MUTLIPLE ALTERATIONS OF CANALICULAR MEMBRANE TRANSPORT ACTIVITIES IN RATS WITH CCl\textsubscript{4}-INDUCED HEPATIC INJURY

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ABSTRACT:

The influence of CCl\textsubscript{4}-induced experimental hepatic injury (CCl\textsubscript{4}-EHI) on the expression and transport activities of primary active transporters on the canalicular membrane, including P-glycoprotein (P-gp), a bile salt export pump (Bsep) and a multidrug resistance associated protein2 (Mrp2), was assessed. CCl\textsubscript{4}-EHI was induced by an intraperitoneal injection of CCl\textsubscript{4} to rats at a dose of 1 ml/kg 24 h prior to the preparation of canalicular liver plasma membrane (cLPM) vesicles and pharmacokinetic studies. The expression of each transporter was measured for the vesicles via Western blot analysis at 6, 12, 24, 36, and 48 h after the injection of CCl\textsubscript{4}. The in vivo canalicular excretion clearance (CL\textsubscript{exc}) of [\textsuperscript{3}H]daunomycin, [\textsuperscript{3}H]taurocholate and [\textsuperscript{3}H]17\textbeta-estradiol-17\textbeta-0-glucuronide (E\textsubscript{2}17\textbetaG), representative substrates of P-gp, Bsep, and Mrp2, respectively, was determined following an i.v. infusion to rats. The uptake of each substrate into cLPM vesicles in the presence of ATP was also measured by a rapid filtration technique. As the result of the CCl\textsubscript{4}-EHI, the protein level of transporters was altered as a function of time in multiple manners; it was increased by 3.6-fold for P-gp, unchanged for Bsep, and decreased by 73% for Mrp2 at 24 h. The in vivo CL\textsubscript{exc} and the intrinsic uptake clearance into cLPM vesicles (CL\textsubscript{int}) at 24 h after the CCl\textsubscript{4} injection (CCl\textsubscript{4}-EHI\textsubscript{24 h}) were also influenced by the EHI in a similar manner; they were increased by 1.8- and 1.9-fold for daunomycin, unchanged for taurocholate, and decreased by 41 and 39% for E\textsubscript{2}17\textbetaG, respectively, consistent with multiple alterations in the expression of the relevant transporters.

Primary active transporters in the liver canalicular membrane, which contain the ATP binding cassette (ABC\textsuperscript{1}), play an important role as efflux pumps in the excretion of endogenous bile constituents or xenobiotics into the bile canaliculi (Hooiveld et al., 2001). As might be expected, certain types of liver diseases have an influence on this hepatobiliary excretion. Experimental hepatic injury (EHI) induced by a single administration of carbon tetrachloride (CCl\textsubscript{4}) has been widely used as a pathological model for liver diseases, since it is known that CCl\textsubscript{4} produces acute hepatocellular injury with centrilobular necrosis and steatosis (Recknagel, 1967). The effects of CCl\textsubscript{4}-EHI on biochemical characteristics such as increased lipid peroxidation (Recknagel, 1967) and the activities (Mourelle et al., 1987; Romero et al., 1994) of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) have been widely studied. Our earlier study revealed that transport systems for some organic cations on the sinusoidal membrane are prone to damage as the result of CCl\textsubscript{4}-EHI, as demonstrated by a decrease in the in vitro maximal rates of hepatic uptake and efflux without any influence on in vivo canalicular excretion clearance (Hong et al., 2000). In addition, a dose-dependent inhibition of Na\textsuperscript{+}/taurocholate cotransport (Ntcp) into sinusoidal membrane vesicles by the presence of trichloroethene and 1,1,2-trichloro-1,2,2-trifluoroethane (all CCl\textsubscript{4} analogs) has been reported (Nehgab et al., 1996). Recently, Geier et al. (2002) reported that hepatobiliary organic anion transporters on the sinusoidal membrane were regulated differently in acute toxic liver injury induced by CCl\textsubscript{4}; mRNA levels were significantly decreased for Ntcp, Oatp1 (organic anion transporting polypeptide1), and Oatp2, whereas the level remained unchanged for Oatp4.

Contrary to the cases of transport systems on the sinusoidal membranes, considerably less information is available on the effects of CCl\textsubscript{4}-EHI on the canalicular transport system, which is believed to be a key step in the vectorial transport of various xenobiotics from the portal blood to the bile (Bosser et al., 1993; Han et al., 1999). Thus, the objective of this study was to examine the effect of CCl\textsubscript{4}-EHI on the expression and functional activity of representative ABC transporters on the canalicular membrane, including P-glycoprotein (P-gp), a bile salt export pump (Bsep), and a multidrug resistance associated protein2 (Mrp2). Western blot analysis was performed to evaluate the expression of the transporters, and in vivo canalicular excretion and in

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vitro uptake into canalicular liver plasma membrane (cLPM) vesicles for daunomycin, taurocholate, and 17β-estradiol-17β-α-glucuronide (E$_2$17βG) were measured to estimate the functional activity of the relevant transporters. These compounds were selected because they represent substrates for P-gp (Kamimoto et al., 1989; Hooiveld et al., 2001), Bsep (Gerloff et al., 1998; Hooiveld et al., 2001), and Mrp2 (Morikawa et al., 2000; Hooiveld et al., 2001), respectively, and are extensively excreted into bile via relevant transporters in rats [i.e., approximately 36% of an i.v. dose (10 μmol/kg) for daunomycin (Catapano et al., 1988), 87% of an i.v. dose (54 μmol/kg) for taurocholate (Ring et al., 1994), and 77% of an i.v. dose (180 pmol/kg) for E$_2$17βG (Morikawa et al., 2000)].

Materials and Methods

Materials. [3H]Taurocholate (2 Ci/mmol), [3H]17β-estradiol-17β-α-glucuronide (E$_2$17βG, 44 Ci/mmol) and [3H]daunomycin (4.4 Ci/mmol) were purchased from PerkinElmer Life Science Inc. (Boston, MA), C219 and M$_2$ III-6, monoclonal antibodies to P-gp and Mrp2, respectively, were purchased from Alexix Biochemical Co. (San Diego, CA), and Antisigg, a polyclonal antibody to Bsep, was purchased from Kamiya Biomedical Co. (Seattle, WA). All other chemicals including reagents for Western blot analysis and the vesicle uptake study were purchased from Sigma-Aldrich (St. Louis, MO).

Induction of Experimental Hepatic Injury by CCl$_4$. Male Sprague Dawley rats (250–300 g; Dae-Han Biolink, Taejon, Korea) were injected intra-peritoneally with a single dose of CCl$_4$ (1 ml/kg) as a 50% (v/v) solution in olive oil and then fasted for 6, 12, 24, 36, or 48 h. Water was fed ad libitum. Control animals received a corresponding dose of olive oil using the same experimental protocol. The activities of sGPT and sGOT were measured (Reitman and Frankel, 1957) using a commercial colorimetric determination kit (Yeong Dong Pharm Co., Seoul, Korea). Experimental protocols involving animal studies were reviewed by the Animal Care and Use Committee in College of Pharmacy, Seoul National University according to the National Institutes of Health guidelines (National Institutes of Health publication number 86–23, revised 1985) “Guide for the Care and Use of Laboratory Animals”.

Preparation of cLPM Vesicles. cLPM vesicles were prepared from four normal and CCl$_4$-EHT rats according to the method of Inoue et al. (1983) as described previously (Song et al., 1999). The purity of the vesicle preparations was routinely assessed by measuring the relative enrichment of the activity of sGPT and sGOT, expressed using the liver substrate (Lowry et al., 1951) using bovine serum albumin as a standard. To estimate the proportion of the inside-out vesicles, the concentrations of the exposed sialic acid of the vesicles were determined (Warren, 1959). Immediately after their preparation, the cLPM vesicles were suspended in a membrane suspension buffer (MSB), which contained 250 mM sucrose, 10 mM Hepes, 10 mM Tris, 10 mM MgCl$_2$, and 0.2 mM CaCl$_2$ (pH 7.4), to yield a protein concentration of 8 to 10 mg/ml. The suspension was stored at −70 °C, for up to 2 weeks, until the uptake studies and Western blot analysis were carried out.

Western Blot Analysis. The vesicle preparations were diluted in a buffer consisting of 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 50 mM Tris-HCl (pH 6.8) to yield a total protein concentration of 1.25 μg/μl. A 16-μl aliquot of the suspension (equivalent to 20 μg of total protein) and 10 μl of a solution of molecular weight markers (High Mw-SDS calibration kit, Amersham Biosciences Inc.) were subjected to electrophoresis on a 6% polyacrylamide gel with 0.1% SDS, and electrotransferred to a nitrocellulose membrane (0.45 μm pore size; Amersham Biosciences Inc.). The membrane was blocked with a phosphate-buffered saline solution (pH 7.4) containing 0.1 (w/v) % Tween 20 (PBST) and 5% (v/v) nonfat dry milk (Seattle Milk Co., Seoul, Korea) for 1 h at 37°C, and then probed for 18 h at 4°C with the primary antibodies (dilution 1:1000 each) C219 (Lee et al., 1996), M$_2$ III-6 (Kool et al., 1997), and Antisigg (Lecureur et al., 2000) that recognize P-gp, Mrp2, and Bsep, respectively. After washing three times with 60 μl of PBST, the membrane was incubated with secondary antibodies [i.e., horseradish peroxidase-conjugated goat anti-mouse IgG for C219 and M$_2$ III-6 (dilution 1:1000; Zymed Laboratories, South San Francisco, CA), and horseradish peroxidase-conjugated goat anti-rabbit IgG for Antisigg (dilution 1:1000; Amersham)] for 1 h at room temperature. After washing three times with 60 ml of PBST, it was possible to visualize all proteins recognized by the antibodies using an enhanced chemiluminescence detection system. Band intensities were analyzed by densitometry using a software program, Quantity One (version 4.1; Bio-Rad, Hercules, CA).

In Vivo Biliary Excretion across Canalicular Membranes. After light anesthesia with ketamine (50 mg/kg as i.p. dose; Yuhan Pharm. Co., Kyonggido, Korea), the femoral arteries and veins of rats were cannulated with PE-50 polyethylene tube and bile ducts with PE-10 polyethylene tube. Normal rats and CCl$_4$-pretreated (for 24 h rats [i.e., CCl$_4$-EHT, EHT rats] were used throughout the in vivo experiments. For the estimation of canaliculal excretion clearance (CL$_{ex}$) of daunomycin, [3H]taurocholate and [3H]E$_2$17βG, rats received i.v. injection at bolus dose of 10 μmol/kg, 400 pmol/kg, and 160 pmol/kg, followed by i.v. infusion at rates of 10 μmol/min/kg, 100 pmol/kg/min, and 160 pmol/kg/min (48 μCi), for respective compounds. Blood samples (0.3 ml) were taken from the femoral artery at 30-min intervals over a 3-h period, and bile was collected for 30-min period up to 3 h. Plasma samples were separated from the blood samples by centrifuging at 3,000 rpm for 10 min. At 3 h (i.e., at the steady state) after each infusion start, rats were sacrificed and the liver was isolated immediately. A 20% liver homogenate was then prepared using normal saline, centrifuged at 3,000 rpm for 10 min, and aliquots (100 μl) of the supernatant were collected for the determination of the respective compounds. The in vivo CL$_{ex}$ of a compound was calculated by dividing the biliary excretion rate by the liver substrate concentration at the steady state.

Assay of Taurocholate, Daunomycin, and E$_2$17βG in In Vivo Study. The concentrations of taurocholate in plasma, liver (homogenates), and bile samples were quantified by liquid scintillation counting (LSC, Wallac 1409; PerkinElmer Life Science Inc.). The concentration of daunomycin, which is known to be metabolized in the body to daunomycinol (Pea et al., 2000), was determined by high performance liquid chromatography (HPLC). A 100-μl aliquot of the biological samples (i.e., plasma, bile, and supernatant of the liver homogenate) was deproteinized by the addition of MeOH (250 μl) containing Adriamycin (internal standard, 1 μM). Ethyl acetate (1 ml) was then added, the suspension was vigorously mixed for 5 min and then centrifuged at 10,000 rpm for 5 min. An aliquot (1.2 ml) of the supernatant was transferred and evaporated using a Spinvac (Hanil Science, Seoul, Korea), and the residue was reconstituted with the mobile phase (150 μl) used for HPLC (acetonitrile: 0.01 M phosphoric acid = 7:3 v/v, pH 3.0). A 50-μl aliquot of the reconstituted solution was injected into the HPLC system, which consisted of a Hitachi L-6200 pump (Hitachi, Japan) and Hitachi C-R6A integrator. The mobile phase consisted of 2-propanol, acetonitrile, and water (53:47:1, v/v). The eluent resulted in sharp and well-resolved peaks corresponding to daunomycin, Adriamycin (internal standard), and possible metabolites. The retention times of Adriamycin and daunomycin were 4.9 and 8.3 min, respectively, under the conditions used. Calibration curves of daunomycin were linear over the concentration range of 0.1–4 μM for plasma, bile, and liver samples, with respective correlation coefficients of over 0.999.

The concentration of E$_2$17βG, which is known to be metabolized mainly to 17β-estradiol-3-sulfate-17-glucuronide (Meyers et al., 1980; Morikawa et al., 1989) is much lower in the body, was determined by thin-layer chromatography (TLC) followed by LSC. Plasma (100 μl) and supernatants of the liver homogenates (100 μl) were deproteinized with methanol (250 μl), centrifuged at 10,000 rpm for 5 min, and 300-μl aliquots were evaporated using a Spinvac. The residues were dissolved in methanol (20 μl), and 5-μl aliquots were spotted onto silica gel coated TLC plates (10 × 20 cm) and developed using chloroform/methanol/acetic acid (7:2:1 v/v) as the irrigant. RF values of E$_2$17βG and its major metabolites, 17β-estradiol-3-sulfate-17-glucuronide, were 0.45 and 0.1, respectively. The recovery of E$_2$17βG from plasma, liver, and bile samples in the TLC was over 95% for the concentration.
range examined (0.1–40 nM) regardless of the nature of the biological samples (i.e., plasma, bile, and the supernatant of liver homogenate). Zonae on the TLC plates that correspond to an Rf of E₁₇βG were collected (by scraping) and the radioactivity determined by LSC. The radioactivity associated with E₁₇βG was 85 to 90, 45 to 50, and 65 to 70% of total radioactivity loaded on the TLC plates for plasma, liver and bile samples, respectively, and the remaining radioactivity could be attributed to a major metabolite of E₁₇βG, probably 17β-estradiol-3-sulfate-17-glucuronide. No other substances exhibiting significant radioactivity were detected on the TLC plates.

In Vitro Vesicle Uptake Studies. The uptake of [³H]daunomycin, [³H]taurocholate, and [³H]E₁₇βG into cLPM vesicles was measured by a rapid filtration technique (Song et al., 1999) using vesicles prepared from normal and CCl₄-EHI₂₄ h rats. The uptake of [³H]daunomycin was measured as follows. A frozen vesicle suspension was quickly thawed by immersion in a 37 °C water bath, re-vascularized by passing it through a 25 gauge needle 20 times, and appropriately diluted with MSB to give 3 to 4 mg/ml of protein. Ten microliters of the diluted suspension was preincubated in a test tube at 37 °C for 4 min, and 40 μl of MSB, which contained 0.2 μM daunomycin (0.035 μCi) with or without the ATP-regenerating system (1.2 mM ATP, 3 mM phosphocreatine, and 3.6 μg/100 μl creatine phosphokinase), was then added to the diluted vesicle suspension. At predetermined times, the uptake was quenched by the addition of 1 ml of an ice-cold solution of MSB containing 20 μM daunomycin. The entire sample was then rapidly filtered through a MF-MEMB filter (0.45-μm pore size, 25-mm diameter; Seoul Science, Seoul, Korea), which had been presoaked for 2 h in ice-cold MSB. The tube was rinsed again with 1 ml of ice-cold MSB and then filtered. After washing with 10 ml of ice-cold MSB, the filter was dissolved in 4 ml of scintillation cocktail (UltimaGold; PerkinElmer Life Science Inc.), and the radioactivity of the mixture was determined by LSC. Presoaking and rinsing the filter with the ice-cold MSB, which contains 20 μM daunomycin, resulted in a very small level of nonspecific binding of daunomycin to the filter (i.e., negligible radioactivity in the filter, data not shown). The amount of daunomycin in the vesicles (expressed as picomoles per milligrams of protein) was plotted against time. The ATP-dependent fraction that was taken up was estimated by the level of nonspecific binding of daunomycin to the filter (i.e., negligible radioactivity in the filter, data not shown). The amount of daunomycin in the vesicles (expressed as picomoles per milligrams of protein) was plotted against time. The ATP-dependent fraction that was taken up was estimated by the level of nonspecific binding of daunomycin to the filter (i.e., negligible radioactivity in the filter, data not shown). The amount of daunomycin in the vesicles (expressed as picomoles per milligrams of protein) was plotted against time. The ATP-dependent fraction that was taken up was estimated by the level of nonspecific binding of daunomycin to the filter (i.e., negligible radioactivity in the filter, data not shown). The amount of daunomycin in the vesicles (expressed as picomoles per milligrams of protein) was plotted against time. The ATP-dependent fraction that was taken up was estimated by the level of nonspecific binding of daunomycin to the filter (i.e., negligible radioactivity in the filter, data not shown).

The initial uptake rate of the substrates (expressed as picomoles per milligrams of protein per 30 seconds), S, is the initial concentration of substrates in the medium (μM). V₀ is the initial uptake rate of the substrates (expressed as picomoles per milligrams of protein per 30 seconds), S is the initial concentration of substrates in the medium (μM). V_max and K_m represent the maximal uptake rate and the medium concentration at half of maximal uptake rate, respectively. The intrinsic clearance for the uptake (CL_{int}) was obtained from V_{max}/K_{m}.

### Data Analysis

All data are expressed in the form of the mean ± S.D. A two-way analysis of variance was performed to test differences in the temporal uptake of each substrate between the transport conditions and treatments (i.e., normal and CCl₄-EHI). The student’s t test was used to test differences in the mean kinetic parameters (i.e., for in vitro and in vivo experiments) between the treatments. In all cases, P < 0.01 was accepted as denoting a statistical difference.

### Results

**Pathophysiological and Biochemical Changes by CCl₄-EHI.** The effects of CCl₄-EHI₂₄ h (i.e., EHI at 24 h after the CCl₄ administration) on various pathophysiological parameters are summarized in Table 1. The body weights were similar in both rats, the liver weight was increased in CCl₄-EHI₂₄ h rats by 15%. EHI was confirmed by 11 and 14-fold increases in the activities of sGOT and sGPT, respectively. The yield of protein from liver homogenates was decreased by 19% as a result of the CCl₄-EHI₂₄ h, which is consistent with the previously reported decrease in protein synthesis (Romero et al., 1994). The ALP activity of the homogenate was unaffected by the CCl₄-EHI₂₄ h.

**Basic parameters for cLPM vesicles are summarized in Table 2.** The protein yield and ALP activity of cLPM vesicles were decreased by a factor of 43 and 54%, respectively, by the CCl₄-EHI₂₄ h, consistent with previously reported data (Mourre et al., 1987). The ALP activities in the vesicles from normal and CCl₄-EHI₂₄ h rats were enriched by 52- and 63-fold, respectively, compared with the corresponding liver homogenates (compare Tables 1 and 2). In spite of the differences in protein levels and ALP activity, the uptake of mannitol by cLPM vesicles was about the same for both the normal and CCl₄-EHI₂₄ h rats (Table 2), indicating a similar and negligible leakage of vesicle membranes. The proportion of inside-out configuration of the vesicles was not affected by the CCl₄-EHI₂₄ h, consistent with our previous reports (Song et al., 1999).

**Western Blot Analysis.** The expression of ABC transporters that mediate ATP binding cassette (ABC) on canalicular membranes was measured for cLPM vesicles by a Western blot analysis (Fig. 1). Representative immunoblots for P-gp in cLPM vesicles, prepared from livers of normal and CCl₄-EHI rats, are shown in Fig. 1A. Bands of approximately 170 kDa, the molecular size of P-gp (Kamimoto et al., 1989), was observed for all the vesicle samples. The intensity of the band varied with time (6, 12, 24, 36, and 48 h) after the CCl₄ administration, compared with cLPM vesicles from the normal liver (i.e., control). The expression levels at 12 h, however, exhibited a marked decrease (i.e., 2.1-fold of the control value). A maximal expression (i.e., 3.6-fold increase) was observed at 24 h after the CCl₄ administr-
of the transporters was examined. Since the rats exhibited maximal change in the expression of P-gp and Mrp2 at 24 h after the CCl4 pretreatment (Fig. 1), subsequent in vivo experiments were performed using rats at 24 h after the pretreatment. An i.v. infusion of taurocholate and E2 17G was performed at tracer doses to avoid their possible interactions with endogenous taurocholate and E2 17G (Morikawa et al., 2000) in canalicular excretion. As the result, the plasma concentration of these compounds remained in the nanomolar range (Table 3). The well known cholestatic effect of E2 17G (Meyers et al., 1980; Morikawa et al., 2000) was not apparent at this plasma level, demonstrated by comparable bile flow rates among normal rats [i.e., 65.5 ± 11 μl/min/kg (mean ± S.D., n = 6)] for daunomycin, 55.5 ± 9.5 μl/min/kg (mean ± S.D., n = 6) for taurocholate, 52.2 ± 14 μl/min/kg (mean ± S.D., n = 6) for E2 17G, and 58.4 ± 6.7 μl/min/kg (mean ± S.D., n = 3) for control]. Moreover, bile flow was not influenced by the CCl4-EH124 h, regardless of the compounds infused (data not shown).

A steady-state plasma concentration and biliary excretion of daunomycin, taurocholate and E2 17G were attained within 60 min after the initiation of constant rate i.v. infusion and a simultaneous i.v. bolus injection of the compounds in both normal and CCl4-EH124 h rats (Fig. 2). Thus, the steady-state value for each rat was read from the average of the last four data points and is expressed in Table 3 as the mean ± S.D. of six rats. No significant differences in the steady state plasma concentration and biliary excretion rate of daunomycin, a representative substrate for P-gp (Kamimoto et al., 1989; Hooiveld et al., 2001), were found between the normal and CCl4-EH124 h rats. However, the CLexc (i.e., in vivo canalicular excretion clearance calculated from dividing biliary excretion rate by liver concentration at the steady state) of daunomycin was increased by 1.6-fold, whereas the steady-state liver concentration of daunomycin was decreased by 42% by the EHI (Table 3).

The steady-state plasma concentration of taurocholate, a representative substrate for Bsep (Gerloff et al., 1998; Hooiveld et al., 2001), was increased by 1.3-fold, but no changes in liver concentration or the biliary excretion rate were detected by the CCl4-EH124 h, resulting in a constant value for CLexc. On the other hand, the CCl4-EH124 h increased the plasma (1.7-fold) and liver concentrations (1.9-fold) of E2 17G, a representative substrate for Mrp2 (Morikawa et al., 2000b; Hooiveld et al., 2001), without influencing biliary excretion rate of the compound, thereby resulting in 41% decreases in the CLexc of the compound (Table 3). In summary, changes in the in vivo CLexc of these substrates were generally consistent with changes in the expression of relevant transporters CCl4-EH124 h (Fig. 1), except for the CLexc of daunomycin, which exhibited a less significant increase (i.e., 1.8-fold) than expected from the 3.6-fold increase in the expression of P-gp (Fig. 1).

**Alterations in the ATP-dependent Uptake of Daunomycin, Taurocholate and E2 17G into cLPM Vesicles.** Certain pathophysiological changes by EHI, such as decreased metabolic activity (Olatunde Farombi, 2000) and an increased plasma level of endogenous compounds (e.g., corticosterone; Huang et al., 2001), might interfere with the in vivo transport of relevant substrates. To exclude this possibility, the uptake of these substrates into cLPM vesicles from normal and CCl4-EH124 h rats was examined. Figure 3A shows the result for daunomycin. The uptake of daunomycin was significantly increased by the presence of ATP. The uptake in the absence of ATP was not influenced by the CCl4-EH124 h, whereas the uptake in the presence of ATP was increased significantly for cLPM vesicles from CCl4-EH124 h rats. Figure 3A shows the concentration (5–500 μM) dependence of the ATP-dependent uptake rate of daunomycin. Consistent with Fig. 3A, a much higher rate of uptake was observed by the

<table>
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<tr>
<th>TABLE 2</th>
<th>Changes in the basic parameters of cLPM vesicles at 24 h after CCl4 administration (1 ml/kg) to normal rats a b</th>
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<tr>
<td>Normal</td>
<td>CCl4-EH124h</td>
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<td>-------------------------------------------------</td>
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<tr>
<td>Protein (mg/g liver)</td>
<td>0.140 ± 0.05</td>
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<tr>
<td>ALP (mmol/mg prot./h)</td>
<td>2.28 ± 0.94</td>
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<tr>
<td>Mannitol uptake (% of medium)</td>
<td>3.85 ± 0.98</td>
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<tr>
<td>Inside-out configuration (%)</td>
<td>30.2 ± 8.6</td>
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a All data represent the means ± S.D. (n = 6).

b Statistically different from normal cLPM vesicles (P < 0.01).

**Fig. 1. Representative immunoblots of P-gp (A), Bsep (B), and Mrp2 (C) proteins, and the relative densities of immunostained bands of P-gp (A'), Bsep (B'), and Mrp2 (C') in cLPM vesicles from normal and CCl4-EH124 h rats (Fig. 2).**

cLPM vesicles were prepared from pooled liver homogenates from normal (n = 4) and CCl4-EH124 h rats (n = 4) at 6, 12, 24, 36, and 48 h after an intraperitoneal injection of CCl4 (1 ml/kg). Three different batches of vesicles were prepared using different liver homogenate pools (i.e., the number of rats at each time point = 12, the total number of rats used = 72). Lanes were loaded with each membrane vesicle preparation (20 μg of total protein). The right lane was loaded with a high molecular weight size marker. The target protein was detected using antibodies such as C219, M3, 3-6, and Antispgp that recognize P-gp, Mrp2, and Bsep, respectively. Each data point in the right column is expressed as the mean ± S.D. for three different batches of cLPM vesicle preparations.

In 36 h, the expression decreased to levels similar to those of the control. Representative immunoblots for Bsep in cLPM vesicles are shown in Fig. 1B. Bands of approximately 150 kDa, the molecular size of Bsep (Gerloff et al., 1998), were observed for all the vesicle samples. Contrary to the case for P-gp, no change in the expression of Bsep was observed by the CCl4 treatment up to 48 h (Fig. 1, B and B'). The strongest band of approximately 190 kDa (Mottino et al., 2000) was observed for Mrp2 (Fig. 1C). The density of the band decreased as a function of time after CCl4 administration, exhibiting a maximal decrease to 27% of control at 24 h and partial recovery to 60% in 48 h (Fig. 1, C and C').

**In Vivo Canalicular Excretion Clearance.** To address the issue of whether the altered expression of ABC transporters (Fig. 1) is reflected on the in vivo functional activity of the transporters, the effect of CCl4-EH124 on the canalicular excretion of representative substrates...
A kinetic analysis revealed a 1.8-fold increase in the value of $V_{\text{max}}$ for the uptake of daunomycin into cLPM vesicles from the CCl$_4$-EHI 24 h rats, compared with those from normal rats, whereas the values of $K_{m}$ for vesicles from both rats were comparable (Table 4). As a result, a 1.9-fold increase in the value of CL int was observed by the CCl$_4$-EHI 24 h for daunomycin (Table 4), highly consistent with the increase in CL exc in the in vivo study (i.e., a 1.8-fold increase).

The increase in the value of $V_{\text{max}}$ under a constant value of $K_{m}$ by the CCl$_4$-EHI 24 h appears to be consistent with the increase in the expression of P-gp by the CCl$_4$-EHI 24 h (Fig. 1, A and A'), although it is less prominent than expected from the 3.6-fold increase in the expression of P-gp (Fig. 1).

Temporal profiles for the uptake of 1/H$_9$262 M taurocholate into cLPM vesicles are shown in Fig. 3B. The uptake of taurocholate was greatly increased by the presence of ATP. However, no differences in the uptake of taurocholate were observed between normal and CCl$_4$-EHI 24 h rats, regardless of the presence of ATP. The uptake of taurocholate in the presence of ATP was determined for a taurocholate concentration range of 5 to 500 M. Consistent with Fig. 3B, no apparent effect of CCl$_4$-EHI 24 h was observed in the concentration-uptake rate profiles (Fig. 3B'). As a result, similar kinetic parameters (i.e., $K_{m}$, $V_{\text{max}}$, and CL int) were obtained for the vesicular uptake of taurocholate for both normal and CCl$_4$-EHI 24 h rats (Table 4). The similarity in $V_{\text{max}}$ values between normal and CCl$_4$-EHI 24 h rats is consistent with the results of the Western blot analysis for Bsep (Fig. 1, B and B').

### Table 3

Summary of the Effects of CCl$_4$ on the Pharmacokinetic Parameters of daunomycin, taurocholate, and E$_2$ 17$eta$

<table>
<thead>
<tr>
<th></th>
<th>Daunomycin (10 pmol/h/kg)</th>
<th>Taurocholate (400 pmol/h/kg)</th>
<th>E$_2$ 17$eta$ (160 pmol/h/kg)</th>
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<tr>
<td></td>
<td>Normal</td>
<td>CCl$<em>4$-EHI$</em>{36}$h</td>
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<tr>
<td>Plasma concentration (P)</td>
<td>2.30 ± 0.24 μM</td>
<td>2.00 ± 0.13 μM</td>
<td>0.288 ± 0.027 nM</td>
</tr>
<tr>
<td>Liver concentration (L)</td>
<td>36.1 ± 3.3 μM</td>
<td>21.1 ± 4.0 μM*</td>
<td>1.39 ± 0.30 nM</td>
</tr>
<tr>
<td>Biliary excretion rate</td>
<td>7.14 ± 0.79 pmol/min/kg</td>
<td>7.99 ± 1.5 pmol/min/kg</td>
<td>5.20 ± 0.62 pmol/min/kg</td>
</tr>
<tr>
<td>CL$_{\text{int}}$ (ml/min/kg)$^b$</td>
<td>0.198 ± 0.015</td>
<td>0.353 ± 0.061*</td>
<td>3.74 ± 0.91</td>
</tr>
</tbody>
</table>

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$^a$ All data represent the means ± S.D. (n = 6).

$^b$ CL$_{\text{int}}$ was calculated from dividing the excretion rate by the liver substrate concentration at the steady state.

$^*$ Significantly different compared to normal rats (P < 0.01).
Temporal profiles of the uptake of $[^3H]E_217\beta G$ into cLPM vesicles from normal and CCl$_4$-EHI 24 h rats are shown in Fig. 3C. The uptake was greatly increased by the presence of ATP. The uptake in the absence of ATP was not influenced by the CCl$_4$-EHI 24 h, whereas the ATP-dependent uptake was much greater than the ATP-independent uptake and was decreased significantly by the CCl$_4$-EHI 24 h. A concentration dependence in the ATP-dependent uptake of $[^3H]E_217\beta G$ was found for the concentration range of 0.5 to 200 $\mu$M (Fig. 3C). A kinetic analysis of the ATP-dependent uptake revealed a 39% decrease in the value of $V_{\text{max}}$ without affecting the value of $K_m$, leading to a 39% decrease in the value of $CL_{\text{int}}$ as a result of the CCl$_4$-EHI 24 h (Table 4). This result appears to be consistent with the results of the Western blot analysis for Mrp2, which exhibited a 73% decrease in the expression (Fig. 1, C and C').

In summary, the CCl$_4$-EHI 24 h increased the vesicular uptake of daunomycin, did not influence the uptake of taurocholate, and decreased the uptake of $E_217\beta G$ in the presence of ATP, all highly consistent with the effect of the CCl$_4$-EHI 24 h on the expression of the responsible transporters (i.e., P-gp, Bsep and Mrp2, respectively, Fig. 1) and the in vivo canalicular excretion (i.e., $CL_{\text{exc}}$, Table 3).

**Effect of Direct Contact of CCl$_4$ on the Uptake of Substrates into cLPM Vesicles.** To examine the issue of whether the above
observed effects of CCl4-EHI 24 h are attributable to direct interactions of the responsible transport systems with CCl4, the uptake of 0.2 µM daunomycin, 1 µM taurocholate, and 1 µM E217βG into cLPM vesicles, following the incubation of normal cLPM vesicles with 1.5 mM CCl4 at 37°C for 15 min, was assessed in the presence and absence of ATP. The uptake of daunomycin into normal vesicles was decreased significantly by incubation in the presence of CCl4 and ATP (Fig. 4A). This is contrary to the results obtained for the uptake of daunomycin into cLPM vesicles that were prepared from CCl4-EHI 24 h rats (Fig. 3A), indicating that CCl4, in the body, affects the ABC transporters on the canalicular membrane in an indirect manner. On the other hand, the effect of direct contact of CCl4 on the uptake of taurocholate and E217βG into cLPM vesicles was similar to that of CCl4-EHI 24 h on the uptake of these substrates, regardless of the presence of ATP (Fig. 3, B and C). Despite this similarity, however, CCl4-EHI seems to influence the ABC transporters on the canalicular membrane, except for Bsep, proteins such as Mdr2 and Bsep (Van Den Elsen et al., 1999). The fact that the level of Mdr2 appeared to be increased in 24 h after the CCl4 pretreatment (from the mRNA level of mdr2; Nakasukasa et al., 1993) suggests that immunostaining using C219 might lead to an overestimation of P-gp levels. In other words, a 3.6-fold increase in the level of P-gp by the CCl4-EHI 24 h (Fig. 1) might be an overestimation, at least in part.

The effect of the CCl4-EHI 24 h, i.e., changes in the expression of the ABC transporters on the canalicular excretion of representative substrates of the transporters was examined in vivo. The CLexc values for the substrates were altered multiply (Table 3), consistent with the changes in the expression level of relevant transporters (Fig. 1). However, the change in the CLexc value for daunomycin (i.e., 1.9-fold increase; Table 3) was not as significant compared with the changes in the expression of P-gp (i.e., 3.6-fold increase; Fig. 1A). This discrepancy could be related to the cross-reactivity of C219 (Van Den Elsen et al., 1999) or to interference by certain endogenous factors in CCl4-EHI 24 h rats on canalicular excretion (Olatunde Farombi, 2000; Huang et al., 2001).

To exclude possible interference by endogenous factors, the in vitro uptake of relevant substrates into cLPM vesicles was examined. Results from the in vitro uptake study were fairly consistent with the expression of relevant transporters (Fig. 1) as demonstrated by Vmax and Kn values in Table 3. The Clint was 1.9-fold increased for daunomycin, remained unchanged for taurocholate, and was 39% decreased for E217βG. An analysis of the uptake studies revealed that the changes in the Clint are solely attributable to changes in the Vmax.

### Table 4

| Kinetic parameters for the ATP-dependent uptake of daunomycin, taurocholate, and E217βG by cLPM vesicles* |
|-----------------|-----------------|-----------------|
|                 | Normal          | CCl4-EHI 24 h   |
| Daunomycin Vmax (nmol/mg protein/30 s) | 3.69 ± 0.36     | 6.72 ± 0.66*    |
| Ks (µM)         | 49.8 ± 15       | 44.9 ± 4.1      |
| Clint (µmol/mg protein/30 s) | 77.4 ± 15       | 149 ± 3.7*      |
| Taurocholate Vmax (nmol/mg protein/30 s) | 0.950 ± 0.79    | 1.02 ± 0.45     |
| Ks (µM)         | 75.9 ± 4.2      | 80.4 ± 3.8      |
| Clint (µmol/mg protein/30 s) | 12.5 ± 0.49     | 12.7 ± 0.07     |
| E217βG Vmax (nmol/mg protein/30 s) | 0.166 ± 0.017   | 0.102 ± 0.016*  |
| Ks (µM)         | 4.22 ± 0.88     | 4.66 ± 2.3      |
| Clint (µmol/mg protein/30 s) | 40.6 ± 9.2      | 24.6 ± 8.0*     |

* All data represent the means ± S.D. (n = 3).
* Clint was calculated by dividing Vmax by Ks.
* Statistically different from normal cLPM vesicles (P < 0.01).

**Discussion**

Western blot analysis (Fig. 1) indicates that the expression level of ABC transporters on the canalicular membrane, except for Bsep, varies as a function of time after the administration of CCl4 to rats, exhibiting maximal changes at 24 h (Fig. 1). Most interestingly, the effect of CCl4-EHI 24 h on the expression levels differed significantly depending on the canalicular transporters; the expression level was increased for P-gp, remained unchanged for Bsep, and was decreased for Mrp2. These results for P-gp and Bsep are consistent with previous reports (Huang et al., 2001; Geier et al., 2002), whereas the result for Mrp2 is contrary to data reported by Geier et al. (2002), in which no change in the expression level of Mrp2 was reported for the liver microsomes of CCl4-EHI 24 h rats. In the present study, Western blot analysis of transporters was performed for cLPM vesicles instead of the liver microsomes of CCl4-EHI 24 h rats. In the present study, Western blot analysis of transporters was performed for cLPM vesicles instead of the liver microsomes (Geier et al., 2002). This difference (i.e., vesicles and microsomes) might be involved in the discrepancy between the two studies in the expression level of Mrp2.

In the present study, Antispgp (Lecureur et al., 2000), MIII-6 (Kool et al., 1997), and C219 (Lee et al., 1996) were used as antibodies for the immunostaining of Bsep, Mrp2, and P-gp, respectively. Antispgp does not cross react with the mdr1a gene product (i.e., P-gp) (Lecureur et al., 2000), and MIII-6 does not with gene products of mdr1a and mrp2 isoforms (i.e., Mrp1, Mrp3, and Mrp5) (Kool et al., 1997). C219, the most widely used antibody for P-gp (Lee et al., 1996), on the other hand, exhibits a partial cross reactivity to other proteins such as Mdr2 and Bsep (Van Den Elsen et al., 1999). The fact that the level of Mdr2 appeared to be increased in 24 h after the CCl4 pretreatment (from the mRNA level of mdr2; Nakasukasa et al., 1993) suggests that immunostaining using C219 might lead to an overestimation of P-gp levels. In other words, a 3.6-fold increase in the level of P-gp by the CCl4-EHI 24 h (Fig. 1) might be an overestimation, at least in part.

The effect of the CCl4-EHI 24 h, i.e., changes in the expression of the ABC transporters on the canalicular excretion of representative substrates of the transporters was examined in vivo. The CLexc values for the substrates were altered multiply (Table 3), consistent with the changes in the expression level of relevant transporters (Fig. 1). However, the change in the CLexc value for daunomycin (i.e., 1.9-fold increase; Table 3) was not as significant compared with the changes in the expression of P-gp (i.e., 3.6-fold increase; Fig. 1A). This discrepancy could be related to the cross-reactivity of C219 (Van Den Elsen et al., 1999) or to interference by certain endogenous factors in CCl4-EHI 24 h rats on canalicular excretion (Olatunde Farombi, 2000; Huang et al., 2001).

To exclude possible interference by endogenous factors, the in vitro uptake of relevant substrates into cLPM vesicles was examined. Results from the in vitro uptake study were fairly consistent with the expression of relevant transporters (Fig. 1) as demonstrated by Vmax and Kn values in Table 3. The Clint was 1.9-fold increased for daunomycin, remained unchanged for taurocholate, and was 39% decreased for E217βG. An analysis of the uptake studies revealed that the changes in the Clint are solely attributable to changes in the Vmax.
and independent of changes in corresponding \( K_m \) values (Table 3), suggesting that the alteration in the quantity of relevant transporters, rather than the alteration of their affinity for their substrates, is responsible for the altered \( CL_{int} \) of the relevant substrates. Neither the inside-out proportion nor leakage (mannitol uptake) of the vesicles (Table 2) appears to be related to the altered \( CL_{int} \) since they were not influenced by the \( CCl_4 \)-EHI. In \( CCl_4 \)-EHI rats, the composition of plasma membrane lipid is changed, possibly increasing the fluidity of the lipid bilayer (Recknagel, 1967; Mourelle et al., 1987). The increase in the membrane fluidity might accelerate the functional activity of ABC transporters (Hyogo et al., 2001). However, the contribution of membrane fluidity-associated alteration is likely to be minimal considering the unchanged or decreased \( CL_{int} \) values for taurocholate and \( E_2 \)17\( \beta \)G (Table 3). The above results suggest that the altered expression of relevant transporters on the canicular membrane is reflected on the canicular excretion of the relevant substrates in vivo.

The effect of \( CCl_4 \)-EHI 24 h on the expression and functional activity of relevant transporters (in vivo and in vitro) is summarized in Fig. 5. In general, the effect of \( CCl_4 \)-EHI 24 h on the ABC transporters, with respect to the expression and functional activity, were different depending on the transporters. The alteration in the expression and functional activity of each transporter was in an identical direction for all the three transporters. However, a closer relationship was observed between \( CL_{int} \) (or \( V_{max} \)) and \( CL_{exc} \), compared with the relationship between the expression of transporters and \( CL_{int} \) or \( CL_{exc} \). This might be related to the cross-reactivity of primary antibodies used in the Western blot analysis (especially for P-gp). It is also consistent with the hypothesis that interference by the endogenous factors in \( CCl_4 \)-EHI 24 h on the relevant transport is not so profound.

The above effects of the \( CCl_4 \)-EHI 24 h appear to be distinct from the result of direct interactions of the membrane or transport systems with \( CCl_4 \), since the incubation of normal cLPM vesicles with 1.5 mM \( CCl_4 \) decreased the uptake of daunomycin to the vesicles in the presence of ATP, for example (Fig. 3), contrary to the increased uptake of the compound into the cLPM vesicles that were prepared from the \( CCl_4 \)-EHI 24 h rats (Fig. 2A). Thus the \( CCl_4 \)-radical, which is formed from \( CCl_4 \) in the body and attacks macromolecules such as proteins and lipid bilayers (Recknagel, 1967; Nelson, 1995), appears to represent the mechanism involved in the indirect effect of the \( CCl_4 \) pretreatment.

Although the expression of transporters was changed by the \( CCl_4 \)-EHI (Fig. 1), the expression does not appear to be necessarily parallel with the level of relevant mRNAs, since the level of mdr 1a and mdr 1b transcripts was reported to be continuously increased for up to 5 days after the \( CCl_4 \) administration (Nakasukasa et al., 1993), whereas the expression of the relevant transporter, P-gp, exhibited a maximal expression at 24 h after the \( CCl_4 \) administration in the present study (Fig. 1, A and A’). Thus, post-transcriptional regulation appears to influence, at least in part, the level of transporters on the canicular membrane. Similar discrepancies in the expression of transporters and responsible mRNAs have been reported for Bsep and Mrp2 in ethynylestradiol-induced cholestasis rats (Trauner et al., 1997; Lee et al., 2000).

One of the most important findings of the present study is the multiple alterations in the expression and activities of canicular membrane transporters by the \( CCl_4 \)-EHI 24 h (Fig. 5). A multiple effect of \( CCl_4 \) has also been demonstrated for the expression of mRNAs and proteins of the sinusoidal transporters (i.e., Ntcp, Oatp1, Oatp2, and Oatp4, Geier et al., 2002) as described in the Introduction. The physiological meaning of this type of influence of \( CCl_4 \)-EHI, especially as host defense mechanisms of biological systems against diseases and xenobiotics, should be carefully considered. Regardless of the underlying mechanisms, the multiple (i.e., transporter and membrane selective) effects of the \( CCl_4 \)-EHI should be taken into account in understanding of hepatic injuries. Special care should be taken concerning the increase in the expression and functional activity of certain transporters (i.e., P-gp, in this study), because it might be contrary to our general understanding that hepatic injuries may damage membrane transporters both in terms of expression and function. In addition, it is noteworthy that the expression of ABC transporters on the canicular membrane may vary depending on the types of EHI, as evidenced by the decrease in the level of both Bsep and Mrp2 by ethynylestradiol-induced EHI (Bossard et al., 1993; Lee et al., 2000).

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