Defective ATP-dependent bile canicular transport of organic anions in mutant (TR⁻) rats with conjugated hyperbilirubinemia

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ABSTRACT TR⁻ mutant Wistar rats secrete markedly fewer organic anions other than bile acids from the liver into the bile than do control rats. Fluorescence-image analysis of isolated normal and TR⁻ hepatocyte “doublets”, which retain a bile canaliculus between them, revealed that normal hepatocytes readily transport a fluorescent bile acid (fluorescein isothiocyanate glycocholate) and a nonbile acid organic anion (carboxydichlorofluorescein diacetate) into the canaliculus. Hepatocyte doublets from TR⁻ rats also transported fluorescein isothiocyanate glycocholate normally, but transport of carboxydichlorofluorescein diacetate into the canaliculus was negligible. Vesicles derived from the canicular domain of the plasma membrane of hepatocytes (CMV) from control and TR⁻ rats were used to characterize the transport process for ³⁵S-labeled bromosulphthalein and ³⁵S-labeled bromosulphthalein glutathione, which represent nonbile acid organic anions. CMV from normal rat hepatocytes had an ATP- and temperature-dependent, saturable transport process for these ³⁵S-labeled compounds that was absent in CMV from TR⁻ rats. CMV from TR⁻ rats retained normal ATP-dependent transport of daunomycin, and immunologic blots with a monoclonal antibody against the multidrug resistance gene product, P-glycoprotein, revealed no difference between normal and TR⁻ CMV. These studies reveal that the bile canaliculus in normal rats contains an ATP-dependent organic anion transport system that is functionally absent in TR⁻ mutant rats. The defect in TR⁻ mutant rats is phenotypically similar to that seen in mutant Corriedale sheep and in the Dubin–Johnson syndrome in man.

The large plasma-to-bile concentration gradients for organic anions suggest that specific transport mechanisms for these ligands exist between the hepatocyte and bile canaliculus. A candidate bile acid transporter (100 kDa) has been identified in canalicular membranes and was functionally reconstituted in proteoliposomes (1). Recently P-glycoprotein, the product of the multidrug resistance gene, was also localized to bile canalicular membranes, where it functions as an ATP-dependent efflux pump with broad specificity for amphipathic, mainly cationic, compounds of 350–800 Da (2).

Jansen *et al.* (3) described homozygous mutant Wistar rats that manifest conjugated hyperbilirubinemia. Organic anions other than bile acids have markedly restricted appearance in bile, as compared with results in normal rats (4, 5).

From studies that use fluorescence-image analysis of hepatocyte pairs (“doublets”) and canalicular membrane vesicles (CMV) from rat liver, we now report that the defect in mutant Wistar rats resides in an ATP-dependent transport system that is specific for organic anions other than bile acids and is distinct from that involving P-glycoprotein.

MATERIALS AND METHODS

Materials. Fluorescein isothiocyanate glycocholate (FITC-GC), a bile acid analogue, was synthesized according to Sherman and Fisher (6), and its ring-OH-linked structure was established by ¹³C NMR. [³H]Daunomycin (4.9 Ci/mmol; 1 Ci = 37 GBq) and [^γ-³²P]ATP (3000 Ci/mmol) were purchased from DuPont/NEN. ³⁵S-labeled bromosulphthalein (BSP) (specific activity, 4034 μCi/μmol) was prepared (7) and used in synthesis and purification of ³⁵S-labeled bromosulphthalein glutathione (BSP-GSH) (specific activity, 2955 μCi/μmol) (8). Carboxydichlorofluorescein diacetate (CFA) was obtained from Molecular Probes. P-Glycoprotein purified from the hamster LZ cell line and monoclonal antibody (C219) against P-glycoprotein were provided by Victor Ling (Ontario Cancer Institute, Toronto). Type I collagenase and modified Eagle's medium were obtained from Sigma. All other chemicals were of analytical grade.

Animals. Male TR⁻ and control Wistar rats weighing 200–250 g were shipped from the Academic Medical Center (Amsterdam) to Tufts University (Boston) where they were housed with alternating 12-hr light–dark cycles and maintained on Purina rat chow and water ad libitum.

Methods. After i.p. anesthesia of TR⁻ and control rats with sodium pentobarbital (5 mg/100 g), the common bile duct was cannulated, and bile was collected in tared tubes every 10 min for 1 hr. CFA/20% dimethyl sulfoxide/80% ethanol was perfused into the jugular vein at 500 nmol/min per kg. FITC-GC (1.7 nmol/g) was injected into other rats as a bolus into the femoral vein. Aliquots of bile were measured by scintillation or fluorescence spectroscopy. Background fluorescence in bile was negligible.

Monolayer cultures of rat hepatocytes were prepared, according to Selgen (9). Intraportal perfusion was performed with Leffert's buffer (10) for 10 min at 40 ml/min followed by perfusion with 0.05% type I collagenase for 10 min. After isolation, cells were filtered through nylon gauze and incubated at 3 × 10⁵ cells per dish on glass coverslips in 35-mm Falcon culture dishes. Viability was assessed by trypan blue exclusion; experiments were done with cells that showed viability of >90%. Cells were maintained in modified Eagle's medium/10% fetal calf serum/penicillin at 100 units/ml/streptomycin at 100 μg/ml. Hepatocyte doublets were identified by phase microscopy after cells had been cultured at 37°C for 4 hr in 5% CO₂. Approximately 8% of hepatocytes appeared in pairs that enclosed a circular space easily identified as a bile canaliculus (11, 12).

Rat CMV were isolated from liver homogenates by using nitrogen cavitation and calcium precipitation methods, as described (13). As compared with homogenates, preparations for normal and TR⁻ rats were enriched 48- and 40-fold in

Table 1. Rate of accumulation of CFA and FITC-GC in bile canaliculus and cytoplasm of hepatocyte doublets for 9 min

	Fluorescence units per min		
	Normal	TR ⁻	Significance
CFA			
Bile canaliculus	1.60 ± 0.31	-2.04 ± 0.43	<i>P</i> < 0.005
minus cytoplasm	(<i>n</i> = 7)	(<i>n</i> = 10)	
Cytoplasm	7.3 ± 0.65	8.5 ± 0.61	NS
	(<i>n</i> = 7)	(<i>n</i> = 10)	
FITC-GC			
Bile canaliculus	0.79 ± 0.19	0.57 ± 0.16	NS
minus cytoplasm	(<i>n</i> = 9)	(<i>n</i> = 8)	
Cytoplasm	2.1 ± 0.30	3.1 ± 0.28	NS
	(<i>n</i> = 9)	(<i>n</i> = 8)	

Hepatocyte doublets from normal and TR⁻ rats were incubated with 0.1 μ M CFA or 0.5 μ M FITC-GC. Fluorescence intensity was recorded every 2 min, and rates were calculated by linear regression. From 7 to 10 doublets from at least two rats were examined in each experiment. The values are mean $\pm$ SEM. NS, not significant.

γ -glutamyl transpeptidase and leucine aminopeptidase activities, respectively, which are markers of the canalicular membrane (13), and ouabain-inhibitable Na⁺,K⁺-ATPase activity, which is a marker of the sinusoidal membrane (13), was not detected. Protein recovery was 4.85 $\pm$ 0.3 and 5.0 $\pm$ 0.8 μ g/g of liver in normal and TR⁻ CMV, respectively. Membranes were stored in 10 mM Hepes-Tris buffer, pH 7.4/0.25 M sucrose/0.2 mM CaCl₂ (buffer A) at -70°C before

use. Protein concentration was determined by the method of Lowry with bovine serum albumin as a standard.

Daunomycin, BSP, and BSP-GSH transport were measured by rapid filtration (2, 13). Transport medium contained [³H]daunomycin (12 μ M), ³⁵S-labeled BSP (1 μ M) or ³⁵S-labeled BSP-GSH, and an ATP-regenerating system (3 mM creatine phosphate/3.6 μ g of creatine phosphokinase) with or without 1.2 mM ATP in buffer B (10 mM Hepes-Tris buffer, pH 7.4/0.25 M sucrose/0.2 mM CaCl₂/10 mM MgCl₂). Transport was initiated by adding 20 μ l of vesicles (38–70 μ g of protein) suspended in buffer A to 0.1 ml of incubation medium that had been preincubated for 10 min at 37°C. Aliquots of 20 μ l were removed at intervals and diluted to 1 ml with ice-cold buffer B. The vesicles were filtered through glass microfiber filters (Whatman, 0.45- μ m pore size), which were washed three times with 6 ml of cold buffer B. Radioactivity on filters was measured in a scintillation spectrometer. All values were corrected for radioactivity found on the filters in the absence of membrane vesicles. The filter blank ranged from 8–12% of BSP or BSP-GSH added.

Cultured rat hepatocytes on cover slips were placed in a Dvorak-Stotler culture chamber (Nicholson Precision Instruments, Gaithersburg, MD) at 37°C and perfused with Leffert's buffer containing 0.1 μ M CFA or 0.5 μ M FITC-GC at 0.3 ml/min. The chamber was installed in a fluorescence microscope (Zeiss) with a stabilized arc lamp as a light source and SITcam (DAGE-MTI, Michigan City, IN) fluorescence detector. The microscope was connected to a Microvax II computer (Digital) and M-1550 printer (C. Itoh Electronics).

FIG. 1. Fluorescence microscopy of hepatocyte doublets from TR⁻ rats. (A) Phase microscopy reveals a bile canaliculus between attached hepatocytes. (B) CFA was not secreted into canaliculus within 9 min after incubation. (C) After addition of FITC-GC and incubation for 30 min, fluorescence appeared in the canaliculus.

Wavelengths of 490 nm for excitation and 525 nm for emission were used. Fluorescence intensity was serially quantitated in user-selected regions of uniform pixel value, electronically corrected for background, and integrated by using an algorithm developed by the Image Analysis Laboratory, Tufts-New England Medical Center. Autofluorescence was not detectable at the setting for gain and sensitivity used. Bleaching was negligible because exposure time was 0.5 sec. Images were digitized, stored, and printed.

Cultured hepatocyte doublets from TR⁻ and normal rats were also examined using an MRC 500 confocal imaging system (Bio-Rad Lasersharp) with a Zeiss fluorescence microscope. The system was connected to a Nimbus AX computer. Doublet cells were incubated with 0.5 μ M CFA or FITC-GC at 37°C for various intervals 3–7 min before examination by confocal microscopy.

Accumulation and release of fluorescence by hepatocyte doublets were fitted to a straight line by linear regression analysis, and the rate was calculated from the slope. Statistical analysis was performed using Student's *t* test. All results are expressed as mean $\pm$ SEM.

RESULTS

Within 60 min, biliary secretion of CFA was 13.7% of the injected dose in control rats and 0.03% in TR⁻ rats (400-fold difference). In contrast, the biliary secretion of FITC-GC in control rats was 96.7% and 21.4% in TR⁻ rats (4-fold difference). Background fluorescence in normal bile was negligible.

Ten doublet preparations from normal and from TR⁻ rats were separately incubated with FITC-GC and CFA, and the proportion that secreted fluorescence into the bile canaliculus within 9 min was determined. Ninety percent of normal and 80% of TR⁻ doublets secreted FITC-GC. Seventy percent of normal and none of TR⁻ doublets secreted CFA.

Incubation of hepatocyte doublets from control and TR⁻ rats with FITC-GC resulted in similar rates of accumulation of fluorescence in the cytoplasm and bile canaliculi, which were linear for 9 min (Table 1). When doublets were incubated with CFA, the rates of accumulation of fluorescence in the cytoplasm were similar in control and TR⁻ rats. CFA fluorescence was significantly reduced in canaliculi from TR⁻ rats (Table 1). In each of three experiments, subsequent incubation with FITC-GC for 30 min resulted in canalicular secretion of fluorescence (Fig. 1). In each of four experiments, incubation of TR⁻ doublets with CFA for 40 min failed to reveal canalicular secretion (Fig. 2).

The bile canaliculus in doublet hepatocytes is a closed sphere surrounded by plasma membrane and cytoplasm, which must be corrected for to measure canalicular fluorescence by conventional microscopy (Table 1). To offset this effect, confocal microscopy was performed in 12 normal and 12 TR⁻ doublets after incubation for various times from 3 to 10 min with CFA or FITC-GC. From 3 to 7 min in all preparations, FITC-GC was present in the canaliculus in control and TR⁻ doublets, and CFA was detected only in the canaliculus of normal doublets. Fig. 3 is representative of three experiments in each group done 7 min after incubation.

The time course of ³⁵S-labeled BSP and ³⁵S-labeled BSP-GH transport in CMV from control rats is shown in Fig. 4. ATP stimulated vesicle-associated ³⁵S-labeled BSP and ³⁵S-labeled BSP-GSH $\approx$ 2-fold in CMV. A component of the initial uptake (0–0.5 min.) apparently represents binding to CMV. ATP-dependent transport was abolished by incubation of CMV at 25°C or in 0.1% deoxycholate, which unseals vesicles (2). ATP-dependent vesicle-associated BSP transport decreased stepwise when osmolality was increased by incubation in raffinose (0.1–2.0 M). The values obtained by extrapolation to infinite high osmolality indicate that 68% of

FIG. 2. Control study of the experiment shown in Fig. 1. (A) Phase-contrast microscopy of TR⁻ doublets. (B) CFA was not secreted into the canaliculus after incubation for 40 min.

BSP associated with canalicular vesicles represents specific uptake into the intravesicular space. Twelve similar studies using six different preparations of CMV from TR⁻ rats revealed the absence of ATP-dependent transport of ³⁵S-labeled BSP and ³⁵S-labeled BSP-GSH (Fig. 5). Increasing the concentration of ATP 10-fold did not alter the results.

CMV from TR⁻ rats were sealed as evidenced by ATP-dependent daunomycin transport in CMV from control and TR⁻ rats (Fig. 6). Verapamil (100 μ M) inhibited ATP-dependent daunomycin transport in CMV from normal and TR⁻ rats but had no effect on ATP-dependent BSP transport by CMV from control rats. Immunoblots (Western) with C219 anti-P-glycoprotein antibody and subsequent densitometry revealed that CMV from control and TR⁻ rats contain equal amounts of immunoreactive protein of $\approx$ 160 kDa.

DISCUSSION

Homozygous TR⁻ rats have an autosomally inherited abnormality characterized by defective hepatobiliary secretion of organic anions, such as bilirubin glucuronides, BSP-GSH, cysteinyl leukotriene, and reduced and oxidized glutathione (3–5, 14–17). In contrast, the biliary secretion of the bile acids, tauro- and glycocholate, and of organic cations is normal (5, 14). Secretion of dinitrophenyl glutathione by isolated hepatocytes from TR⁻ rats is severely impaired, indicating that the transport defect resides in the hepatocyte (16).

The present studies confirm that the defect involves transfer of organic anions other than bile acids from the hepatocyte

FIG. 3. Confocal microscopy. Hepatocyte doublets from normal (A and C) and TR⁻ (B and D) rats were incubated with 0.5 μ M CFA (A and B) or FITC-GC (C and D) for 7 min and examined by confocal microscopy. In A, C, and D, the fluorescence accumulated in the canaliculus more than in the cytoplasm; in B, CFA was not secreted into the canaliculus despite its abundance in cytoplasm.

into the bile. Biliary excretion of FITC-GC was reduced in TR⁻ rats, although to a lesser degree than was CFA excre-

FIG. 4. Time course of BSP and BSP-GSH transport in normal CMV. BSP ($\square$, $\blacksquare$) and BSP-GSH (Δ , $\blacktriangle$) transport were measured in 10 mM Hepes-Tris buffer, pH 7.4/0.25 M sucrose/0.2 mM CaCl_2 /10 mM MgCl_2 containing ^{35}S -labeled BSP (1 μ M) or ^{35}S -labeled BSP-GSH and an ATP-regenerating system (3 mM creatine containing 3.6 μ g of creatine phosphokinase) with 1.2 mM ATP ($\blacksquare$, $\blacktriangle$) or without ATP ($\square$, Δ) in a total volume of 0.1 ml. Each point represents the mean value of duplicate determinations in five experiments.

tion, which may result from competition for transport in TR⁻ liver *in vivo* but not *in vitro*. Studies *in vivo* measured total biliary secretion, whereas studies in doublets and CMV measured transport rates. Studies with TR⁻ doublets reveal

FIG. 5. Time course of BSP and BSP-GSH transport in TR⁻ CMV. BSP ($\square$, $\blacksquare$) and BSP-GSH (Δ , $\blacktriangle$) transport were measured in the medium described for Fig. 4 with 1.2 mM ATP ($\blacksquare$, $\blacktriangle$) or without ATP ($\square$, Δ). Each point represents the mean value of duplicate determinations in five experiments.

FIG. 6. Time course of daunomycin transport in normal (Δ , $\blacktriangle$) and TR⁻ ($\square$, $\blacksquare$) CMV. Transport was measured in the medium described for Fig. 4, which contained 12 μ M [3 H]daunomycin instead of 35 S-labeled BSP or 35 S-labeled BSP-GSH with 1.2 mM ATP ($\blacksquare$, $\blacktriangle$) or without ATP ($\square$, Δ). Each point represents the mean value of duplicate determinations in five experiments.

that bile acid transport, as investigated with FITC-GC, appears normal, whereas CFA transport is impaired. Confocal microscopy provides further evidence that there is virtually no secretion of CFA into the bile canaliculi of TR⁻ hepatocyte doublets, which, nevertheless, transport FITC-GC. These results are consistent with *in vivo* studies of biliary excretion of FITC-GC, which reveal little difference between normal and TR⁻ rats.

BSP and BSP-GSH were transported in normal CMV by a saturable, temperature- and ATP-dependent process that requires a vesicular space. Previous studies of the transport of organic anions other than bile acids by CMV suggested that the process does not require ATP and is driven by the electrochemical gradient (18). The relatively low specific activity of the organic anions used in these earlier investigations necessitated performance at organic anion concentrations that approach equilibrium and, therefore, obscured the transport process we have observed. CMV from normal and TR⁻ rats similarly bound BSP and BSP-GSH, but CMV from TR⁻ rats lacked ATP-dependent transport of these ligands. ATP-dependent transport of daunomycin was demonstrated in the same CMV preparations from TR⁻ rats, indicating that the vesicles are intact and sealed. ATP-dependent daunomycin transport by CMV occurs only in inside-out vesicles (2) and was quantitatively similar in CMV from normal and TR⁻ rats (2, Fig. 6). We postulate that ATP-dependent transport of BSP and BSP-GSH by CMV is similarly unidirectional because of the requirement for ATP, which should be accessible only from the cytoplasmic domain of the plasma membrane.

Other evidence suggests that the P-glycoprotein system is intact in TR⁻ rats. Immunoblots using C219 antibody revealed similar presence of P-glycoprotein in normal and TR⁻ CMV but not in sinusoidal membrane vesicle preparations. Transport by the P-glycoprotein system is inhibited by ve-

rapamil (2), which did not inhibit ATP-dependent transport of BSP or BSP-GSH by CMV from normal rats.

The functional abnormality in TR⁻ rats resembles the defect in mutant Corriedale sheep (19) and in patients with the Dubin-Johnson syndrome (20, 21). In the ovine and human mutants, the defect is transmitted with the characteristics of an autosomal recessive trait that involves transfer of organic anions other than bile acids from hepatocyte to bile. In addition, the liver is black, which results from accumulation of anionic metabolites of tryptophan, tyrosine, and phenylalanine in hepatocellular lysosomes producing a melanin-like pigment. The liver in TR⁻ rats is not black; however, we have demonstrated that biliary secretion of epinephrine metabolites, which are precursors of the hepatic pigment in mutant sheep, is profoundly reduced (data not shown).

These studies reveal that TR⁻ rats lack an ATP-dependent transport system for nonbile acid organic anions in the bile canaliculi. It is likely that the defect is related to that present in mutant Corriedale sheep and in the human Dubin-Johnson syndrome.

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