Biliary Excretion of 17β-Estradiol 17β-D-Glucuronide Is Predominantly Mediated by cMOAT/MRP2

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Purpose. The mechanism for the biliary excretion of 17β-estradiol 17β-D-glucuronide ($E_2$17βG), a cholestatic metabolite of estradiol, is still controversial. The purpose of the present study is to examine the transport of $E_2$17βG across the bile canalicular membrane.

Methods. We examined the uptake of [^{3}H]$E_2$17βG by isolated canalicular membrane vesicles (CMVs) prepared from Sprague-Dawley (SD) rats and Eisai Hyperbilirubinemic rats (EHBR) whose canalicular multispecific organic anion transporter/multidrug resistance associated protein 2 (cMOAT/MRP2) function is hereditarily defective. Also, *in vivo* biliary excretion of intravenously administered [^{3}H]$E_2$17βG was examined.

Results. In CMVs prepared from SD rats, but not from EHBR, a marked ATP-dependent uptake of [^{3}H]$E_2$17βG was observed. Moreover, $E_2$17βG competitively inhibited the ATP-dependent uptake of [^{3}H]2,4-dinitrophenyl-S-glutathione (DNP-SG). In addition, no significant inhibitory effect of verapamil (100 μM) and PSC-833 (5 μM) on the uptake of [^{3}H]$E_2$17βG was observed. *In vivo*, the biliary excretion of intravenously administered [^{3}H]$E_2$17βG was severely impaired in EHBR while the biliary excretion of [^{3}H]$E_2$17βG in SD rats was reduced by administering a cholestatic dose (10 μmol/kg) unlabeled $E_2$17βG, but not by PSC-833 (3 mg/kg).

Conclusions. The transport of $E_2$17βG across the bile canalicular membrane is predominantly mediated by cMOAT/MRP2.

KEY WORDS: cMOAT/MRP2; biliary excretion; 17β-estradiol 17β-D-glucuronide; P-glycoprotein.

INTRODUCTION

17β Estradiol 17β-D-glucuronide ($E_2$17βG), a metabolite of 17β-estradiol, has been identified as one of the most potent agents which can induce cholestasis in pregnancy (1). Indeed, the pathophysiology of cholestasis in pregnancy has been studied extensively by the administration of ethinylestradiol (EE), a synthetic steroid whose plasma half-life is much longer than that of estradiol. (2,13). It has been shown that EE-treatment reduces the activities of primary active transporters located on the bile canalicular membrane such as bile salt exporting pump (BSEP) and canalicular multispecific organic anion transporter/multidrug resistance associated protein 2 (cMOAT/MRP2), which are responsible for bile acid-dependent and independent bile flow formation, respectively (2,3). In particular, by comparing the kinetic parameters for the ATP-dependent transport of BSEP and cMOAT/MRP2 substrates (such as taurocholate and 2,4-dinitrophenyl-S-glutathione (DNP-SG), respectively) in the isolated bile canalicular membrane vesicles (CMVs), it has been shown that the number of these transporters is reduced during cholestasis (2,3). Moreover, Western blot analysis indicated the down-regulation of cMOAT/MRP2 protein under such cholestatic conditions (3). However, the finding that the absolute value for the decrease in bile acid-independent bile flow rate induced by EE was comparable in normal and mutant rats (Groningen Yellow and Transport deficient rats (GY/TR)) whose cMOAT/MRP2 function is defective suggests the reduction in cMOAT/MRP2 activity is not necessarily related to the cholestatic condition induced by the administration of this steroid (4).

In contrast to its well characterized cholestatic nature, the transport mechanism of $E_2$17βG in hepatocytes is not fully understood. Recently, it was suggested the hepatocellular uptake of $E_2$17βG is predominantly mediated by organic anion transporting polypeptide 1 (5,6) in exchange for the efflux of reduced glutathione (7). Although it is well established that the biliary excretion of many xenobiotics and their metabolites is mediated by primary active transporters located on the bile canalicular membrane (such as cMOAT/MRP2 and MDR1 P-glycoprotein (P-gp)) (8–11), the mechanism for excretion of $E_2$17βG across this membrane is still controversial. The findings that i) the total radioactivity excreted into bile after the administration of [^{3}H]$E_2$17βG is significantly reduced in mutant rats deficient in cMOAT/MRP2 expression (Eisai hyperbilirubinemic rats; EHBR), and ii) the amount of [^{3}H]estradiol glucuronides with or without sulfate excreted into the bile after the injection of [^{3}H]estradiol is also decreased in EHBR, suggests $E_2$17βG is excreted into the bile via cMOAT/MRP2 (12). Concerning the pathogenesis of EHBR, we have previously found the defect of cMOAT/MRP2 in EHBR is ascribed to the introduction of a premature stop codon within the open reading frame by point mutation (13). Moreover, Büchler *et al.* (14) indicated the loss of cMOAT/MRP2 protein from the canalicular membrane of EHBR. Collectively, these observations are consistent with the finding that $E_2$17βG is transported via MRP1 (15,16), whose substrate specificity is quite similar to that of cMOAT/MRP2 (8–11).

Although Vore *et al.* demonstrated mutual inhibition by $E_2$17βG and DNP-SG of the uptake into CMVs, they also found the transport of $E_2$17βG is inhibited by several P-gp substrates, such as PSC-833, cyclosporin A, and daunorubicin, along with C219, a monoclonal antibody to this efflux pump (17). In addition, they showed the transport of [^{3}H]daunorubicin is also competitively inhibited by $E_2$17βG (17). Recently, it has been shown the uptake of [^{3}H]$E_2$17βG into membrane vesicles isolated from Sf9 cells is stimulated by human MDR1 gene transfection (18). These results are consistent with the hypothesis

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ABBREVIATIONS: $E_2$17βG, 17β-estradiol 17β-D-glucuronide; SD, Sprague-Dawley; EHBR, Eisai Hyperbilirubinemic rats; cMOAT, canalicular multispecific organic anion transporter; MRP, multidrug resistance associated protein; P-gp, P-glycoprotein; CMVs, canalicular membrane vesicles; DNP-SG, 2,4-dinitrophenyl-S glutathione; K_m, Michaelis constant; K_i, inhibition constant; V_{max}, maximum transport velocity; P_{dif}, nonspecific diffusion clearance; CL_{bile}, biliary clearance; CL_{urine}, urinary clearance.

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that P-gp significantly contributes to the biliary excretion of $E_2$17βG.

The purpose of the present study is to clarify the mechanism for the bile canalicular transport of $E_2$17βG. Since the substrates of cMOAT/MRP2 have been identified by comparing the transport activity between the normal and cMOAT/MRP2-deficient rats (8,10,11), the transport of $E_2$17βG was thoroughly investigated in *in vitro* experiments with CMVs as well as *in vivo* experiments in SD rats and EHBR.

MATERIALS AND METHODS

Materials

[^{3}H]$E_2$17βG (55 μCi/nmol) was purchased from New England Nuclear (Boston, MA). Unlabeled and labeled DNP-SG (50.0 μCi/nmol) were synthesized as described previously (19). $E_2$17βG, ATP, creatine phosphate, and creatine phosphokinase were purchased from Sigma Chemical Co. (St. Louis, MO). Verapamil was purchased from Wako Pure Chemical Ind. Ltd. (Tokyo, Japan). PSC-833 was supplied by Novartis Pharma Ltd. (Basel, Switzerland). All other chemicals used were commercially available and of reagent grade.

Male SD rats (250–300 g) were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and male EHBR (270–360 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan).

Preparation of CMVs

CMVs were prepared from the liver of male SD rats and EHBR as described previously (20) and kept as a suspension in 50 mM Tris buffer (pH 7.4) containing 250 mM sucrose. The membrane vesicles were then frozen in liquid N_2 and stored at −100°C until used. To check the purity of the prepared CMVs, the activity of alkaline phosphatase was determined as described previously (20) and protein concentrations were determined as reported previously (20), using an assay kit (Bio Rad Laboratories, Richmond, CA) with bovine serum albumin as a standard.

Uptake Study of [^{3}H]$E_2$17βG and [^{3}H]DNP-SG

The uptake study of [^{3}H]$E_2$17βG and [^{3}H]DNP-SG was performed as reported previously (20). The transport medium (10 mM Tris, 250 mM sucrose and 10 mM $MgCl_2 \bullet 6H_2O$, pH 7.4) contained the isotopically labeled ligand, 5 mM ATP or 5 mM AMP, and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml creatine phosphokinase). A 16 μl aliquot of transport medium was mixed rapidly with 4 μl vesicle suspension (10 μg protein). PSC-833 was dissolved in DMSO and diluted to a final concentration (0.2% DMSO) with transport buffer. In the control study, the same concentration of DMSO was also added to the transport medium. The transport reaction was stopped by the addition of 1 ml ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.4). The stopped reaction mixture was filtered through a 0.45 μm HA filter (Millipore Corp., Bedford, MA), and then washed twice with 5 ml stop solution. The radioactivity retained on the filter and in the reaction mixture was measured in a liquid scintillation counter (LS 6000SE, Beckman Instruments, Fullerton, CA) following the addition of scintillation cocktail (Clearsol I, Nacalai Tesque, Tokyo, Japan). Ligand uptake was normalized in terms of the amount of membrane protein.

The ATP-dependent uptake was determined by subtracting the uptake in the presence of AMP from that in the presence of ATP. The kinetic parameters were estimated as described previously (20).

In Vivo Study

The bile duct was cannulated using polyethylene tubing (Becton Dickison Primary Care Diagnostics, Sparks, MD) and the bladder was catheterized using a different type of polyethylene tubing (No.8; O.D. 2.33 mm, Hibiki Co., Tokyo, Japan). The body temperature of rats was maintained under suitable lighting. [^{3}H]$E_2$17βG (180 pmol/2 ml/kg; 10 μCi/2 ml/kg), with or without unlabeled $E_2$17βG, dissolved in distilled water: polyethylene glycol: ethanol (10:4: 1 v/v), was injected intravenously. PSC-833 (3 mg/800 μl/kg) was administered intravenously 30 min prior to the injection of [^{3}H]$E_2$17βG. Bile specimens were collected at 0–10, 10–20, 20–30, 30–40, 40–50, 50–60, 60–90, 90–120 min, and stored on ice. Blood was collected at 1, 5, 10, 30, 60 min. The total radioactivity in plasma, bile, and urine was measured in a liquid scintillation counter (LS 6000SE, Beckman Instruments, Fullerton, CA).

TLC Analysis

Plasma and urine samples were deproteinized with methanol, then aliquots were transferred to Eppendorf tubes and lyophilized. The dried residues were dissolved in 20 μl methanol and spotted onto high performance thin-layer chromatography (HPTLC) plates (10 × 10 cm, Whattman, New Jersey). Bile specimens were spotted directly onto HPTLC plates. HPTLC was carried out with chloroform:methanol:acetic acid (7:2:1 v/v). For bile specimens, the radioactivity associated with each spot on the HPTLC plates was quantified with an image analyzer (BAS-1500; Fuji Photo film, Tokyo, Japan). For plasma and urine specimens, the radioactivity was measured in a liquid scintillation counter (LS 6000SE, Beckman Instruments, Fullerton, CA) after scraping off the silica-gel from the HPTLC plates.

Calculation of *In Vivo* Kinetic Parameters

The area under the plasma concentration-time curve (AUC) was estimated by the trapezoidal rule using the plasma data from 0 to 60 min. The clearance values of bile and urine (CL_{bile} and CL_{urine}) were determined by dividing the amounts of $E_2$17βG excreted into bile and urine from 0 to 120 min by the AUC from 0 to 60 min.

RESULTS

CMV Study

The time profile for the uptake of [^{3}H]$E_2$17βG by CMVs prepared from SD rats and EHBR was examined (Fig. 1). In CMVs from SD rats, a marked ATP-dependence was observed, and the uptake of [^{3}H]$E_2$17βG in the presence of ATP increased linearly up to 2 min. In contrast, in CMVs from EHBR, no significant ATP-dependent transport of [^{3}H]$E_2$17βG was

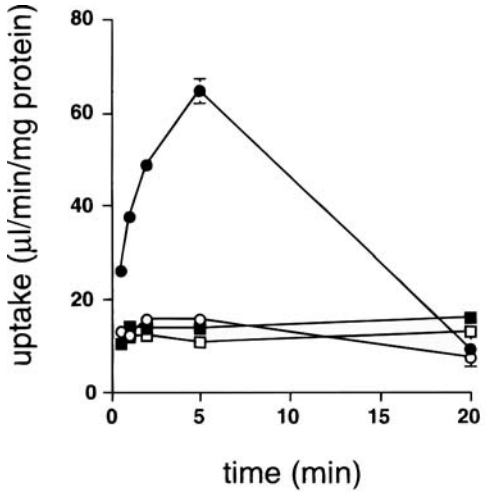

Fig. 1. Time-dependent uptake of [^{3}H]$E_2$17βG by CMVs. CMVs prepared from SD rats (circles) and EHBR (squares) were incubated at 37°C with (closed circles) or without (open circles) ATP and ATP-regenerating system in the medium. The concentration of [^{3}H]E_2 17βG was 0.05 μM. Each point and vertical bar represent the mean ± S.E. of three different experiments.

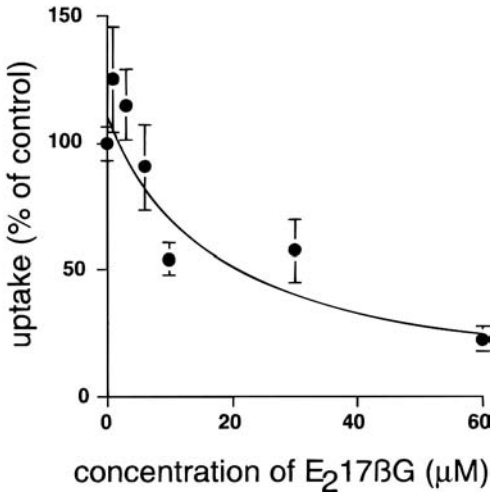

Fig. 3. Effect of $E_2$17βG on the ATP-dependent uptake of [^{3}H]DNP-SG by CMVs prepared from SD rats. CMVs prepared from SD rats were incubated at 37°C for 2 min, with or without ATP and ATP-regenerating system in the medium containing [^{3}H]DNP-SG (1 μM) and unlabeled $E_2$17βG. The ATP-dependent uptake was obtained by subtracting the value in the absence of ATP from that in its presence. Each point and vertical bar represent the mean ± S.E. of three different experiments. The solid line represents the fitted line which was obtained by fitting the data to uptake (% of control) = $1/(1 + I/K_i)$

observed (Fig. 1). Kinetic analysis revealed that the ATP-dependent uptake of [^{3}H]$E_2$17βG into CMVs from SD rats could be described by one saturable (K_m = 6.32 ± 2.39 μM, V_{max} = 81.3 ± 29.2 pmol/min/mg protein) and one non-saturable component (P_{dif} = 2.68 ± 0.69 μM) (Fig. 2). Then, the effect of $E_2$17βG on the ATP-dependent uptake of [^{3}H]DNP-SG by CMVs from SD rats was examined (Fig. 3). The uptake of [^{3}H]DNP-SG was inhibited by $E_2$17βG in a concentration-dependent manner, with an IC_{50} of 16.5 ± 5.3 μM. Moreover, $E_2$17βG competitively inhibited the ATP-dependent uptake of [^{3}H]DNP-SG (Fig. 4). The K_m value of [^{3}H]DNP-SG and the K_i value of $E_2$17βG for the uptake of [^{3}H]DNP-SG were calculated as 20.9 ± 4.3 μM and 5.78 ± 1.20 μM, respectively. The statistical analysis indicated the significant correlation

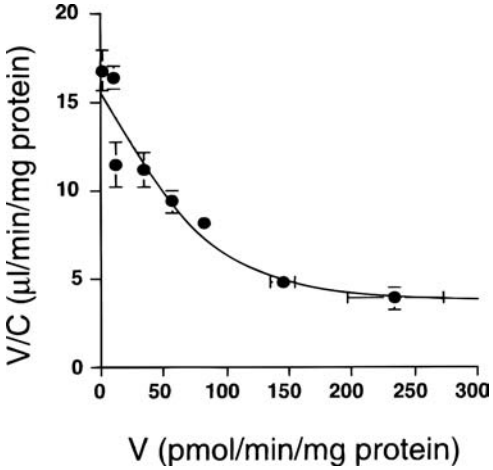

Fig. 2. Eadie-Hofstee plot of the uptake of [^{3}H]$E_2$17βG by CMVs prepared from SD rats. CMVs prepared from SD rats were incubated at 37°C for 2 min, with or without ATP and ATP-regenerating system in the medium. The ATP-dependent uptake was obtained by subtracting the value in the absence of ATP from that in its presence. The concentration of [^{3}H]$E_2$17βG was 0.1 μM. Each point and bar represent the mean ± S.E. of three different experiments. The solid line represents the fitted line which was obtained by fitting the data to $V/C = V_{max}/(K_m + C)$.

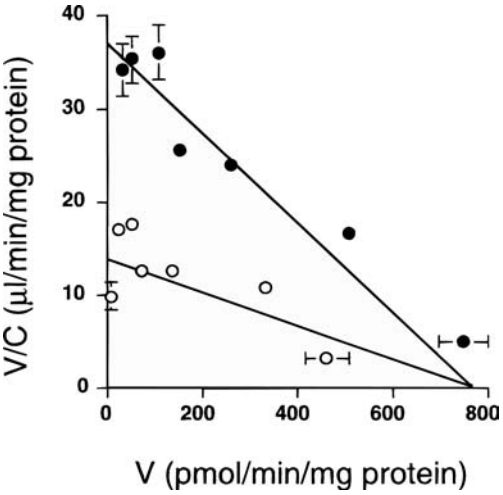

Fig. 4. Eadie-Hofstee plot of the uptake of [^{3}H]DNP-SG in the presence and the absence of $E_2$17βG by CMVs prepared from SD rats. CMVs prepared from SD rats were incubated at 37°C for 2 min, with or without ATP and ATP-regenerating system in the medium in the absence (closed circles) and presence (open circles) of $E_2$17βG (10 μM). The ATP-dependent uptake was obtained by subtracting the value in the absence of ATP from that in its presence. The concentration of [^{3}H]DNP-SG was 1 μM. Each point and bar represent the mean ± S.E. of three different experiments. The solid lines represents the fitted lines which was obtained by simultaneously fitting the data to $V/C = V_{max}/(K_m + C)$ (in the absence of $E_2$17βG) and $V/C = V_{max}/\{K_m(1 + I/K_i) + C\}$ (in the presence of $E_2$17βG).

between the vertical and horizontal axes of Eadie-hofstee plot for the transport of DNP-SG in the presence ($p < 0.05$) and the absence ($p < 0.001$) of $E_2$17βG.

The Inhibitory effect of P-gp inhibitors on the ATP-dependent uptake of both [^{3}H]DNP-SG and [^{3}H]$E_2$17βG was minimal; PSC-833 and verapamil had no significant effect on the transport of these ligands, up to concentrations of 5 μM and 100 μM, respectively (Fig. 5).

In Vivo Study

The biliary excretion of [^{3}H]$E_2$17βG in SD rats and EHBR *in vivo* was also examined after intravenous administration of tracer (180 pmol/kg) and cholestatic (10 μmol/kg) doses. The time-profiles of the plasma concentration (Fig. 6A and B) and the cumulative amount of [^{3}H]$E_2$17βG excreted into the bile (Fig. 6D and E) and urine were examined to calculate CL_{bile} (Fig. 7A and B) and CL_{urine} (Fig. 7D and E). The plasma concentration of [^{3}H]$E_2$17βG in EHBR was slightly higher than that in SD rats after administration of 180 pmol/kg, but no significant difference was observed between the two strains of rats after administration of 10 μmol/kg (Fig. 6). The cumulative biliary excretion of $E_2$17βG in EHBR was markedly lower than that in SD rats; after administration of a tracer dose, more than 98% of the dose (77% $E_2$17βG and 21% $E_2$17βG-3-sulfate) was excreted into the bile up to 60 min in SD rats, whereas, the corresponding amount was only 9% in EHBR (Fig. 6). As a consequence, the calculated CL_{bile} in EHBR was only 10% that in SD rats. After administration of a cholestatic dose of $E_2$17βG, CL_{bile} in SD rats was reduced compared with that after the tracer dose, whereas, in EHBR, this parameter was the same for the two doses (Fig. 7). Moreover, the biliary excretion of $E_2$17βG was not affected by PSC-833 (3 mg/kg) in both SD rats and EHBR. Also, CL_{urine} was similar in the two rat strains, and unaffected by administration of PSC-833 (3 mg/kg) (Fig. 7).

After the tracer dose, the bile flow rate (g/hr) in SD rats and EHBR was 1.0 ± 0.1 and 0.83 ± 0.11 from 0 min to 60 min, and 0.71 ± 0.08 and 0.63 ± 0.09 from 60 min to 120 min, respectively. After the cholestatic dose, the bile flow rate (g/hr) in SD rats and EHBR was 0.34 ± 0.06 and 0.55 ± 0.14 from 0 min to 60 min, and 0.69 ± 0.07 and 0.31 ± 0.13 from 60 min to 120 min, respectively. After the administration of PSC-833, the bile flow rate (g/hr) in SD rats and EHBR was 0.86 ± 0.10 and 0.58 ± 0.08 from 0 min to 60 min, and 0.79 ± 0.08 and 0.40 ± 0.10 from 60 min to 120 min, respectively.

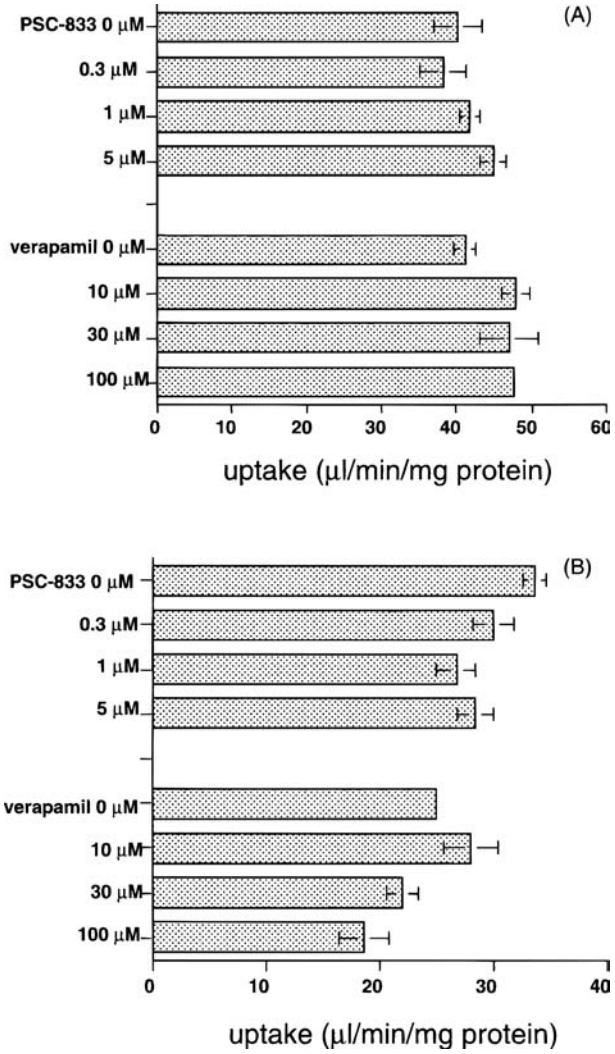

Fig. 5. Effect of verapamil and PSC-833 on the ATP-dependent uptake of [^{3}H]DNP-SG (panel A) and [^{3}H]$E_2$17βG (panel B) by CMVs prepared from SD rats. CMVs prepared from SD rats were incubated at 37°C for 2 min, with or without ATP and ATP-regenerating system in the medium containing several concentrations of verapamil or PSC-833. The ATP-dependent uptake was obtained by subtracting the value in the absence of ATP from that in its presence. The concentration of [^{3}H]DNP-SG and [^{3}H] $E_2$17βG was 1 μM and 0.1 μM, respectively. Each bar represents the mean ± S.E. of three different experiments. Statistical comparisons made by ANOVA followed by Dunnett's test indicated that the effect of verapamil and PSC-833 on the uptake of DNP-SG and E_2 17βG was not significant.

DISCUSSION

$E_2$17βG causes cholestasis in pregnancy (1). In the present study, we investigated the mechanism for the transport of $E_2$17βG across the bile canalicular membrane by comparing the transport properties in SD rats and EHBR. The time-profile for the uptake of [^{3}H]$E_2$17βG by CMVs prepared from SD rats and EHBR was examined (Fig. 1). In CMVs from SD rats, but not from EHBR, marked ATP-dependence and overshoot phenomena were observed, suggesting that $E_2$17βG is predominantly transported via cMOAT/MRP2 (Fig. 1). Previously, we reported that DNP-SG is transported almost entirely by cMOAT/MRP2, since the ATP-dependent uptake of DNP-SG was almost completely impaired in CMVs from EHBR (20). In the present study, we examined the concentration-dependent inhibitory effect of $E_2$17βG on the uptake of DNP-SG into CMVs from SD rats (Fig. 3). The ATP-dependent uptake of [^{3}H]DNP-SG was inhibited by $E_2$17βG in a concentration-dependent manner; in the presence of 60 μM $E_2$17βG, the uptake of [^{3}H]DNP-SG was almost completely abolished (Fig. 3). Moreover, the K_i value of $E_2$17βG for the uptake of [^{3}H]DNP-SG was calculated as 5.78 ± 1.20 μM (Fig. 4), which was in the same range with the K_m value of [^{3}H]$E_2$17βG (6.32 ± 2.39 μM; Fig. 2), indicating that $E_2$17βG competitively inhibits the uptake of [^{3}H]DNP-SG. The K_m value of [^{3}H]DNP-SG determined in the

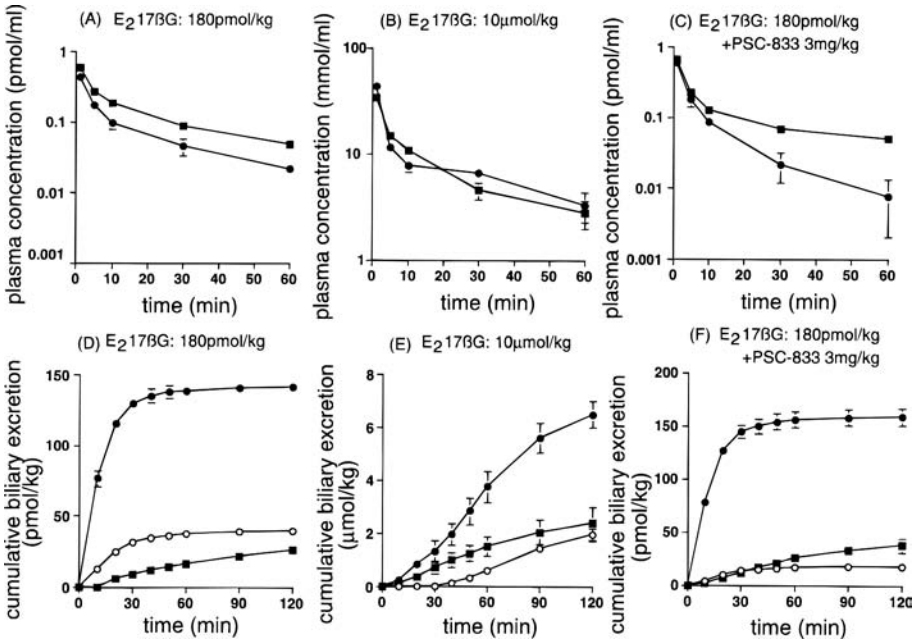

Fig. 6. Plasma concentrations and cumulative biliary excretion of [^{3}H]$E_2$17βG and [^{3}H]$E_2$17βG3S in SD rats and EHBR. The time-profiles of the plasma concentrations (upper panel) and cumulative biliary excretion (lower panel) of [^{3}H]$E_2$17βG were determined after i.v. administration of a tracer dose (180 pmol/kg) (panels A and D) and a cholestatic dose (10 μmol/kg) (panels B and E). The time-profiles were also determined in rats which received an i.v. injection of PSC-833 (3 mg/kg) prior to administration of a tracer dose of [^{3}H] $E_2$17βG (180 pmol/kg) (panels C and F). Closed circles and squares represent the data in SD rats and EHBR, respectively. Open circles represent the biliary excretion of [^{3}H] $E_2$17βG3S in SD rats and EHBR. $E_2$17βG3S, 17β-estradiol-3-sulfate-17β-D-glucuronide is a sulfate conjugate of $E_2$17βG. Each point and vertical bar represent the mean ± S.E. of three different experiments.

present study (20.9 ± 4.3 μM) was similar to the value previously determined in our laboratory (17.6 ± 4.9 μM) (20). These results suggest $E_2$17βG and DNP-SG share a common transporter, cMOAT/MRP2.

In addition, in order to examine the contribution of P-gp to $E_2$17βG uptake, we examined the inhibitory effect of P-gp inhibitors (verapamil and PSC-833). Since verapamil and PSC-833 are also known to inhibit cMOAT/MRP2 at higher concentrations (21), we compared the inhibitory effect of these P-gp inhibitors on the uptake of $E_2$17βG and DNP-SG, the latter being transported predominantly by cMOAT/MRP2, but not by P-gp (20). The uptake of both $E_2$17βG and DNP-SG was not inhibited by either verapamil or PSC-833 up to concentrations of 100 μM and 5 μM, respectively (Fig. 5). It has been reported that 100 μM verapamil inhibits the uptake of daunomycin, a typical substrate of P-gp into CMVs to 26% of the control (22). In addition, we had previously found that the ATP-dependent uptake of octreotide, a P-gp substrate, into CMVs was reduced to 25% of the control by 100 μM verapamil, and to approximately 20% of the control by 1 μM PSC-833 (23). Also, P-gp-mediated transport was completely inhibited by 20 μM PSC-833 (23). These results were consistent with the previous observation that the K_i value of PSC-833 for P-gp is 0.3 μM (21). Thus, the concentration of verapamil and PSC-833 used in the present study was high enough to inhibit P-gp activity. Moreover, Böhme *et al.* reported that the K_i value of PSC-833 for cMOAT/MRP2 was 29 μM (21), which is consistent with our data showing that 5 μM PSC-833 did not significantly inhibit the uptake of $E_2$17βG (Fig. 5). Collectively, these *in vitro* data suggest that $E_2$17βG is predominantly transported across the bile canalicular membrane via cMOAT/MRP2.

To support the results of the CMV study, we also performed investigations *in vivo*. The main elimination route of $E_2$17βG was the biliary excretion (Fig. 7). The cumulative biliary excretion of [^{3}H]$E_2$17βG after a tracer dose was markedly reduced in EHBR (Fig. 6) and the CL_{bile} was also reduced in EHBR compared with SD rats (Fig. 7), suggesting that cMOAT/MRP2 predominantly mediates the biliary excretion of this cholestatic steroid. The finding that the biliary excretion of $E_2$17βG-3-sulfate was detectable in SD rats, but not in EHBR (Fig. 6), is consistent with the previous findings (12). The CL_{bile} in SD rats after a cholestatic dose was lower than that after a tracer dose (Fig. 7), resulting from saturation of the uptake pathway from blood to hepatocytes (K_m = 12.9 ± 1.3 μM) (5,6) or the excretion pathway from hepatocytes to bile (K_m = 6.32 ± 2.39 μM) (Fig. 2). Since the CL_{bile} in EHBR was not reduced in a dose-dependent manner (Fig. 7), the reduced CL_{bile} in SD rats might be due to saturation of the biliary excretion. The delayed biliary excretion of $E_2$17βG and its metabolite (Fig. 6) may result from the reduction in bile flow rate following cholestasis (see Results).

The cumulative biliary excretion of $E_2$17βG and CL_{bile} in SD rats and EHBR were not altered by administration of PSC-833 (3 mg/kg). Since we previously reported that the CL_{bile} of P-gp substrates (such as vincristine and digoxin) in SD rats was significantly reduced by 3 mg/kg PSC-833 (24), the dose

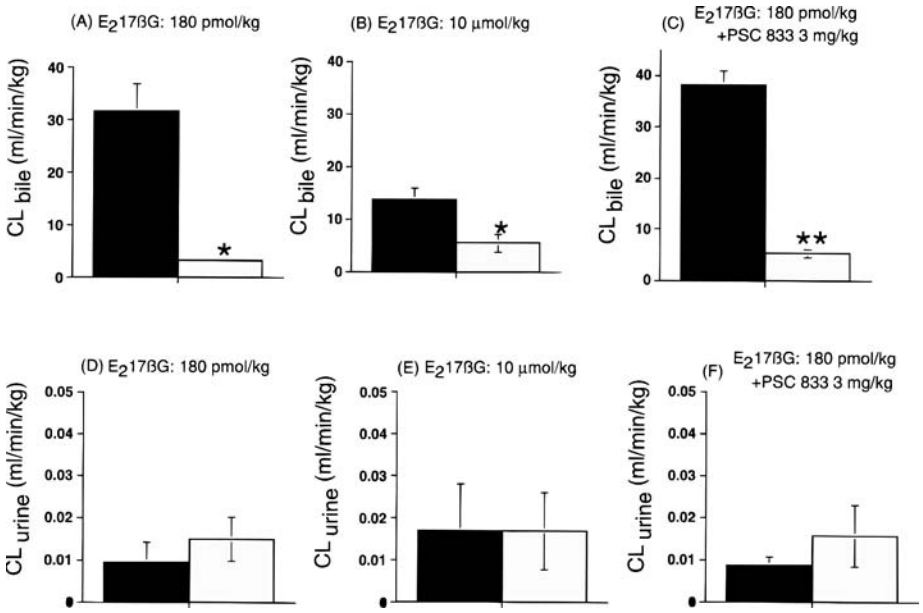

Fig. 7. Biliary and urinary excretion clearance of [^{3}H]$E_2$17βG. CL_{bile} (panels A and B) and CL_{urine} (panels D and E) were determined after i.v. administration of a tracer dose (180 pmol/kg) and a cholestatic dose (10 μmol/kg) to SD rats (closed bars) and EHBR (open bars). The effect of PSC-833 (3 mg/kg) on the disposition of [^{3}H] $E_2$17βG was also determined (panels C and F) in SD rats (closed bars) and EHBR (open bars). Each bar represents the mean ± S.E. of three different experiments. Statistical comparisons were made using student's t tests. * $P < 0.05$, ** $P < 0.01$, respectively.

of PSC-833 used in the present study should be high enough to inhibit P-gp activity. These *in vivo* results also suggest that P-gp makes only a minor contribution to the biliary excretion of $E_2$17βG. The analysis of CL_{urine} indicated that the contribution of cMOAT/MRP2 and P-gp to the renal clearance of $E_2$17βG is minor (Fig. 7).

Previously, we proposed a hypothesis that transporter(s) other than cMOAT/MRP2 is (are) also involved in the biliary excretion of certain kinds of organic anions, since the ATP-dependent uptake of E3040 (6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole)-glucuronide was detectable in CMVs from EHBR, although the extent was approximately one-third that in CMVs from SD rats (20). In the present study, since the ATP-dependent uptake of $E_2$17βG was almost completely abolished in CMVs from EHBR, such transporter(s) may not be involved in the excretion of $E_2$17βG. In addition, although we have indicated that the hepatic expression of MRP3 is observed in EHBR, but not in SD rats (25), and that MRP3 accepts $E_2$17βG as a good substrate (26), MRP3 may contribute not to the biliary excretion of substrates but rather to their export in the blood stream, since it was recently demostrated that this transporter is located on the basolateral membrane (27,28).

The mechanism for $E_2$17βG-induced cholestasis is still controversial. Vore *et al.* (1) and Stieger *et al.* (29) hypothesized that the highly accumulated $E_2$17βG in the bile inhibit the function of P-gp and bile salt export pump, respectively, to induce the cholestasis. The present finding that $E_2$17βG is excreted into the bile predominantly via cMOAT/MRP2 may provide the important information in understanding the mechanism of cholestasis induced by $E_2$17βG.

In conclusion, our *in vivo* and *in vitro* studies with CMVs show that $E_2$17βG is predominantly transported by cMOAT/MRP2. Although it has been established that the transport of $E_2$17βG is partly mediated by P-gp, the contribution of P-gp to the hepatobiliary excretion of $E_2$17βG seems minor.

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