes. Despite this impairment, phenylephrine-induced Ca^{2+} and IP_3 signaling potentiated secretin-induced cholangiocyte cAMP synthesis and secretion in IBDUs from BDL rats. The involvement of Ca^{2+}-sensitive PKC isoforms was indicated by a sensitivity of phenylephrine to Gö6976, and membrane translocation of PKC-α and -β-II. Our data suggest that the potentiation of secretin-stimulated ductal secretion by phenylephrine is mediated by the activation of the Ca^{2+}-dependent isoforms PKC-α and -β-II. Because a PKC-β-II specific inhibitor is not commercially available, our studies do not provide conclusive evidence on the role of PKC-β-II in phenylephrine stimulation of secretin-stimulated choleresis. In support of the notion that different PKC isoforms can be activated differentially, studies have shown[7] that gastrin inhibits ductal hyperplasia and secretion via activation of PKC-α. Insulin and ursodeoxycholate inhibition of secretin-stimulated choleresis is associated with activation of PKC-α.[21,42] D2 dopaminergic receptor agonists inhibit secretin-stimulated ductal secretion via activation of PKC-γ.[11] The differential cross-talk between $[Ca^{2+}]_i$ and adenylyl cyclase (which leads to stimulatory or inhibitory effects on secretin-stimulated cAMP levels and ductal secretion)[7,9,11,21,22,42] depends on the type of receptor (M3 acetylcholine,[9] D2 dopaminergic,[11] insulin,[21] or gastrin[7]) or transporter (apical bile acid transporter[22,42,43]) that is up- or downregulated.[22,42] Although there is activation of $[Ca^{2+}]_i$ but not PKC with acetylcholine,[9] there is activation of intracellular IP_3/Ca^{2+} and specific PKC isoforms with D2 dopaminergic agonists (PKC-γ),[11] gastrin (PKC-α),[7] insulin (PKC-α),[21] or ursodeoxycholate (PKC-α).[42] These interactions can induce a differential cross-talk between $[Ca^{2+}]_i$ and specific adenylate cyclase isoforms leading to inhibition or stimulation of adenylate cyclase and secretin-stimulated ductal secretion.

Adenylyl cyclase isoforms (more than 12) are regulated differently by Ca^{2+} and PKC depending on the cell types and which agonist or antagonist is involved. Type II adenylate cyclase is positively modulated by PKC-α.[44] Adenylyl cyclase 8 is stimulated by Ca^{2+}-calmodulin pathways, and through this mechanism muscarinic agents increase agonist-induced cAMP release in different cells. In other cells, adenylyl cyclase isoforms 5 and 6 are inhibited by Ca^{2+} agonists through a calmodulin-independent mechanism, which leads to decreased cAMP release.[45,46]

In summary, cholangiocyte secretion is positively modulated by the cholinergic system via acetylcholine M3 receptors[5,9] and the adrenergic system via α-1 adrenergic receptor agonists. However, although acetylcholine positively modulates adenylate cyclase through Ca^{2+} and calmodulin-dependent but PKC-insensitive

pathways, α-1 adrenergic receptor agonists act through the activation of PKC-α and PKC-β-II. In contrast to the cholinergic and adrenergic system, the dopaminergic innervation of intrahepatic bile ducts negatively modulates secretin-stimulated choleresis through a downregulation of adenylate cyclase, which is mediated by the activation of PKC-γ.[11] Cholangiocyte proliferation is a typical hallmark of cholangiopathies,[2] where enhanced secretion of proliferating cholangiocytes compensates for bile duct damage. Coordinated stimulation of ductal secretion by secretin (via cAMP) and adrenergic agonists (via Ca^{2+}/PKC) may induce maintaining maximal bicarbonate excretion in proliferating ducts, thus compensating for the impairment of secretion in damaged ducts.

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Nerve Growth Factor Modulates the Proliferative Capacity of the Intrahepatic Biliary Epithelium in Experimental Cholestasis

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Background & Aims: We evaluated the expression of neurotrophins in rat cholangiocytes and the role and mechanisms by which nerve growth factor (NGF) modulates cholangiocyte proliferation. *Methods:* The expression of neurotrophins and their receptors was investigated by immunohistochemistry in liver sections and reverse-transcription polymerase chain reaction and immunoblots in isolated cholangiocytes. In vitro, the effect of NGF on cholangiocyte proliferation and signal transduction was investigated by immunoblotting for proliferating cell nuclear antigen, phosphorylated AKT (p-AKT), phosphorylated extracellular signal–regulated kinase 1/2 (p-ERK1/2), phosphorylated c-Jun-N-terminal kinase, and phosphorylated p38. In vivo, rats that had undergone bile duct ligation (BDL) were treated with an anti-NGF antibody to immunoneutralize NGF and bile duct mass, proliferation, apoptosis, and inflammation were investigated by immunohistochemistry. *Results:* NGF and its TrkA receptor were expressed by normal rat cholangiocytes and up-regulated following BDL. Cholangiocytes secrete NGF, and secretion is increased in proliferating BDL cholangiocytes. In vitro, NGF stimulated cholangiocyte proliferation, which was associated with enhanced p-AKT and p-ERK1/2 expression. NGF proliferation in vitro was partially blocked by the MEK inhibitor (UO126) and completely ablated by the phosphatidylinositol 3-kinase inhibitor (wortmannin). In vitro, NGF and estrogens have an additive effect on cholangiocyte proliferation by acting on phosphorylated TrkA and p-ERK1/2. In vivo, immunoneutralization of NGF decreased bile duct mass in BDL rats, which was associated with depressed proliferation and enhanced apoptosis and with increased portal inflammation. *Conclusions:* Cholangiocytes secrete NGF and express NGF receptors. NGF induces cholangiocyte proliferation by activating the ERK and, predominantly, the phosphatidylinositol 3-kinase pathway and exerts an additive effect in combination with estrogens on proliferation.

Cholangiocytes are the epithelial cells lining intrahepatic bile ducts that are characterized by marked proliferative capacities, which are evidenced under experimental conditions as well as during human cholangiopathies.[1–3] At the experimental level, dietary manipulations, partial hepatectomy, or bile duct ligation (BDL) lead to marked proliferation of intrahepatic cholangiocytes.[1–3] The BDL rat is the most widely used experimental model, in which a typical and selective cholangiocyte proliferation leads to a marked increase in intrahepatic bile duct mass.[1–3] In the human pathology, cholangiocyte proliferation is a typical hallmark of all cholangiopathies, which condition, as a repair and compensatory mechanism, the evolution of the disease toward the terminal ductopenic stage.[1–3] During proliferation, cholangiocytes display enhanced secretory activities and responsiveness to the secretory stimulus of various hormones and peptides, including secretin and acetylcholine.[1–5] Furthermore, proliferating cholangiocytes acquire phenotypical features of neuroendocrine epithelium, including (1) expression of neuroendocrine markers (chromogranin A, glycolipid A2-B4, S-100 protein, neural cell adhesion molecule) and acquisition of neuroendocrine granules[6,7]; (2) expression of the parathyroid hormone–related peptide, which is encoded by a

Abbreviations used in this paper: BDL, bile duct ligation; ERK, extracellular signal–regulated kinase; JNK, c-jun-N-terminal kinase; NGF, nerve growth factor; p-AKT, phosphorylated AKT; PCNA, proliferating cellular nuclear antigen; p-ERK1/2, phosphorylated extracellular signal–regulated kinase 1/2; PI3-kinase, phosphatidylinositol 3-kinase; p-TrkA, phosphorylated TrkA; RT-PCR, reverse-transcription polymerase chain reaction; t-TrkA, total TrkA; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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growth factor–regulated early-response gene and is involved in the growth and differentiation of the cell[8]; (3) increased expression and response to endothelin[9]; and (4) enhanced response to hormones/neuropeptides such as secretin, somatostatin, and acetylcholine.[1–5,10]

Nerve growth factor (NGF) is a member of the neurotrophin family, which regulates growth and differentiation of target tissues by acting on specific tyrosine kinase receptors of the Trk family (NTr).[11,12] Different NTr have been identified, including TrkA, TrkB, TrkC, and the low-affinity p75 receptor.[11,12] NGF and the related receptors have recently been identified in different non-nervous tissues where this neurotrophin plays a role in the regulation of proliferation, differentiation, remodeling, and inflammation.[11,12] However, no information exists regarding the role of neurotrophins in the modulation of cholangiocyte pathophysiology. The aims of this study were to evaluate (1) the expression of NGF, other neurotrophins, and the related receptors in cholangiocytes of normal and BDL rats and (2) the role and mechanisms of NGF in the regulation of cholangiocyte proliferation.

Materials and Methods

Male 344 Fischer rats (225–250 g) were purchased from Charles River Italia (Calco, Italy) and fed ad libitum in a light- and temperature-controlled environment. The study protocols were in compliance with the institution's guidelines. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. BDL was performed as previously described.[5] Normal and 1-week BDL rats were used for immunohistochemistry in liver sections as well as for isolation of cholangiocytes.

Isolation of Cholangiocytes

Cholangiocytes were isolated from normal or 1-week BDL rats by immunomagnetic separation[4,5,9] with a viability >90% (trypan blue exclusion). The purity of cholangiocyte preparations was assessed by (1) γ-glutamyltransferase–positive staining,[13] (2) glucose-6-phosphatase staining[14] and reverse-transcription polymerase chain reaction (RT-PCR) for albumin (hepatocyte markers), (3) RT-PCR for fucose receptor (Kupffer cell marker), and (4) RT-PCR for von Willebrand factor (endothelial cell marker). All isolated cells were γ-glutamyltransferase positive, whereas glucose-6-phosphatase–positive cells were absent and RT-PCR for von Willebrand factor, fucose receptor, and albumin was negative in cell preparations from both normal and BDL livers, indicating absolute purity of cholangiocyte preparations. NGF was measured in the rat serum and in supernatant of isolated cholangiocytes from normal and 1-week BDL rats, suspended in 1× hepes buffer saline (HBS), by using a Sandwich ELISA Kit (Chemicon International, Inc. Temecula, CA) according to the manufacturer's instructions. In the supernatant of isolated cholangiocytes from normal and 1-week BDL rats, we also measured K^+ concentration as a marker of cell permeability by using QuikLYTE Integrated Multisensor Technology (Dade Behring, Inc., Deerfield, IL). To evaluate whether NGF secreted by proliferating cholangiocytes may induce cell proliferation, the supernatant of normal and BDL cholangiocytes (after 4 hours of incubation) was transferred into plates containing normal quiescent, freshly isolated cholangiocytes. After 4 hours of incubation in the presence or absence of an immunoneutralizing anti-NGF antibody (150 pg/mL of supernatant), the protein expression of proliferating cell nuclear antigen (PCNA) (Western blot) in cholangiocytes was measured as a marker of cell proliferation.

Immunohistochemical Studies on Liver Samples

Formalin-fixed, paraffin-embedded liver sections were stained with H&E for routine examination. To determine the degree of portal inflammation, inflammatory grading was blindly performed by an independent pathologist as previously described.[15,16] For NGF and TrkA immunodetection, formalin-fixed, paraffin-embedded liver sections were obtained from normal or BDL rats. Sections were immersed in 0.01 mol/L citrate buffer (pH 6.0) and irradiated in a microwave oven for 15 minutes. Liver sections were then incubated with or without the polyclonal primary antibody anti-NGF or anti-TrkA (1:20 dilution in phosphate-buffered saline; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at room temperature, and the reactive sites were detected using a Dako LSAB kit (according to the manufacturer's instructions) followed by incubation for 5 minutes with phosphate-buffered saline containing 0.06% diaminobenzidine and 0.01% H_2O_2 added just before use. To control the specificity of anti-NGF and anti-TrkA antibodies, the primary antibody was combined with a 5-fold excess of blocking peptide (Santa Cruz Biotechnology) and incubated overnight at 4°C (as recommended by the manufacturer).

RT-PCR Analysis of Neurotrophins and Related Receptors in Isolated Cholangiocytes

Total cellular RNA was extracted from cholangiocytes immunoisolated from normal or 1-week BDL rats by using the Micro-Fast Track II Kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Total RNA (1 μg) was used for first-strand complementary DNA synthesis by AMV reverse transcriptase (Roche Diagnostics, Mannheim, Germany). Degenerate oligonucleotide primers were synthesized based on the published sequence for the rat neurotrophins and related receptors as follows. NGF: 5′-CCAAGGACGCAGCTTTCTAT-3′ (forward), 5′-CTCCGGTGAGTCCTGTTGAA-3′ (reverse); brain-derived neurotrophic factor: 5′-ATTTGTCCGAGGTGGTAGTACTTCATC-3′ (forward), 5′-AGGAGGCTCCAAAGGCACTTGACT-3′ (reverse); neurotrophin 3: 5′-TTACAGGTGAACAAGGTGAT-3′ (forward), 5′-ACGAGTTTGTTGTTTTCTGA-3′ (reverse); neurotrophin 5: 5′-CCCTGCGT-

CAGTACTTCTTCGAGAC-3′ (forward), 5′-CTGGACGT-CAGGCACGGCCTGTTC-3′ (reverse); TrkA: 5′-CTGAG-GTCTCTGTCCAAGTC-3′ (forward), 5′-CCCAAAAG-GTGTTTCGTCCT-3′ (reverse); TrkB: 5′-GGCCAAGAATGA-ATATGGGAA-3′ (forward), 5′-TTGAGCTGG-CTGTTGGT-GAT-3′ (reverse); TrkC: 5′-AGCTGCTCACTAACCTGCAG-CATG-3′ (forward), 5′-GCTAAAGATCTCCCAAAGAA-TAAC-3′ (reverse); low-affinity NGF receptor: 5′-TGCTGCT-GCTGC-TGATTCTA3′ (forward), 5′-GACCTTGGGATC-CATCGAC-3′ (reverse); neurophilin 1: 5′-CGCCTGGTGAGC-CCTGTGGTCTATT-3′ (forward), 5′-TGTTCTTGTCGCC-TTTCCCTTCTTC-3′ (reverse); albumin (hepatocytes): 5′TTGC-CTTTTCCCAGTATCTCCA-3′ (forward), 5′ACACTCGTT-TCTTTCGGGCT3′ (reverse); von Willebrand factor (endothelial cells): 5′GAGCGGTGCTCC-TTTGAGGA-3′ (forward), 5′-CACGTAGTCCTCGTGACAGC-3′ (reverse); fucose receptor (Kupffer cells): 5′GGAGGATGAAGGAGGCGGAACTG-3′ (forward), 5′GCCCCCAAGCAACTGCACC-3′ (reverse). Albumin, von Willebrand factor, and fucose receptor were used as markers of purity, whereas glyceraldehyde-3-phosphate dehydrogenase was used as housekeeping gene. Rat brain RNA was used as positive control in all cases with the exception of albumin, von Willebrand factor, and fucose receptor, where total rat liver was used as positive control. Standard RT-PCR conditions were used (35 step cycles: 60 seconds at 94°C, 45 seconds at 55°C, and 60 seconds at 72°C). PCR products were subcloned and sequenced.

Western Blot Analysis

For Western blot analysis, cholangiocytes were solubilized in lysis buffer containing 0.125 mol/L Tris HCl (pH 6.8), 10% sodium dodecyl sulfate, 2 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L benzamidine, and 1% aprotinin at 4°C for 1 hour. After centrifugation at 10,000*g* for 30 minutes at 4°C, the supernatant was recovered and protein concentration was determined with the Lowry method.[17] Cell extracts (100 μg) were diluted in 2× Laemmli sample buffer containing 0.3 mol/L 2-mercaptoethanol and resolved by 7.5% or 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Western blotting was performed as described[4,5,9] by using the following antibodies (obtained from Santa Cruz Biotechnology): (1) anti-PCNA mouse monoclonal antibodies (1:400 dilution), (2) anti-NGF rabbit polyclonal antibody (1:100 dilution), (3) anti-total TrkA (t-TrkA) rabbit polyclonal antibody (1:100 dilution), (4) anti-phosphorylated TrkA (p-TrkA) mouse monoclonal antibody (1:200 dilution), (5) anti-total extracellular signal–regulated kinase (ERK) 1/2 rabbit polyclonal antibody (1:1000 dilution), (6) anti–p-ERK1/2 (tyrosine-threonine diphosphorylated ERK 1/2 mouse monoclonal antibody, 1:1000 dilution), (7) anti-total AKT mouse monoclonal antibody (1:100 dilution), (8) anti-phosphorylated AKT (p-AKT) rabbit polyclonal antibody (1:250 dilution), (9) anti-total c-jun-N-terminal kinase (JNK) mouse monoclonal antibody (1:100 dilution), (10) anti-phosphorylated JNK mouse monoclonal antibody (dilution 1:100), (11) anti-total p38 mouse monoclonal antibody (dilution 1:200), and (12) anti-phosphorylated p38 mouse monoclonal antibody (1:200 dilution). For β-actin, we used a mouse monoclonal antibody obtained from Sigma Chemical Co. (1:10,000 dilution). As secondary antibodies, anti-mouse immunoglobulin G peroxidase conjugated (1:2000; Sigma Chemical Co.) or anti-rabbit immunoglobulin G peroxidase conjugated (1:10,000; Sigma Chemical Co.) were used. The intensity of the bands was determined by scanning video densitometry (Ultra Violet Products, Cambridge, England). Western blot analysis for t-TrkA and p-TrkA was also performed in cholangiocyte apical and basolateral plasma membranes prepared by isopyknic centrifugation on a 3-step sucrose gradient (38%, 34%, and 31% wt/wt) according to the technique previously described by Tietz et al.[18] The purity of the membrane preparations was tested by measuring the activity of specific markers for the basolateral (i.e., Na^+, K^+–adenosine triphosphatase) and apical (i.e., alkaline phosphatase) domain of cholangiocyte membranes as described.[18]

Treatment of BDL Rats With Anti-NGF Antibody

Immediately after BDL, 200 μL of polyclonal neutralizing NGF antibody (400 μg/dose)[19,20] (Sigma Chemical Co.) was administered intraperitoneally every day for 7 days (n = 8). BDL controls (n = 8) received nonimmune serum with the same modalities. In BDL rats treated with anti-NGF antibody or nonimmune serum, we determined (1) bile duct mass after immunohistochemical staining for γ-glutamyltransferase as previously described,[4,5,9] (2) apoptosis in situ in the liver by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) analysis as described,[4,5,9] and (3) PCNA protein expression in isolated cholangiocytes by immunoblotting. In addition, inflammation score was determined according to Ishak et al.[15]

Statistical Analysis

Data are presented as arithmetic mean ± SEM. Statistical analysis was conducted using the paired or unpaired Student *t* test as appropriate or analysis of variance when multiple comparisons were performed.

Results

Neurotrophins and Neurotrophin Receptors in Isolated Cholangiocytes and In Situ in the Liver

RT-PCR analysis. The expression of neurotrophins and neurotrophin receptors was investigated by RT-PCR in pure preparations of cholangiocytes isolated from normal (n = 3 cell preparations) or 1-week BDL rats (n = 3) by using gene-specific primers (Figure 1). NGF and TrkA (the preferred NGF receptor) were expressed in cholangiocytes isolated from both normal and BDL rat livers. Cholangiocytes isolated from normal rats also express the neurotrophin 4/5, whereas its preferred receptor TrkB was not de-

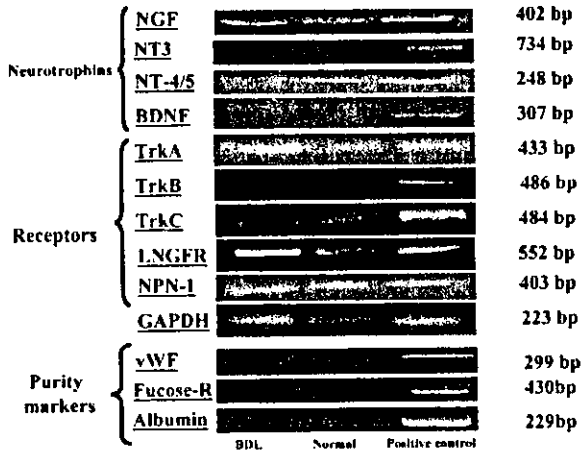

Figure 1. RT-PCR analysis of neurotrophins and related receptors in immunoisolated cholangiocytes from normal and 1-week BDL rat livers. Agarose gel 1.2% electrophoresis of RT-PCR products amplified using gene-specific primers for different neurotrophins and neurotrophin receptors. For each reaction, 1 μg of total RNA was used as template in standard RT-PCR conditions. Glyceraldehyde-3-phosphate dehydrogenase was used as housekeeping gene. Brain was used as positive control. Albumin (hepatocytes), von Willebrand factor (endothelial cells), and Fucose receptor (Kupffer cells) were tested as markers of purity and, in these cases, total liver was used as positive control. Figures are representative of n = 3 cell preparations.

tectable. The neurotrophin receptor TrkC and the low-affinity NGF receptor p75 were also expressed in normal and BDL rat cholangiocytes. In contrast, neurotrophin 3 and the brain-derived neurotrophic factor were absent in both normal and BDL rat cholangiocytes. Because neurophilin 1, the receptor for semaphorins, is involved in the modulation of NGF effects in different cells and tissues,[11,12] its expression in rat cholangiocytes was also evaluated; this showed that neurophilin-1 messenger RNA was expressed by both normal and BDL rat cholangiocytes. RT-PCR for albumin (hepatocyte marker), von Willebrand factor (endothelial cell marker), and fucose receptor (Kupffer cell marker) was negative in both normal and BDL cholangiocytes (Figure 1), and this confirms the absolute purity of our cholangiocyte preparations.

Western blot analysis of NGF and TrkA receptor in normal and BDL cholangiocytes. NGF and its preferred receptor TrkA (t-TrkA and p-TrkA) were analyzed by Western blot in pure preparations of cholangiocytes isolated from normal and 1-week BDL (n = 9) rat livers. NGF, t-TrkA, and p-TrkA were significantly increased ($P < 0.02$) in proliferating cholangiocytes isolated from BDL rats as compared with cholangiocytes isolated from normal rats (Figure 2). In cholangiocyte plasma membrane subfractions, we found that t-TrkA was expressed (Western blot) in the basolateral fraction of both normal and BDL rat cholangiocytes, whereas p-TrkA was found only in the basolateral fraction of BDL rat cholangiocytes (Figure 2). The apical membranes of both normal and BDL cholangiocytes showed undetectable levels of both t-TrkA and p-TrkA (Figure 2).

Measurement of NGF in the supernatant of isolated cholangiocyte suspensions and in the rat serum. Cholangiocytes were isolated from normal or BDL rat livers, sus-

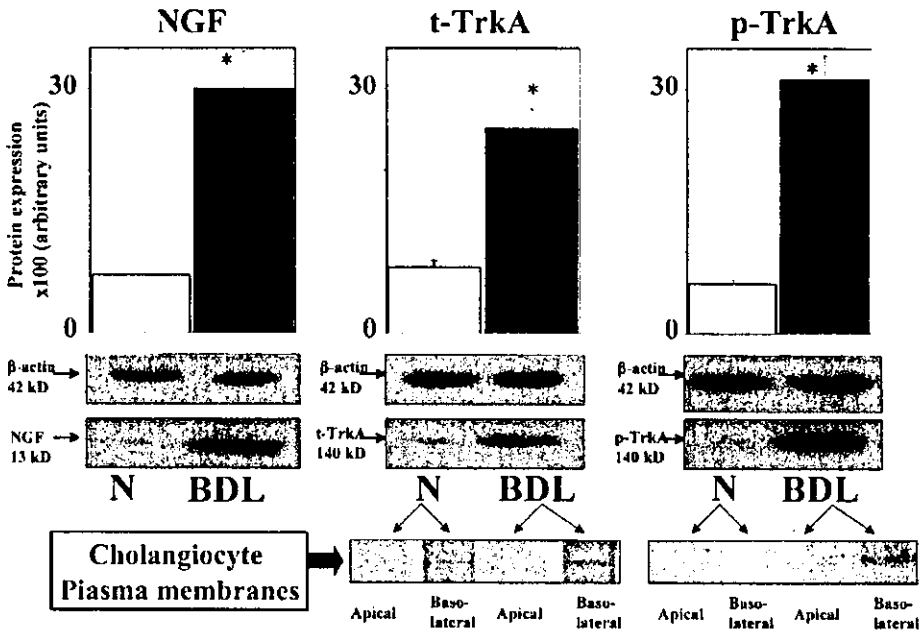

Figure 2. Western blot analysis of NGF, t-TrkA, and p-TrkA in cholangiocytes isolated from normal and 1-week BDL rat livers. Cholangiocytes from BDL rats showed a higher expression of NGF, t-TrkA, and p-TrkA ($P < 0.02$) than cholangiocytes isolated from normal rat livers. Protein expression of β-actin was similar between normal and BDL cholangiocytes. Figures are representative of n = 9 cell preparations. *$P < 0.02$. In cholangiocyte plasma membrane subfractions, t-TrkA was expressed in the basolateral fraction of both normal and BDL rat cholangiocytes while p-TrkA was expressed only in the basolateral fraction of BDL rat cholangiocytes. The apical membranes of both normal and BDL cholangiocytes showed undetectable levels of both t-TrkA and p-TrkA. Figure is representative of n = 3 membrane subfraction preparations.

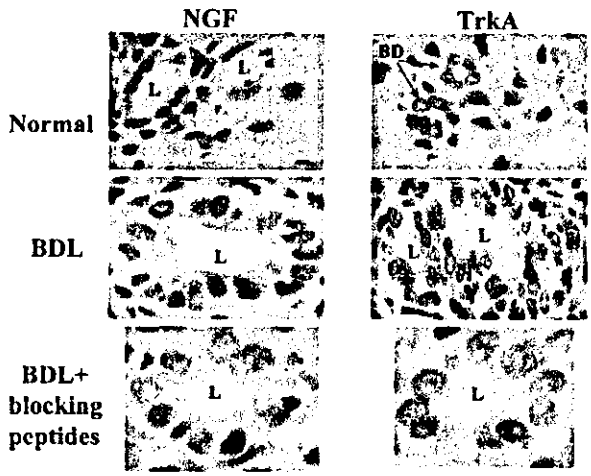

Figure 3. Immunohistochemistry for NGF and TrkA in 1-week BDL and sham-operated BDL rat livers. Cholangiocytes of proliferating bile ducts (BDL) showed a strong positivity (*middle panels*) after incubation with anti-NGF or anti-TrkA antibodies, whereas no staining was observed in the BDL negative control liver sections when primary antibodies were neutralized with blocking peptides (*lower panels*). In sham-operated rats (normal, *upper panels*), bile ducts showed absence of a specific reaction after incubation with either NGF or TrkA antibodies. BD, bile ducts; L, bile duct lumen. (Original magnification 150×.)

pended in 1× HBS, and incubated for 4 hours. Subsequently, NGF levels were measured in the supernatants. NGF concentration in the supernatant was 3 times higher for cholangiocytes isolated from 1-week BDL rats (34.7 ± 2.1 pg/mL; n = 7 cell preparations; 100 million cells/100 μL supernatant) in comparison with normal rats (12.9 ± 3.7 pg/mL; n = 7 cell preparations; 100 million cells/100 μL supernatant). The K^+ concentration in the supernatant (basal = 4.9 mEq/L) was similar, after 4 hours, between normal (5.21 ± 0.05 mEq/L) and BDL (5.24 ± 0.07 mEq/L) cholangiocytes, thus excluding that the increased NGF concentration in the supernatant was just an expression of an increased permeability of BDL cholangiocytes. NGF concentration in the serum of BDL rats was 4-fold higher than in normal rats (208 ± 72 vs. 51 ± 9 pg/mL; $P < 0.02$; n = 8).

Immunohistochemistry for NGF and TrkA in the liver. In sham-operated rats, bile ducts showed an absence of a specific reaction after incubation with either NGF or TrkA antibody (Figure 3). In 1-week BDL rat liver, proliferating bile ducts were strongly positive after incubation with anti-NGF antibody (Figure 3). The product of reaction appeared diffusely localized in the cytoplasm of the cholangiocytes. TrkA was also expressed by bile ducts after BDL (Figure 3). In fact, a specific positive reaction appeared in the cytoplasm, mainly in the perinuclear region and in proximity to the plasma membrane. When anti-NGF and anti-TrkA antibodies were neutralized with blocking peptides, BDL liver sections were negative (Figure 3), demonstrating the specificity of the immunohistochemical staining.

In Vitro Effect of NGF on Cholangiocyte Proliferation

Pure preparations of cholangiocytes isolated from normal rat livers were incubated with NGF (100 ng/mL culture medium), and PCNA protein expression was evaluated as a marker of cell proliferation. PCNA was expressed at low levels in cholangiocytes isolated from normal rat liver (which are normally in a quiescent state) but was significantly enhanced after 3 hours of incubation with NGF ($P < 0.02$; n = 8 cell preparations; Figure 4). This indicates that NGF stimulates cholangiocyte proliferation. To investigate the NGF signaling, we measured protein expression of total and phosphorylated ERK1/2, AKT (downstream effector of the phosphatidylinositol 3-kinase [PI3-kinase] pathway[21]), JNK, and p38 after incubation of normal cholangiocytes with NGF. Without significant changes of total protein (total ERK1/2 and total AKT), the protein expression of p-ERK1/2 and p-AKT increases over basal levels starting 5 minutes after incubation with NGF and remaining stable up to 180 minutes ($P < 0.02$ vs. basal; Figure 4; n = 8). The protein expression of phosphorylated JNK and phosphorylated p38 was also enhanced ($P < 0.02$) after incubation with NGF (Figure 4), but their increase occurred later (30–60 minutes) than the increase of p-ERK1/2 and p-AKT. Total JNK and p38 protein expression was unchanged by NGF.

To further explore the involvement of PI3-kinase and ERK pathways in NGF-induced cholangiocyte proliferation, we evaluated how selective inhibitors of MEK (UO126, 10 μmol/L) and PI3-kinase (wortmannin, 25 nmol/L) influence the NGF-induced PCNA protein expression in isolated cholangiocytes. The stimulatory effect of NGF on PCNA protein expression was partially blocked by UO126 ($P < 0.05$; n = 5 cell preparations; Figure 5*A*) and completely abolished by wortmannin ($P < 0.01$; n = 5 cell preparations; Figure 5*A*). The 2 inhibitors completely blocked the phosphorylation of the respective target signaling protein (i.e., p-AKT for wortmannin and p-ERK1/2 for UO126) induced by NGF (Figure 5*B*; n = 5). These findings indicate PI3-kinase as the predominant intracellular signaling pathway mediating the stimulatory effect of NGF on cholangiocyte proliferation.

Proliferation of quiescent normal cholangiocytes was also induced by the supernatant of BDL cholangiocyte suspensions. In fact, when the supernatant of BDL cholangiocytes (obtained after 4 hours of incubation) was transferred into plates containing normal quiescent, freshly isolated cholan-

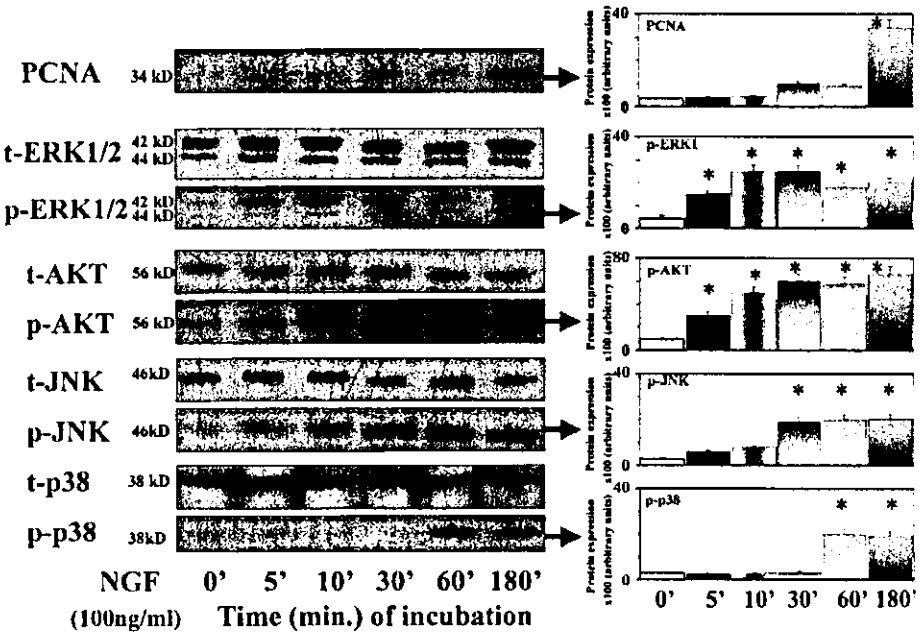

Figure 4. Western blot analysis of PCNA, ERK1/2, AKT, JNK, and p38 in cholangiocytes isolated from normal rat livers and incubated with NGF (100 ng/mL culture medium; 0–180 minutes). PCNA was enhanced after 3 hours of incubation with NGF (n = 8). Without significant changes of total protein (total ERK1/2 and total AKT), the protein expression of p-ERK 1/2 and p-AKT (phosphorylated form) increases over basal levels starting 5 minutes after incubation with NGF and remaining stable up to 180 minutes. Without significant changes of total protein (total JNK and total p38), the protein expression of phosphorylated JNK and phosphorylated p38 was enhanced 30–60 minutes after incubation with NGF. n = 8 cell preparations. $*P < 0.02$ vs. time 0.

giocytes, the protein expression of PCNA in cholangiocytes was significantly ($P < 0.05$; n = 8) increased after 4 hours of incubation, and this effect was partially blocked by neutralizing anti-NGF antibody (150 pg/mL; Figure 6). Supernatant from normal quiescent cholangiocytes showed no effect (Figure 6). These data suggest that the NGF secreted by proliferating cholangiocytes modulates cholangiocyte proliferation by autocrine/paracrine mechanisms.

Additive effect of estrogens and NGF on cholangiocyte proliferation in vitro. Recent data indicate that NGF and estrogen have a synergistic effect in modulating proliferation of cells expressing estrogen receptors.[22,23] To explore this issue, we incubated isolated rat cholangiocytes from normal rats with NGF (100 ng/mL) and/or 17β-estradiol (10 nmol/L) for 3 hours. PCNA, NGF, TrkA, and ERK1/2 protein expression was then evaluated by Western blot. PCNA and p-ERK1/2 protein expression was higher, whereas total ERK1/2 was similar in cholangiocytes incubated with NGF plus 17β-estradiol in comparison with NGF or 17β-estradiol alone ($P < 0.05$; n = 8 cell preparations; Figure 7), indicating that the 2 substances have an additive effect on modulating cholangiocyte proliferation with the effect associated with enhanced ERK phosphorylation. No significant effect of 17β-estradiol and of NGF plus 17β-estradiol on p-AKT protein expression was found (not shown). 17β-estradiol alone showed no significant effect on the protein expression of NGF or p-TrkA. However, when 17β-estradiol was administered simultaneously with NGF, there was an increase of NGF or p-TrkA protein expression in cholangiocytes that was higher in comparison with NGF or 17β-estradiol alone ($P < 0.05$; n = 8 cell preparations; Figure 7).

Effect of long-term administration of anti-NGF antibody on bile duct mass, cholangiocyte proliferation, and inflammation in BDL rats. To evaluate the in vivo role of NGF and related receptors in modulating the proliferative properties of cholangiocytes, we treated BDL rats with an anti-NGF antibody capable of immunoneutralization of endogenous NGF.[19,20] Cholangiocyte proliferative activities were evaluated in comparison with BDL rats treated with nonimmune serum. In BDL rats treated with anti-NGF antibody (n = 8), bile duct mass was significantly decreased in comparison with BDL control rats (n = 8) treated with nonimmune serum (0.57% ± 0.11% vs. 3.03% ± 0.33%; $P < 0.001$; Figure 8*A*) evaluated by quantitative morphometry after γ-glutamyltransferase staining. The decreased bile duct mass induced by anti-NGF antibody was caused by impairment of cholangiocyte proliferation and by increased apoptosis. In fact, immunohistochemistry for PCNA showed that treatment with anti-NGF antibody induced a marked decrease in BDL rats ($P < 0.05$; Figure 8*B*) in the number of PCNA-positive cholangiocytes in comparison with BDL rats treated with nonimmune serum. Consistently, Western blot analysis of PCNA protein expression in cholangiocytes isolated from BDL rats treated with anti-NGF antibody indicated a signif-

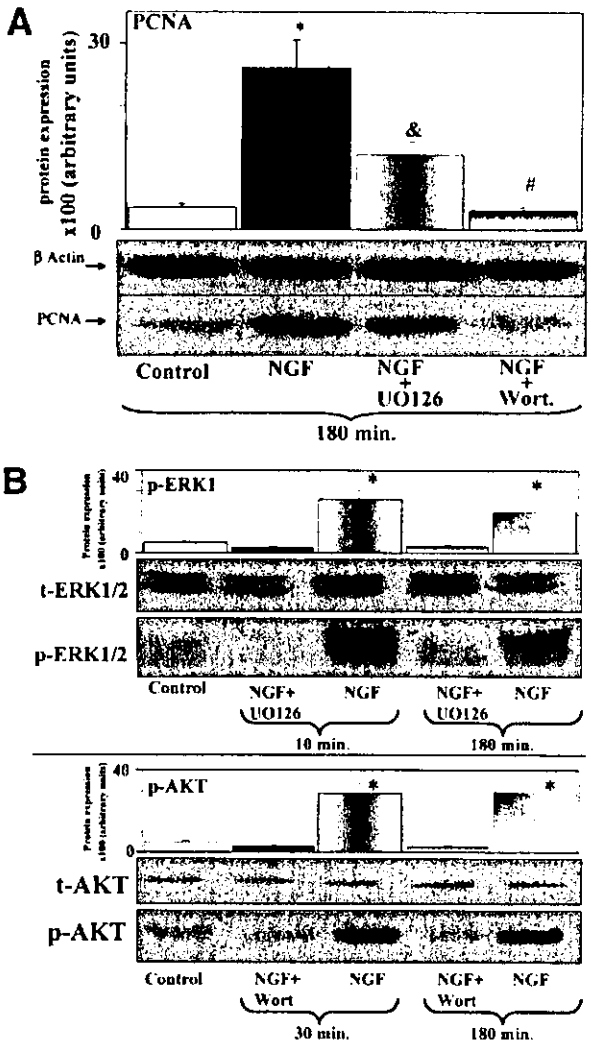

Figure 5. Effect of PI3-kinase and MEK inhibitors on NGF-induced cholangiocyte proliferation. (*A*) Western blot analysis of PCNA in cholangiocytes isolated from normal rat livers and incubated with NGF and/or wortmannin or UO126 for 180 minutes. Cholangiocytes isolated from normal rat livers (quiescent cells) and incubated with NGF (100 ng/mL culture medium) for 3 hours showed a marked increase of PCNA. The MEK inhibitor (UO126; 10 μmol/L; n = 5) partially abolishes the stimulatory effect of NGF on PCNA protein expression. The PI3-kinase inhibitor wortmannin (25 nmol/L; n = 5) totally abolishes the stimulatory effect of NGF on PCNA protein expression. The protein expression of β-actin was similar in the different experimental conditions. $^{*}P < 0.01$ vs. controls; $^{\&}P < 0.05$ vs. NGF alone, $P < 0.05$ vs. controls; $^{\#}P < 0.01$ vs. NGF alone, not significant vs. controls. (*B*) Western blot analysis of total and phosphorylated ERK1/2 and AKT in cholangiocytes isolated from normal rat livers and incubated with NGF and/or wortmannin or UO126. The 2 inhibitors completely blocked the phosphorylation of the respective target signaling protein (i.e., p-AKT for wortmannin and p-ERK1/2 for UO126) induced by NGF. The protein expression of total ERK1/2 and AKT was similar in the different experimental conditions. The time of incubation (i.e., 10 minutes for ERK and 30 minutes for AKT) was chosen by considering the maximal increase of phosphorylation induced by NGF (see Figure 4). $^{*}P < 0.01$ vs. control or NGF plus inhibitors; n = 5.

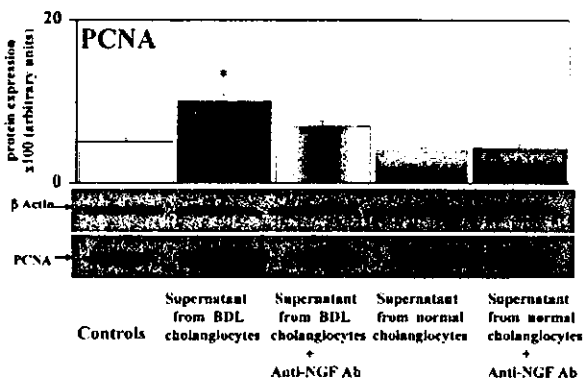

Figure 6. Western blot analysis of PCNA in normal cholangiocytes incubated with the supernatant of normal or BDL cholangiocytes and/or neutralizing anti-NGF antibody. The protein expression of PCNA in normal quiescent cholangiocytes was increased after 4 hours of incubation with the supernatant of BDL cholangiocyte suspensions. This effect was partially blocked by the neutralizing anti-NGF antibody (150 pg/mL). The supernatant of normal quiescent cholangiocytes failed to show any effect. n = 8 cell preparations. $^{*}P < 0.05$ vs. other columns.

icant decrease ($P < 0.05$; Figure 8*C*) of PCNA protein expression (mass) in comparison with BDL rats treated with nonimmune serum. In addition, there was an increase ($P < 0.05$) in the number of TUNEL-positive cholangiocytes (per portal tract) after treatment with neutralizing anti-NGF antibody with respect to appropriate controls (Figure 8*D*). Combined, these findings indicate that neutralization of NGF function through anti-NGF antibody depresses the proliferative response of cholangiocytes to BDL and enhances apoptosis, leading to a decrease in bile duct mass. Immunohistochemistry for NGF and TrkA in BDL rats treated with anti-NGF showed no significant difference when compared with nonimmune serum–treated BDL rats (not shown).

Because NGF has been shown to play a role in modulating the inflammatory processes in different tissues, we also evaluated the inflammation score in portal tracts, showing how the blockage of NGF functions (through anti-NGF) resulted in a worsening portal inflammation score (2.8 ± 0.8 vs. 1.25 ± 0.4; nonimmune serum; $P < 0.001$).

Discussion

The main findings of our study indicate that (1) NGF and TrkA receptor (the preferred NGF receptor) are expressed by normal rat cholangiocytes and overexpressed in cholangiocytes proliferating following BDL; (2) rat cholangiocytes secrete NGF and secretion is increased in cholangiocytes proliferating after BDL, which is associated with enhanced serum NGF levels in BDL