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Hepatic Gene Transfer of Human Aquaporin-1 Improves Bile Salt Secretory Failure in Rats with Estrogen-Induced Cholestasis

Julieta Marrone, Leandro R. Soria, Mauro Danielli, Guillermo L. Lehmann, Maria Cecilia Larocca, and Raúl A. Marinelli

The adenoviral gene transfer of human aquaporin-1 (hAQP1) water channels to the liver of 17α-ethinylestradiol-induced cholestatic rats improves bile flow, in part by enhancing canalicular hAQP1-mediated osmotic water secretion. To gain insight into the mechanisms of 17α-ethinylestradiol cholestasis improvement, we studied the biliary output of bile salts (BS) and the functional expression of the canalicular BS export pump (BSEP; ABCB11). Adenovector encoding hAQP1 (AdhAQP1) or control vector was administered by retrograde intrabiliary infusion. AdhAQP1-transduced cholestatic rats increased the biliary output of major endogenous BS (50%-80%, $P < 0.05$) as well as that of taurocholate administered in choleretic or trace radiolabel amounts (around 60%, $P < 0.05$). Moreover, liver transduction with AdhAQP1 normalized serum BS levels, otherwise markedly elevated in cholestatic animals. AdhAQP1 treatment was unable to improve BSEP protein expression in cholestasis; however, its transport activity, assessed by adenosine triphosphate-dependent taurocholate transport in canalicular membrane vesicles, was induced by 90% ($P < 0.05$). AdhAQP1 administration in noncholestatic rats induced no significant changes in either biliary BS output or BSEP activity. Canalicular BSEP, mostly present in raft (high cholesterol) microdomains in control rats, was largely found in nonraft (low cholesterol) microdomains in cholestasis. Considering that BSEP activity directly depends on canalicular membrane cholesterol content, decreased BSEP presence in rafts may contribute to BSEP activity decline in 17α-ethinylestradiol cholestasis. In AdhAQP1-transduced cholestatic rats, BSEP showed a canalicular microdomain distribution similar to that of control rats, which provides an explanation for the improved BSEP activity. *Conclusion:* Hepatocyte canalicular expression of hAQP1 through adenoviral gene transfer promotes biliary BS output by modulating BSEP activity in estrogen-induced cholestasis, a novel finding that might help us to better understand and treat cholestatic disorders. (HEPATOLOGY 2016; 00:000–000)

Cholestatic liver diseases result in systemic and intrahepatic accumulation of endogenous bile salts (BS) that causes liver injury, ultimately leading to fibrosis and cirrhosis.[1] The canalicular BS export pump (BSEP; ABCB11) is the primary hepatocyte transporter for biliary BS secretion, i.e., the rate-limiting step in the enterohepatic circulation of BS.[2] Impairment of expression and/or function of BSEP has been directly linked to inherited and acquired cholestatic liver diseases.[3-5] Estrogens and

Abbreviations: AdhAQP1, adenovector encoding human aquaporin-1; AQP8, aquaporin-8; BS, bile salts; BSEP, bile salt export pump/ABCB11; EE, 17α-ethinylestradiol; hAQP1, human aquaporin-1; TC, taurocholate.

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L.R. Soria's present address is: Telethon Institute of Genetics and Medicine, Naples, Italy.
G.L. Lehmann's present address is: Margaret Dyson Vision Research Institute, Weill Cornell Medical College, New York, NY.

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their metabolites are causally associated with intrahepatic cholestasis in susceptible pregnancies as well as in postmenopausal hormone replacement therapy and administration of oral contraceptives.[4,6] Experimental cholestasis induced by the synthetic estrogen 17α-ethinylestradiol (EE) is a rodent model widely used to investigate the molecular mechanisms of estrogen-induced bile secretory dysfunction.[7] In this model, cholestasis would ultimately be caused by the impairment of key canalicular solute transporters for bile formation, including BSEP. Canalicular aquaporin-8 (AQP8) channels, which facilitate osmotically driven water transport,[8] are also down-regulated and may contribute to the EE-induced cholestasis.[9] The gene transfer of the archetypal human aquaporin-1 (hAQP1) water channel through the adenoviral vector encoding hAQP1 (AdhAQP1) has been successfully used to restore normal salivary flow to irradiated hypofunctional salivary glands in experimental animals[10] and humans.[11] We have recently provided evidence suggesting that the adenoviral transfer of hAQP1 gene to livers of EE-induced cholestatic rats improves bile flow, at least in part by enhancing the canalicular hAQP1-mediated osmotic water secretion.[12] In an effort to fully understand the mechanisms involved in the hAQP1-induced improvement of estrogen-induced cholestasis, we further studied the biliary output of BS and the functional expression of BSEP.

Materials and Methods

ANIMALS AND TREATMENTS

Adult male Wistar rats were maintained on a standard laboratory diet and water *ad libitum* and housed in a temperature-controlled and humidity-controlled environment under a constant 12-hour light/dark cycle. All animals received humane care, according to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health). Protocols were approved by the local animal welfare committee. EE (Sigma-Aldrich, St. Louis, MO; 5 mg/kg body weight) was administered subcutaneously for 5 consecutive days. Under these conditions, EE induces cholestasis in rats.[9] Noncholestatic control rats received only the EE vehicle (propylene glycol).

ADENOVIRAL VECTORS

AdhAQP1 is a replication-deficient serotype 5 recombinant adenovirus encoding hAQP1 and including a cytomegalovirus promoter and simian virus 40 polyadenylylation site. AdhAQP1 was kindly donated by Dr. Bruce Baum (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD); details of the construction procedures have been described elsewhere.[10] The adenoviral vector used as a control is also a replication-deficient serotype 5 recombinant adenovirus which encodes β-galactosidase.

IN VIVO DELIVERY OF RECOMBINANT ADENOVIRUS TO THE BILIARY TRACT

After the third EE administration, rats were anesthetized by intraperitoneal injection of ketamine (100 mg/kg body weight)/xylazine (15 mg/kg body weight). A 2-cm transversal laparotomy was made just below the xiphoid appendix to expose the duodenum. The duodenal antimesenteric wall was punctured with a 25-gauge needle, and a PE-10 polyethylene catheter (Intramedic; Clay Adams, Parsippany, NJ) was introduced first into the lumen of the duodenum and then through the papilla of Vater 1 cm into the bile duct. A virus dose of 3×10^{10} plaque-forming units per liver

ARTICLE INFORMATION:

From the Instituto de Fisiología Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Raúl A. Marinelli, Ph.D.
Instituto de Fisiología Experimental, Facultad de Ciencias Bioquímicas y Farmacéuticas
Universidad Nacional de Rosario
Suipacha 570
2000 Rosario, Santa Fe, Argentina
E-mail: rmarinel@unr.edu.ar or marinelli@ifise-conicet.gov.ar
Tel: +54-341-4305799

suspended in 300 μL of sterile saline was retrogradely infused into the biliary tract over a period of 3 minutes. In additional control experiments, animals received only 300 μL of sterile saline. The catheter was maintained in place for 15 minutes, to prevent backflow. After the procedure was completed, the catheter was gently removed, the duodenal puncture and abdominal wall were sutured, and the animal was allowed to recover. Initial experiments indicated that the protein expression of hAQP1 in hepatocytes takes 72 hours.[12] Thus, the experiments were performed 72 hours after adenoviral infusion (i.e., on the sixth day after starting EE treatment).

BASAL AND TAUROCHOLATE-STIMULATED BILE SECRETION STUDIES

After treatments, rats were anesthetized as mentioned above and maintained under this condition during bile collection. For this purpose, a middle abdominal incision was made and the common bile duct was cannulated with PE-10 polyethylene tubing. Bile was collected for three 10-minute periods. Taurocholate (TC; Sigma-Aldrich) was intravenous bolus-injected at 8 μmol/100 g body weight to stimulate hepatocyte (canalicular) bile flow, and bile was collected for an additional 10-minute period. Bile flow was determined by gravimetry, assuming a density of the bile of 1.0 g/mL At the end of bile collection, blood samples were taken by cardiac puncture, animals were killed by exsanguination, and the livers were harvested for evaluation.

[^{3}H]TC BILIARY OUTPUT

After intravenous bolus injection of 0.1 μCi [^{3}H]TC (specific activity 5 Ci/mmol; Perkin Elmer, Waltham, MA), bile was collected for 30 minutes in 5-minute periods. Aliquots (50 μL) of bile were taken for scintillation counting. [^{3}H]TC biliary output was calculated from the radioactivity and bile flow values.[13]

SERUM BS

BS concentration was determined in serum using a commercial kit (Randox, Crumlin, UK), according to the manufacturer's instructions.

LIVER SERUM ENZYMES

Serum activities of aspartate transaminase, alanine transaminase, lactate dehydrogenase, and alkaline phosphatase were determined using commercial kits (Wiener Lab, Rosario, Argentina), according to the manufacturer's instructions.

BILIARY BS CONCENTRATION

Total BS concentration was assessed using the 3α-hydroxysteroid dehydrogenase procedure.[14]

BILIARY BS COMPOSITION

Individual biliary BS were identified and quantified by high-performance liquid chromatography (Waters, Milford, MA) by comparison with standard compounds, as reported.[15]

HEPATIC PLASMA MEMBRANE FRACTIONS

Livers were homogenized by 15 up-and-down strokes with a loose-fitting Dounce homogenizer in four volumes of 0.3 M sucrose (MP Biomedicals, Solon, OH), containing 0.1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 0.1 mM leupeptin (Chemicon Millipore, Darmstadt, Germany). Liver homogenates were subjected to low-speed centrifugation to obtain postnuclear supernatants. Fractions enriched in plasma membranes were prepared from liver homogenates by centrifugation at 200,000*g* for 60 minutes on a discontinuous 1.3 M sucrose gradient as described.[16] The plasma membrane band was removed, diluted to 0.3 M, and centrifuged at 200,000*g* for 60 minutes to yield the plasma membrane fraction, which was further purified by centrifugation at 100,000*g* for 90 minutes on a continuous (9%-60%) sucrose gradient.[16] Purified plasma membranes were layered onto a discontinuous sucrose gradient composed of 31%, 34%, and 38% sucrose. After centrifugation at 270,000*g* for 3 hours, the band at the top of the 31% layer (canalicular plasma membrane) was collected, diluted with washing buffer, and spun at 200,000*g* for 1 hour.[9,17] Membrane proteins were determined according to Lowry's method using bovine serum albumin as standard. The specific activity of canalicular marker 5′-nucleotidase in canalicular membranes was comparable to that reported[9] and did not differ among experimental groups (Supporting Fig. S1A). The enrichment of 5′-nucleotidase relative to

homogenate was around 30 in all groups, also comparable to that reported.[9] The activity of 5′-nucleotidase were assessed with a commercial kit (Wiener Lab).[9] Only negligible contamination with basolateral membranes was observed based on immunoblotting detection of the basolateral marker aquaporin-9 (data not shown).

IMMUNOBLOTTING

Western blottings of membrane fractions were performed as described by our laboratory,[9,16] using rabbit affinity-purified AQP1 (1 μg/mL; Alpha Diagnostics International, San Antonio, TX), AQP8 or aquaporin-9 (1 μg/mL; Alpha Diagnostics International), BSEP (1 μg/mL; Kamiya, Seattle, WA), caveolin-1 antibodies (1 μg/mL; Sigma-Aldrich); mouse β-actin (1 μg/mL; Sigma-Aldrich), and clathrin (1 μg/mL; Sigma-Aldrich) antibodies and the corresponding horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific, Rockford, IL). Protein bands were detected by an enhanced chemiluminescence detection system (Thermo Scientific). Autoradiographs were obtained by exposing the membranes to Amersham Hyperfilm ECL (GE Healthcare Limited, Chalfont St. Giles, UK). Densitometric analysis of the developed bands was performed using Image J Software.[18]

CONFOCAL IMMUNOFLUORESCENCE MICROSCOPY AND IMAGE ANALYSIS

Fixed liver sections were permeabilized and blocked with 0.2% Triton X-100/bovine serum albumin 3% in phosphate-buffered saline for 30 minutes and incubated overnight at 4°C with rabbit affinity-purified AQP1 antibodies (10 μg/mL) and mouse β-actin (1 μg/mL; Sigma-Aldrich). After washing, samples were incubated with Alexa 488-conjugated goat antirabbit secondary antibody (Molecular Probes, Eugene, OR) and Alexa 594-conjugated donkey antimouse secondary antibody (Molecular Probes) for 1 hour. Samples were washed, incubated with 4′,6-diamidino-2-phenylindole (Molecular Probes, 50 μM) for 10 minutes, and mounted with Prolong (Molecular Probes). Fluorescence localization was detected by confocal microscopy (Nikon C1PlusSiR with inverted microscope Nikon TE2000). Images were collected with the same confocal settings in each set of experiments. With these settings no autofluorescence was detected. Controls omitting primary or secondary antibodies revealed no labeling. For the sake of clarity, contrast and brightness adjustments were equally applied to the entire images using Adobe Photoshop software.

TC TRANSPORT STUDIES IN LIVER CANALICULAR VESICLES

Uptake of [^{3}H]TC (specific activity 5 Ci/mmol; Perkin Elmer) was measured in canalicular plasma membrane vesicles by a rapid filtration technique.[19] Briefly, frozen canalicular membrane suspensions were rapidly thawed by immersion in a 37°C water bath, diluted to the desired protein concentration (2.5 mg/mL), and vesiculated by passing membranes 10 times through a 27-gauge needle. The tightness of canalicular vesicles was checked by stopped-flow spectrophotometry.[12] We observed constant light scattering for at least 60 seconds, suggesting that the vesicles were mostly tight and stable during TC transport studies. An aliquot (20 μL) of membrane vesicles was incubated for 5 minutes at 37°C, and the uptake was initiated by adding 80 μL of incubation buffer (100 mM sucrose, 100 mM NaCl, 0.2 mM $CaCl_2$, 5 mM $MgSO_4$, and 20 mM 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid-Tris, pH 7.5) containing 0.5 μCi [^{3}H]TC (i.e., 0.1 nmol of TC), with or without 5 mM adenosine triphosphate (Sigma-Aldrich). After 30-second and 1-minute incubations, uptake was terminated by the addition of 3.5 mL of ice-cold stop solution (100 mM sucrose, 100 mM NaCl, 0.2 mM $CaCl_2$, 5 mM $MgSO_4$, and 20 mM 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid-Tris, pH 7.5). Membrane vesicles were separated from the incubation medium by immediate filtration through a polyvinylidene fluoride filter with a 0.45-μm pore diameter (Millipore Corporation, Billerica, MA) that was presoaked in a buffer containing 1 mM TC (Sigma) to diminish nonspecific filter binding. The filter was washed five times with 3.5 mL of stop solution, dissolved in 3 mL of scintillation fluid (Perkin Elmer), and counted for radioactivity using a scintillation counter (RackBeta 1214; Pharmacia Wallac Oy, Turku, Finland). Nonspecific binding to membranes was determined using the same method except that the procedure was conducted at 0°C-4°C. BSEP activity was estimated by the adenosine triphosphate-dependent [^{3}H]TC transport.[19] All incubations were performed in triplicate. TC transport was found to vary linearly up to 1 minute. Observations were confirmed with at least three separate membrane preparations.

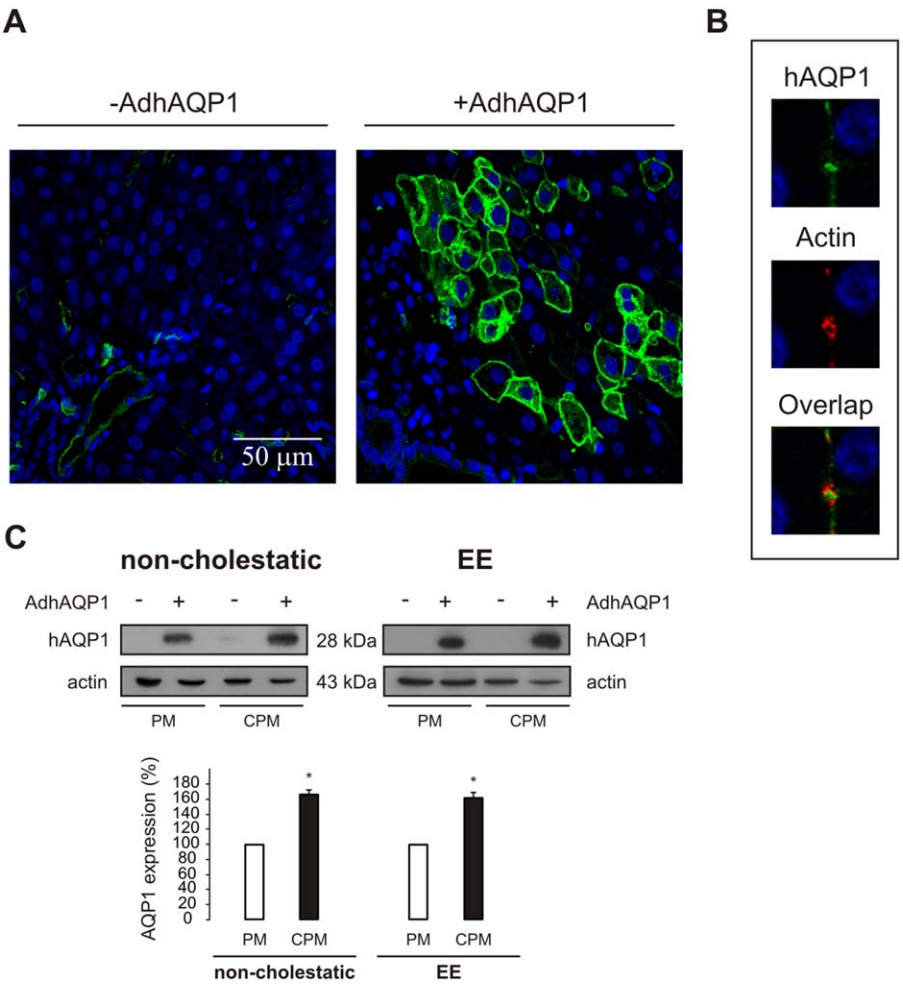

FIG. 1. Hepatocyte expression of hAQP1 in EE cholestatic rats after retrograde intrabiliary AdhAQP1 delivery. The induction of cholestasis by EE and the delivery of AdhAQP1 and control vectors are detailed in Materials and Methods. Experiments were performed 72 hours after adenoviral infusion (i.e., on the sixth day after starting EE treatment). (A) Immunofluorescence localization of AQP1 in liver portal areas of EE cholestatic rats transduced with control vector (-AdhAQP1) or with AdhAQP1 (+AdhAQP1) viewed by laser scanning confocal microscopy. -AdhAQP1 rats showed endogenous AQP1 staining only in peribiliary vascular endothelia and cholangiocytes, while +AdhAQP1 rats showed intense hAQP1 staining in hepatocytes (mostly on plasma membranes). Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). (B) Confocal immunofluorescence localization of hAQP1 to canaliculi (labeled with actin) from EE cholestatic rats transduced with AdhAQP1. Nuclear 4′,6-diamidino-2-phenylindole staining is in blue. (C) Anti-AQP1 immunoblot of hepatic plasma membranes and canalicular plasma membranes from noncholestatic and EE cholestatic rats transduced with AdhAQP1 vector. Lanes were loaded with 20 μg of proteins. Blots were reprobed using anti-β-actin antibody as a control for equal protein loading. Densitometric analysis of three separate experiments in each group. Data (means ± standard error) are expressed as percentage of noncholestatic or EE plasma membrane values. Abbreviations: CPM, canalicular plasma membrane; PM, plasma membrane.

PREPARATION OF DETERGENT SOLUBLE/INSOLUBLE CANALICULAR MICRODOMAINS

Canalicular plasma membranes were resuspended in buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid, and a mixture of protease inhibitors), containing 1% (final concentration) Triton X-100 (Sigma-Aldrich), and incubated on ice for 30 minutes. Insoluble components were spun down at 18,000*g* for 60 minutes, and the supernatants (containing Triton X-100 soluble proteins) were collected in a new tube. The pellets were suspended in solubilization buffer (50 mM Tris-HCl [pH 8.8], 5 mM ethylene diamine tetraacetic acid, and 1% sodium dodecyl sulfate) and homogenized. After acetone precipitation, soluble

TABLE 1. Liver Serum Enzymes and Body and Liver Weights

	Noncholestatic	Noncholestatic +AdhAQP1	EE	EE +AdhAQP1
Serum enzymes				
ALT (U/L)	18 ± 2	15 ± 2	16 ± 3	19 ± 1
AST (U/L)	144 ± 6	144 ± 18	131 ± 28	122 ± 11
LDH (U/L)	739 ± 170	725 ± 159	878 ± 125	827 ± 83
ALP (U/L)	175 ± 14	170 ± 42	483 ± 27*	310 ± 22*#
Weights (g)				
Body (body weight)	339 ± 15	345 ± 16	356 ± 9	324 ± 23
Liver	11.6 ± 0.7	12.2 ± 0.7	15.8 ± 0.3*	15.3 ± 0.7*

Data are means ± standard error (n = 3). *$P < 0.05$ compared with noncholestatic and noncholestatic+AdhAQP1. #$P < 0.05$ compared with EE. Noncholestatic and EE rats received control vector.
Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; LDH, lactate dehydrogenase.

and insoluble fractions were processed for western blotting.[20]

To analyze detergent soluble/insoluble canalicular microdomains by flotation in a sucrose gradient, a Triton X-100 extraction was performed as described above. Thereafter the extract was brought to 40% sucrose, overlaid with a discontinuous 30%-5% sucrose gradient, and centrifuged at 200,000*g* for 18-20 hours.[20,21] After centrifugation, 1-mL fractions were collected from the top to the bottom and analyzed by immunoblotting.

CHOLESTEROL DETERMINATION

Cholesterol was determined spectrophotometrically using commercial kits (Wiener Lab), according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Data are expressed as means ± standard error. Significance was determined by the Student *t* test or one-way analysis of variance, Tukey's test; $P < 0.05$ was considered statistically significant.

Results

HEPATOCYTE EXPRESSION OF HAQP1 IN ADHAQP1-TRANSDUCED EE CHOLESTATIC RATS

As a first step, we confirmed the hepatic expression and subcellular localization of hAQP1 after AdhAQP1 retrograde intrabiliary administration to EE cholestatic rats by confocal immunofluorescence microscopy.[12]

In EE cholestatic rats with retrograde intrabiliary administration of control vector, AQP1 was not observed in hepatocytes (Fig. 1A). In agreement with the reported endogenous hepatic AQP1 expression,[22] AQP1 staining was strong in peribiliary vascular endothelia and weak in cholangiocytes (Fig. 1A). AdhAQP1 administration in EE-treated rats induced the expression of hAQP1 in hepatocytes, which was predominantly located in plasma membranes (Fig. 1A). As we reported,[12] hAQP1 expression was evident in canalicular plasma membranes, the bile secretory pole of hepatocytes (Fig. 1B). Regarding the immunoblotting experiments, hepatic subfractionation showed a band of the expected size (about 28 kDa) that was stronger in canalicular membranes (Fig. 1C). As we reported,[12] the hepatocyte expression and canalicular location of hAQP1 did not differ between EE cholestatic and noncholestatic control rats (Fig. 1C).

In addition, the retrograde biliary infusion of AdhAQP1 to noncholestatic and EE cholestatic rats induced no significant changes in transaminases and lactate dehydrogenase serum activities or body and liver weights (Table 1). However, the increased serum alkaline phosphate activity in EE rats was reduced by AdhAQP1 administration.[12]

BILIARY OUTPUT OF BS IN ADHAQP1-TRANSDUCED EE CHOLESTATIC RATS

Figure 2 shows the basal bile flow as well as the basal biliary concentration and output of total BS in EE cholestatic and noncholestatic rats transduced with AdhAQP1 or control vector. In EE-induced cholestasis, bile flow (Fig. 2A) and biliary BS output (Fig. 2C) were significantly reduced, while biliary BS concentration (Fig. 2B) was not significantly

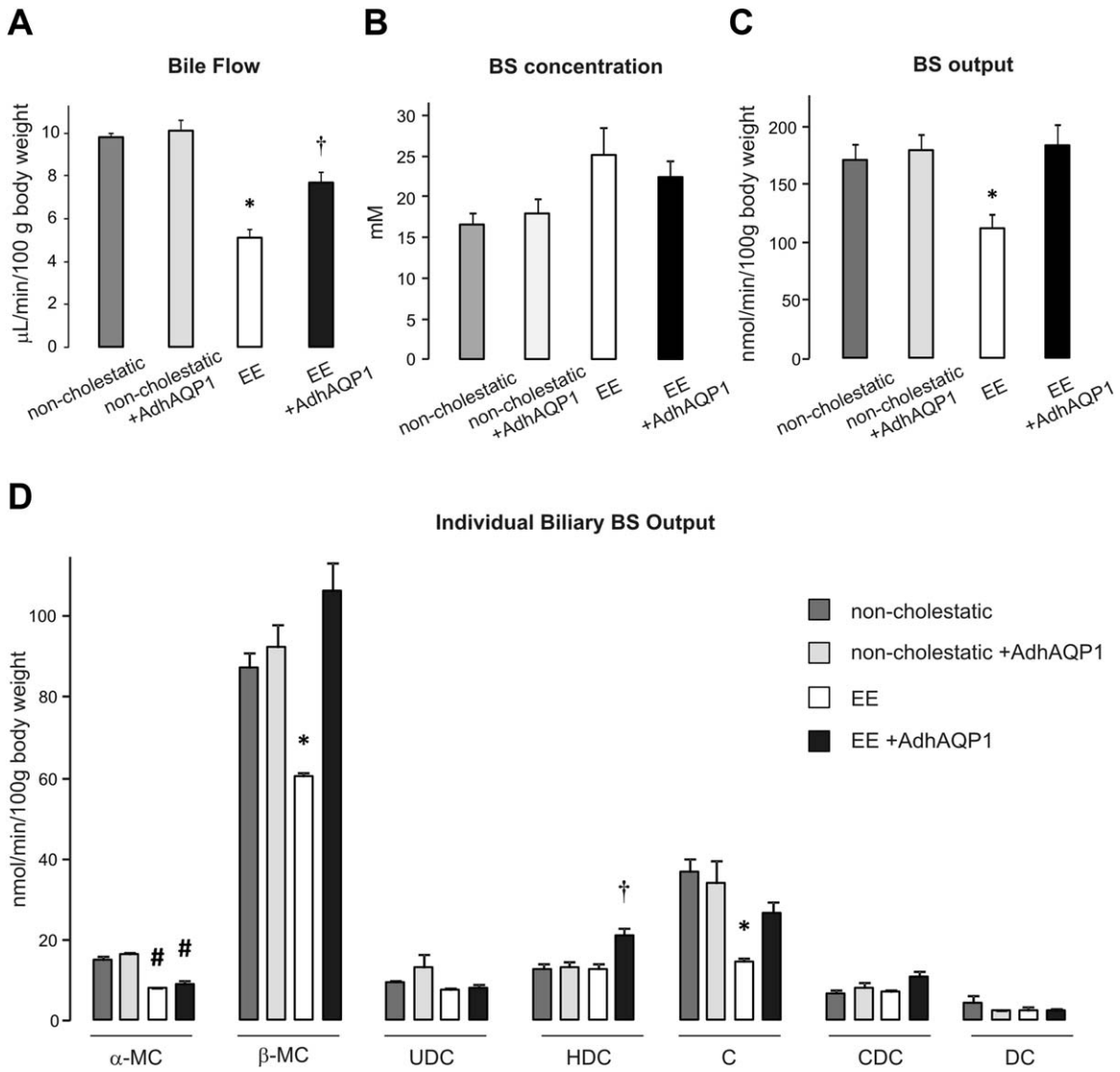

FIG. 2. Basal biliary BS output in AdhAQP1-transduced EE cholestatic rats. The induction of cholestasis by EE and the delivery of AdhAQP1 and control vectors are detailed in Materials and Methods. Experiments were performed 72 hours after adenoviral infusion (i.e., on the sixth day after starting EE treatment). (A) Bile flow, (B) biliary concentration of total BS, and (C) biliary output of total BS from noncholestatic and EE cholestatic rats transduced with control or AdhAQP1 vector. Bile was collected in 30-minute periods under basal, nonstimulated conditions. Data are given as mean ± standard error of six independent experiments per group. (D) Biliary output of individual endogenous BS assessed by high-performance liquid chromatography. Data are given as mean ± standard error of three independent experiments per group. $^{*}P < 0.05$ from noncholestatic controls and EE+Ad*b*AQP1, $^{\#}P < 0.05$ from noncholestatic controls, $^{\dagger}P < 0.05$ from noncholestatic controls and EE. Abbreviations: C, cholate; CDC, chenodeoxycholate; DC, deoxycholate; HDC, hyodeoxycholate; α-MC, α-muricholate; *β*-MC, *β*-muricholate; UDC, ursodeoxycholate.

modified.[23] As described,[12] AdhAQP1 delivery significantly improved bile flow in EE cholestatic rats, whereas it did not significantly affect bile flow in noncholestatic rats (Fig. 2A). AdhAQP1 delivery to EE cholestatic rats caused a significant increase of 65% in biliary BS output, thus reaching noncholestatic control values (Fig. 2C). High-performance liquid chromatographic analysis of biliary BS composition showed that the biliary output of major endogenous BS was significantly increased (up to 80%) in cholestatic rats after transduction with AdhAQP1 (Fig. 2D). AdhAQP1 delivery did not significantly affect biliary output of individual BS in noncholestatic rats (Fig. 2D).

We also studied biliary BS excretion after the intravenous administration of TC, a physiological BS that

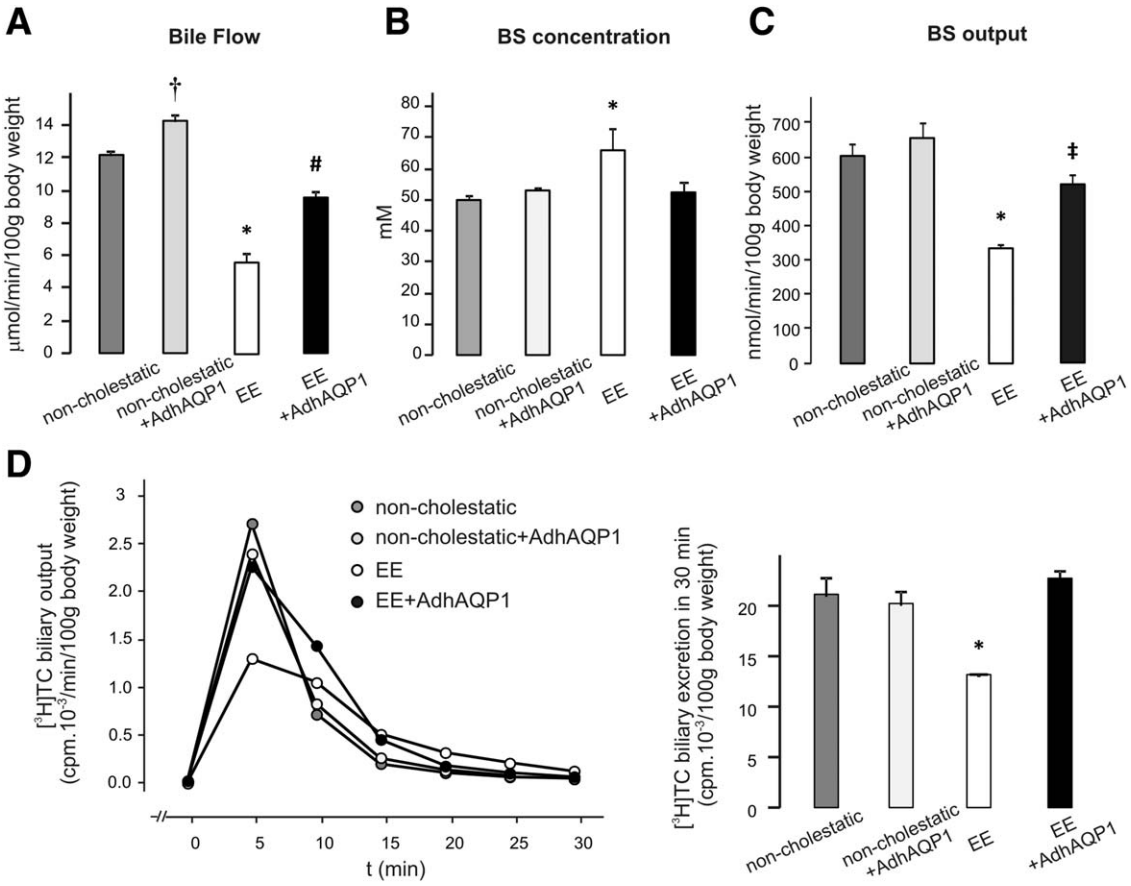

FIG. 3. TC administration to AdhAQP1-transduced EE cholestatic rats. The induction of cholestasis by EE and the delivery of AdhAQP1 and control vectors are detailed in Materials and Methods. Experiments were performed 72 hours after adenoviral infusion (i.e., on the sixth day after starting EE treatment). (A) Bile flow, (B) biliary concentration of total BS, and (C) biliary output of total BS from noncholestatic and EE cholestatic rats transduced with control or AdhAQP1 vector after a single intravenous administration of TC (8 μmol/100 g body weight). Bile was collected in a 10-minute period. Data are given as mean ± standard error of six independent experiments per group. (D) Representative time course of biliary [^{3}H]TC output (left panel) and 30-minute cumulative biliary [^{3}H]TC output (right panel) after a single intravenous administration of 0.1 μCi [^{3}H]TC. Data are given as means ± standard error of four independent experiments per group. $^*P < 0.05$ from noncholestatic controls and EE+AdhAQP1; $^{\#}P < 0.05$ from noncholestatic controls and EE; $^{\dagger}P < 0.05$ from noncholestatic, EE, and EE+AdhAQP1; $^{\ddagger}P < 0.05$ from noncholestatic+AdhAQP1 and EE.

is efficiently taken up and excreted into bile by hepatocytes.[13] Figure 3 shows the TC-stimulated bile flow and biliary concentration and output of total BS in EE cholestatic and noncholestatic rats transduced with AdhAQP1 or control vector. As described[12] and similar to what occurred in basal conditions, AdhAQP1 delivery significantly improved bile flow in EE cholestatic rats subjected to TC overload. AdhAQP1 delivery slightly, but significantly, increased bile flow in noncholestatic rats (Fig. 3A).

In EE-induced cholestasis, biliary BS concentration was slightly increased (Fig. 3B), whereas biliary BS output was markedly decreased (Fig. 3C).[24] Similar to what was observed under basal conditions, AdhAQP1 delivery to EE cholestatic rats, but not to noncholestatic controls, caused a significant increase of about 60% in biliary BS output (Fig. 3C).

TC was also intravenously injected in trace radiolabel amounts. As shown,[13] time course (Fig. 3D, left panel) and cumulative (Fig. 3D, right panel) biliary output of [^{3}H]TC was found to be significantly reduced in EE-induced cholestasis. AdhAQP1 delivery caused a significant increase of 74% in the cumulative biliary output of [^{3}H]TC only in EE cholestatic rats (Fig. 3D, right panel).

Thus, the data indicate that hepatic hAQP1expression through AdhAQP1 administration in EE cholestatic rats increases biliary BS output.

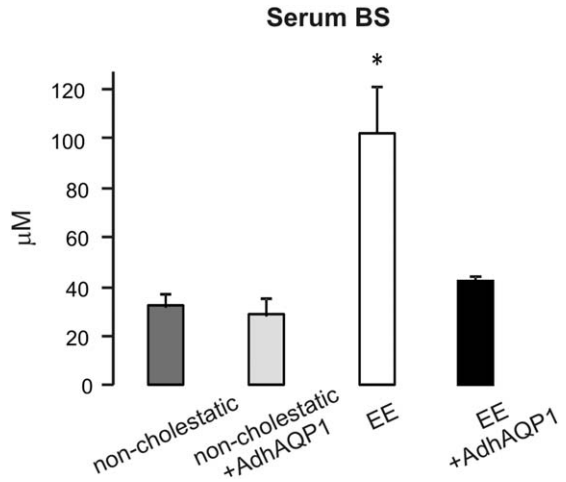

FIG. 4. Serum BS levels in AdhAQP1-transduced EE cholestatic rats. The induction of cholestasis by EE and the delivery of AdhAQP1 and control vectors are detailed in Materials and Methods. Serum BS was assessed 72 hours after adenoviral infusion (i.e., on the sixth day after starting EE treatment). Noncholestatic control and EE rats received control vector. Data are means ± standard error of five independent experiments. $^*P <$ 0.05 from noncholestatic controls and EE+AdhAQP1.

SERUM LEVELS OF BS IN ADHAQP1-TRANSDUCED EE CHOLESTATIC RATS

We next explored whether the enhanced biliary BS output in AdhAQP1-transduced EE cholestatic rats had any impact on the high serum BS levels found in cholestasis. As described,[25] serum BS showed markedly higher levels in EE cholestatic rats (Fig. 4). AdhAQP1 delivery resulted in a striking reduction of BS serum levels in cholestatic rats with no evident effects in noncholestatic control rats (Fig. 4).

HEPATOCYTE EXPRESSION AND TRANSPORT ACTIVITY OF BSEP IN ADHAQP1-TRANSDUCED EE CHOLESTATIC RATS

BSEP is the key BS transporter in hepatocyte bile canalicular plasma membranes. To determine whether BSEP is involved in the augmented bile salt output in AdhAQP1-transduced cholestatic rats, we assessed its canalicular protein expression and transport activity. As reported,[26] canalicular protein expression of BSEP was down-regulated by around 40% in EE-induced cholestasis (Fig. 5). AdhAQP1 delivery did not alter canalicular BSEP protein expression (Fig. 5). Similar to BSEP, the hepatocyte basolateral sodium/TC cotransporting polypeptide (Ntcp; Slc10a1) was found to be down-regulated in EE-induced cholestasis[27] and unaltered by AdhAQP1 delivery (data not shown).

As reported,[28] canalicular BSEP transport activity was reduced in EE-induced cholestasis by 50% (Fig. 6). However, AdhAQP1 delivery to cholestatic rats significantly induced BSEP transport activity by about 90% (Fig. 6). Interestingly, AdhAQP1 administration in noncholestatic control rats induced no significant change in BSEP activity (Fig. 6).

BSEP LOCALIZATION IN CANALICULAR MICRODOMAINS IN ADHAQP1-TRANSDUCED EE CHOLESTATIC RATS

Canalicular BSEP is mostly present in cholesterol-rich microdomains,[29,30] and canalicular membrane

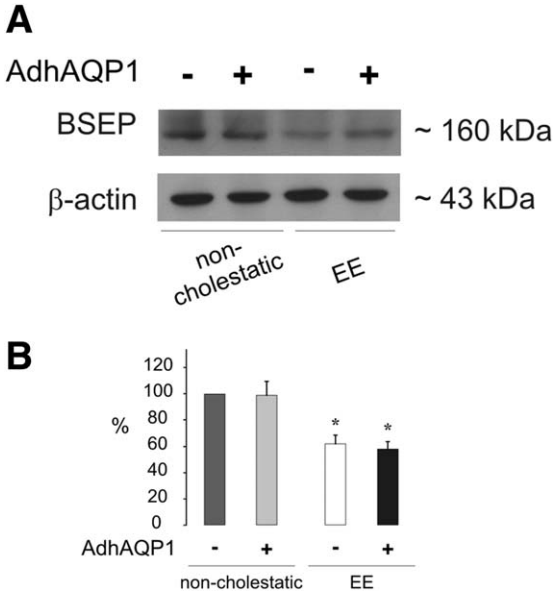

FIG. 5. Canalicular expression of BSEP in AdhAQP1-transduced EE cholestatic rats. The induction of cholestasis by EE and the delivery of AdhAQP1 and control vectors are detailed in Materials and Methods. Experiments were performed 72 hours after adenoviral infusion (i.e., on the sixth day after starting EE treatment). Liver canalicular plasma membrane fractions were isolated and subjected to immunoblotting as described in Materials and Methods. (A) Representative immunoblot for BSEP in canalicular membranes (20 μg proteins/lane). Blots were reprobed using anti-β-actin antibody as a control for equal protein loading. (B) Densitometric analysis of four separate experiments in each group. Data (means ± standard error) are expressed as percentage of noncholestatic controls. $^*P < 0.05$ from noncholestatic controls.

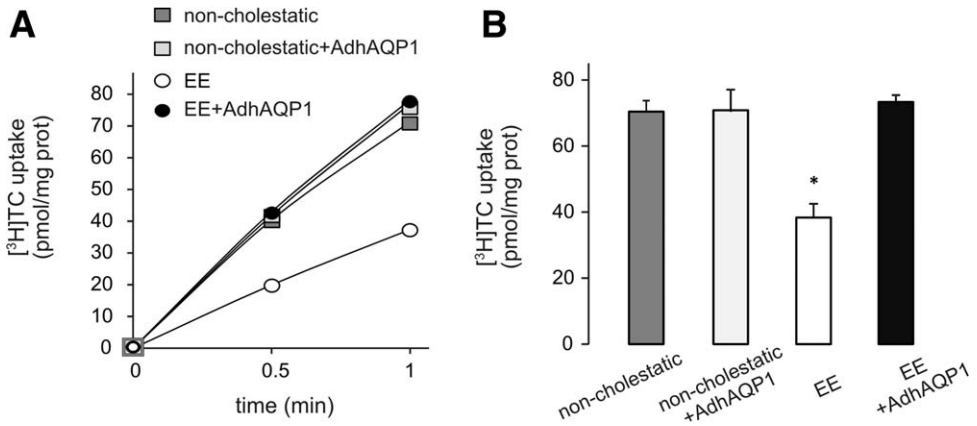

FIG. 6. Canalicular transport activity of BSEP in AdhAQP1-transduced EE cholestatic rats. The induction of cholestasis by EE and the delivery of AdhAQP1 and control vectors are detailed in Materials and Methods. Experiments were performed 72 hours after adenoviral infusion (i.e., on the sixth day after starting EE treatment). BSEP activity was estimated by the adenosine triphosphate-dependent [^{3}H]TC transport in hepatic canalicular plasma membrane vesicles (for details see Materials and Methods). (A) Representative time course of [^{3}H]TC uptake in canalicular plasma membrane vesicles. (B) [^{3}H]TC uptake in canalicular plasma membrane vesicles at 1 minute. Data are means ± standard error of three independent experiments. *$P < 0.05$ from noncholestatic controls and EE+AdhAQP1.

cholesterol content is a critical determinant of BSEP transport activity.[31] In fact, there is a positive correlation between membrane cholesterol and BSEP activity. Thus, to understand the mechanisms involved in the induction of BSEP activity, we assessed BSEP expression in cholesterol-rich canalicular membrane microdomains. We isolated raft (high cholesterol) and nonraft (low cholesterol) canalicular microdomains based on their detergent solubility and enrichment of raft protein caveolin-1 or nonraft protein clathrin, as described in Materials and Methods and shown in Fig. 7.

In noncholestatic rats injected with AdhAQP1 or control vector, canalicular BSEP was more concentrated in cholesterol-rich (about 70%) than non-cholesterol-rich (about 30%) microdomains (Fig. 7A,B). Nevertheless, in EE cholestatic rats with administration of control vector, there was a striking difference, BSEP being about 30% in cholesterol-rich and 70% in non-cholesterol-rich canalicular membrane microdomains (Fig. 7A,B). Similar results were found in EE cholestatic rats with administration of saline instead of control vector (not shown). Interestingly, AdhAQP1 delivery in EE cholestatic rats nearly restored the canalicular microdomain distribution of BSEP to noncholestatic values (Fig. 7A,B). AQP8, another canalicular protein, showed a distribution similar to BSEP in noncholestatic rats, i.e., around 90% in cholesterol-rich and 10% in non-cholesterol-rich microdomains. AQP8 protein, like BSEP, is downregulated in EE-induced cholestasis.[9] However, in clear contrast to the BS transporter, its raft/nonraft canalicular distribution was not significantly altered in EE cholestatic rats with or without AdhAQP1 administration (Fig. 7A,B). Expression of hAQP1 in canalicular membranes was almost entirely restricted to cholesterol-rich raft microdomains (Fig. 7A).

Canalicular raft/nonraft microdomains analyzed by flotation sucrose gradient confirmed that in EE cholestatic rats canalicular BSEP (but not AQP8) shifted from raft to nonraft microdomains, whereas in AdhAQP1-transduced cholestatic rats BSEP showed a canalicular microdomain distribution similar to that of control rats (Fig. 7C).

Discussion

Our data suggest that canalicularly expressed hAQP1, through adenoviral gene transfer, improves hepatocyte BS secretory dysfunction in estrogen-induced cholestasis by increasing BSEP transport activity.

Hepatocyte bile formation is an osmotic secretory process. Osmotically active solutes, primarily BS, through BSEP, and other organic anions, through multidrug resistance-associated protein-2, are transported into and concentrated within the canalicular lumen. This results in transcellular (mainly through

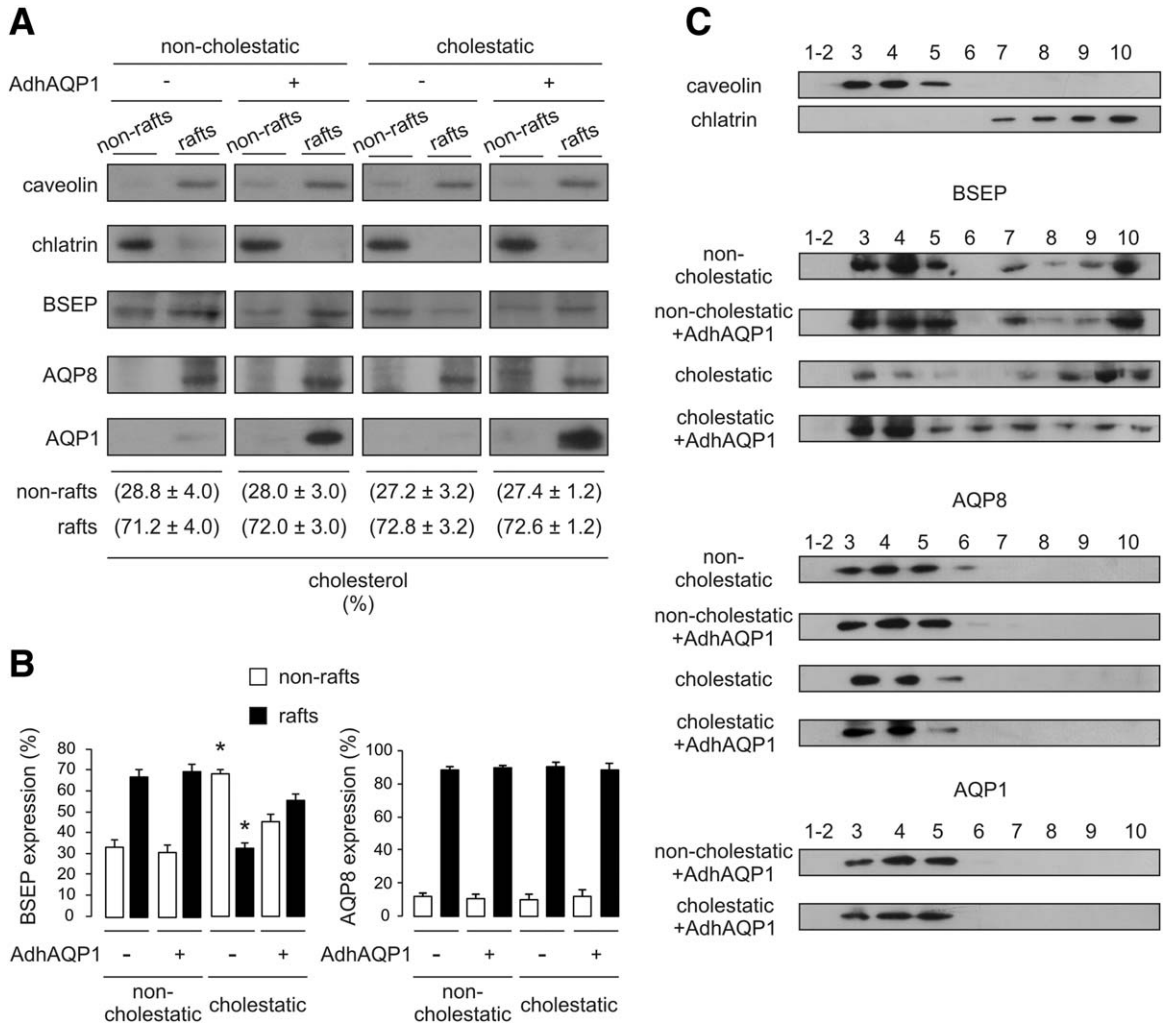

FIG. 7. BSEP localization in canalicular raft/nonraft lipid microdomains in AdhAQP1-transduced EE cholestatic rats. The induction of cholestasis by EE and the delivery of AdhAQP1 and control vectors are detailed in Materials and Methods. Experiments were performed 72 hours after adenoviral infusion (i.e., on the sixth day after starting EE treatment). Raft (high cholesterol) and nonraft (low cholesterol) microdomains were obtained from highly purified liver canalicular plasma membranes as indicated in Materials and Methods. (A) Representative immunoblots for caveolin-1 (raft marker), clathrin (nonraft marker), BSEP, AQP1, and AQP8 in raft and nonraft canalicular microdomains (20 μg proteins/lane). Cholesterol distribution, expressed as percentage of total content (milligrams of cholesterol per milligram of protein), is also shown. (B) Percentage of BSEP and AQP8 in raft and nonraft canalicular microdomains. Data are means ± standard error of four independent experiments. *$P < 0.05$ from noncholestatic controls and cholestatic+AdhAQP1. (C) Canalicular raft/nonraft microdomains prepared by flotation sucrose gradient as detailed in Materials and Methods. Rafts (detergent insoluble fractions) were found at the boundary between 5% and 30% sucrose, corresponding to fractions 3-5 of the gradient, as revealed by caveolin-1. These fractions contain around 70% of the total cholesterol. The gradient distributions of caveolin-1 and the nonraft marker clathrin were identical among the four experimental groups. For the sake of clarity, only those corresponding to noncholestatic rats are shown.

aquaporins) and paracellular entry of water.[2,32-34] Impairment of these basic processes of canalicular fluid secretion is believed to be the primary event in the development of cholestasis induced by estrogens.[3,6,8] In experimental EE-induced cholestasis, canalicular expression of BSEP, multidrug resistance-associated protein-2, and AQP8 is down-regulated.[9,26]

We recently found that canalicularly expressed hAQP1 in EE-induced cholestatic rats improves bile flow.[12] The choleretic efficiency of endogenous BS (i.e., volume of bile per micromole of excreted bile salt) was significantly augmented, thus suggesting that the bile flow was improved to some extent by hAQP1-mediated canalicular water transport.[12] An

unexpected finding in hAQP1-transduced cholestatic rats was the significant improvement in the biliary output of BS (Figs. 2 and 3). This effect was absent in noncholestatic control rats (Figs. 2 and 3), indicating its specificity for rats undergoing EE-induced cholestasis. Taken together, our previous[12] and present data suggest that the adenoviral transfer of hAQP1 gene to livers of EE-induced cholestatic rats improves bile secretory failure by increasing both biliary output and choleretic efficiency of BS.

In EE-induced cholestasis, decreased canalicular disposition of BS is thought to cause their hepatic retention[13] and, consequently, increased serum BS levels. In accordance with the improvement in biliary BS excretion, serum BS concentration was normalized in AdhAQP1-transduced cholestatic rats (Fig. 4). As the increased biliary BS output was not due to an up-regulation of BSEP (or Ntcp) protein expression (Fig. 5), we considered and found an induction of BSEP intrinsic transport activity in hAQP1-transduced cholestatic rats (Fig. 6).

Canalicular BSEP is mostly recruited in cholesterol-rich microdomains or lipid rafts,[30,31] and its transport activity depends on membrane cholesterol content, being reduced or increased with lower or higher cholesterol, respectively.[31,35,36] Kinetic analysis of BSEP transport activity indicates that membrane cholesterol alters maximum velocity but not the Michaelis constant.[31,35,36] Although the actual mechanisms have not been elucidated yet, it is thought that cholesterol either is an allosteric modulator of BSEP or simply acts by changing membrane fluidity.[35]

Cholesterol-enriched hepatocyte plasma membrane microdomains are isolated by their insolubility in cold nonionic detergents. We used a protocol that allows quantitative assessments of protein distribution between raft (high cholesterol) and nonraft (low cholesterol) microdomains.[20,37] As reported,[21] we obtained canalicular membrane microdomains resistant to Triton X-100, enriched in cholesterol, positive for the raft protein caveolin-1 and negative for the nonraft protein clathrin (Fig. 7). Endogenous canalicular AQP8 was present in these microdomains.[21] We found BSEP to be enriched in raft microdomains (Fig. 7), as originally identified by capillary liquid chromatography-tandem mass spectrometry analysis of Triton X-100-resistant proteins from rat liver.[29] BSEP has also been found to be present in cholesterol-enriched microdomains resistant to either the nonionic detergent Lubrol[38] or BS.[39] Thus, different studies coincide in that BSEP normally resides in canalicular cholesterol-enriched microdomains.

Unexpectedly, in EE-induced cholestatic rats, BSEP (but not AQP8) redistributed to nonraft (i.e., detergent soluble, low cholesterol, and clathrin-positive) canalicular microdomains (Fig. 7). In EE-induced cholestasis, canalicular BSEP activity is markedly reduced, maximum velocity, but not Michaelis constant, being affected.[26] Although this can be attributed to the down-regulation of BSEP protein expression, the shift of BSEP to microdomains with lower cholesterol content (Fig. 7) could contribute to the defective BSEP activity in EE-induced cholestasis. In line with this, kinetic analysis of BSEP transport activity predicts a decrease in maximum velocity, with no alteration of Michaelis constant, at lower membrane cholesterol.[31,35,36] Interestingly, in EE cholestasis, maximum velocity for canalicular BSEP showed a 63% reduction,[26,28] while BSEP protein decreased only 47%.[26]

It has been demonstrated that BSEP is constitutively endocytosed by a clathrin-mediated process.[40] In cholestasis induced by the estrogen estradiol 17β-D-glucuronide, canalicular BSEP expression is reduced by enhanced endocytosis.[41,42] Thus, it is tempting to speculate that in estrogen-induced cholestasis BSEP would be first concentrated in nonraft clathrin-enriched microdomains, as described here, before being retrieved in clathrin-coated vesicles. In support of this is the fact that AQP8, which unlike BSEP was retained in raft microdomains in cholestasis (Fig. 7), does not undergo canalicular retrieval in estradiol 17β-D-glucuronide-induced cholestasis.[43]

In AdhAQP1-transduced cholestatic rats, BSEP showed a canalicular microdomain distribution similar to that of control rats, i.e., mostly present in raft (high cholesterol) microdomains (Fig. 7). This should explain, to a certain extent, the up-regulation of BSEP activity (Fig. 6). Nevertheless, the mechanisms by which the presence of hAQP1 stabilizes BSEP in canalicular rafts are unknown. The fact that hAQP1 is almost exclusively located in canalicular rafts (Fig. 7) may suggest an interaction with the raft scaffolding protein caveolin-1. In fact, AQP1 contains a putative caveolin-1 binding site, and several studies have described an association between caveolin-1 and AQP1 in nonhepatic cells.[44] Mice transduced with an adenovector encoding the human caveolin-1 gene have a significant increase in the maximal secretory rate of taurocholate with no changes in BSEP protein expression.[45] As caveolin-1 is a cholesterol-binding protein, caveolin-1 overexpression likely induced changes in the membrane lipid environment of BSEP that favor its transport activity. Nevertheless, a direct activating interaction between caveolin-1

and BSEP, as suggested for another hepatic canalicular transporter,[46] might be possible. We found no significant alteration in canalicular caveolin-1 protein expression with the treatments; i.e., neither EE-induced cholestasis nor AdhAQP1 administration showed any influence (data not shown). Thus, it remains open how hAQP1 would be able to induce a caveolin-mediated retention of BSEP in canalicular rafts of AdhAQP1-transduced cholestatic animals.

It has been reported that under normal or cholestatic conditions the intrabiliary administration of an elevated volume (i.e., 720 μL at a rate of 2 μL/second in 30-g mice) causes disruption of canalicular tight junctions.[47] In our studies, a lower volume (300 μL) was slowly delivered in 320-g rats. Although a transient opening of canalicular tight junctions cannot be excluded, it did not seem to result in disruption of tight junctions and loss of polarized membrane protein localization (Supporting Fig. S1).

Retrograde biliary AdhAQP1 administration induced hAQP1 expression in roughly 20% of hepatocytes,[12] the periportal ones being predominantly transduced, i.e., the primary hepatocytes for bile formation.[48] In addition, to cause no significant adverse effects (Table 1), intrabiliary infusion allows hepatic gene delivery with minimal leak outside the liver because of the anatomical constraints of the biliary tract.[49] Moreover, excess adenovirus is delivered immediately into the duodenum and excreted in the stool. Thus, repetitive transgene hepatic expression could be achieved by intrabiliary infusion without immunosuppression.[50]

In cholestasis, the systemic and intrahepatic accumulation of BS has critical deleterious effects on liver function. As hAQP1 gene transfer clearly improved biliary output and serum levels of BS and retrograde biliary infusion is an approach that would be clinically feasible by means of endoscopic retrograde cholangiography, AdhAQP1 delivery might be effective for the treatment of certain cholestatic disorders.

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Author names in bold designate shared co-first authorship.

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