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Modulation of intestinal transport of 2,4-dinitrophenyl-S-glutathione, a multidrug resistance-associated protein 2 substrate, by bilirubin treatment in rats

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Abstract

The effect of bilirubin treatment on intestinal transport of 2,4-dinitrophenyl-S-glutathione (DNP-SG), a substrate of multidrug resistance-associated protein 2 (MRP2), after application of 1-chloro-2, 4-dinitrobenzene (CDNB), a precursor of DNP-SG, was examined in rat intestine by the in-vitro everted sac, in-situ re-circulating perfusion, and in-situ loop methods. CDNB was taken up rapidly by jejunum and ileum, and the consequent intestinal efflux of DNP-SG, a glutathione conjugated metabolite of CDNB, was significantly higher in jejunum than in ileum in the in-situ and in-vitro studies. Co-administration of bilirubin (100 μM), as well as probenecid (1 mM) or ciclosporin (100 μM), with CDNB decreased the DNP-SG efflux in jejunum significantly, but not in ileum. The suppression of DNP-SG efflux in jejunum was also observed after intravenous administration of bilirubin (85.5 μmol kg^{-1}), in which plasma bilirubin glucuronide levels were approximately 100 μM. In the in-vitro metabolism study, bilirubin exerted no significant effect on CDNB metabolism in the intestinal S9 fraction (supernatant of 9000 *g*). These results suggested that the diseased states accompanied with hyperbilirubinaemia might have increased the intestinal absorption, or oral bioavailability, of MRP2 substrates by suppressing MRP2 function at the proximal intestinal region.

Introduction

The small intestine is often exposed to various harmful exogenous substances, and therefore possesses various metabolic enzymes and efflux transporters as a detoxification system. P-glycoprotein (P-gp), an ATP-dependent efflux transporter, is expressed on the apical membrane of intestinal enterocytes in man and rodents (Terao et al 1996; Suzuki & Sugiyama 2000). Along the small intestinal tract, the expression level of P-gp, or P-gp function, increases from the proximal to the distal region (Trezise et al 1992; Yumoto et al 1999). In addition to P-gp, another ATP-dependent efflux transporter, multidrug resistance-associated proteins (MRPs), such as MRP1, 2 and 3, are also expressed in rat small and large intestine (Cherrington et al 2002; Chan et al 2004). P-gp transports relatively lipophilic compounds such as immunosuppressants, calcium channel blockers, anticancer agents, and steroidal agents out of cells (Hunter et al 1993; Terao et al 1996). MRPs transport relatively hydrophilic compounds including the glucuronide, glutathione, and sulfate conjugates of endogenous and exogenous compounds (Suzuki & Sugiyama 2002; Chan et al 2004). MRP2, also called canalicular multispecific organic anion transporter (cMOAT), is expressed on the apical membrane of enterocytes preferentially in the proximal intestine (Mottino et al 2001). The distribution pattern of MRP2 expression is reportedly similar to those of conjugating enzymes such as UDP-glucuronosyltransferase and glutathione S-transferase in rats (Chowdhury et al 1985; Mottino et al 2000).

Both P-gp and MRPs recognize not only exogenous compounds but also many endogenous compounds (Suzuki & Sugiyama 2002; Uhr et al 2002). Previously, we found the systemic modulation of P-gp function under diseased states such as acute renal failure and acute hepatic failure, due to the alteration in blood concentrations and/or composition of endogenous P-gp inhibitors in rats (Huang et al 2000, 2001;

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Murakami et al 2002). Similarly, it would be possible for MRP function to be modulated under diseased states accompanied by increased endogenous MRP inhibitors.

In this study, we have examined the effect of bilirubin treatment on MRP2 function pharmacokinetically by measuring the transport of 2,4-dinitrophenyl-S-glutathione (DNP-SG), a MRP2 substrate, after application of 1-chloro-2,4-dinitrobenzene (CDNB), a precursor of DNP-SG (Gotoh et al 2000). Bilirubin generated from endogenous haem catabolism was conjugated by bilirubin uridine diphosphate (UDP)-glucuronosyltransferase to form mono- and diglucuronides of bilirubin mainly in the liver (Mottino et al 1988; Kamisato et al 2000). The bilirubin glucuronides are MRP2 substrates and inhibitors, and are excreted into bile across the hepatocyte canalicular membrane by MRP2 (Nishida et al 1992; Jedlitschky et al 1997; Kamisako et al 1999, 2000). Hyperbilirubinaemia is accompanied by obstructive jaundice caused by cancer, liver graft, or Dubin-Johnson syndrome, an inherited MRP2 disorder (Toh et al 1999; Feng et al 2003; Ben-Ari et al 2004). In such diseased states, not only are the unconjugated/conjugated bilirubin levels in blood modulated but other factors are as well, such as hepatic blood flow rate, metabolic enzyme activity, and/or protein binding. Therefore, this study was carried out in normal rats to reveal the effect of unconjugated/conjugated bilirubin itself on intestinal MRP2 function, by eliminating other influencing factors accompanied by hyperbilirubinaemia.

Materials and Methods

Materials

CDNB, glutathione (GSH), and 17β-estradiol were obtained from Wako Pure Chemicals (Osaka, Japan). 1-Fluoro-2, 4-dinitrobenzene (FDNB) and bilirubin were purchased from Tokyo Kasei (Tokyo, Japan) and Kanto Kagaku (Tokyo, Japan), respectively. Probenecid was purchased from Sigma Chemical Co. Ltd (St Louis, MO) and ciclosporin was a generous gift from Novartis Pharma Co. Ltd (Tokyo, Japan).

Synthesis of DNP-SG

DNP-SG, a GSH-conjugated metabolite of CDNB, was synthesized according to Hinchman et al (1991). Briefly, 2.5 mmol FDNB dissolved in 2.5 mL methanol was slowly mixed with 3.75 mmol GSH dissolved in 1 M $KHCO_3$ (12.5 mL) under stirring. After 15-min incubation at room temperature, the solution was filtered and acidified to approximately pH 2 with diluted HCl. The precipitate was collected by vacuum filtration and was washed with a sufficient amount of distilled water to remove any extra GSH.

Animals

Male Sprague-Dawley (SD) rats and Eisai hyperbilirubinaemic rats (EHBR) lacking MRP2 hereditarily were used. The SD rats and EHBR were seven- to nine-weeks old, and three rats were used for each experiment. Experiments with animals were performed in accordance with the "Guide for Animal Experimentation" from Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University.

Effect of bilirubin treatment on DNP-SG efflux in the intestinal everted sac in-vitro

A 20-cm long everted jejunum was prepared from SD rats. CDNB was dissolved at a concentration of 50 μM in pH 6.0 isotonic phosphate buffered saline (PBS (in mM): 20.6 Na_2HPO_4, 2.6 NaH_2PO_4, 129 NaCl, 1.5 KCl, 1.14 glutathione (GSH), 14 glucose) containing 4% dimethyl sulfoxide (DMSO), and 1 mL drug solution was applied to the serosal side of the closed everted sac. The sac was then immersed in 8 mL, pH 6.0 PBS containing 4% DMSO prewarmed at 37°C and pre-oxygenated with 5% CO_2/95% O_2. The bubbling of the incubation medium with a CO_2/O_2 gas was continued throughout the efflux study. The efflux of DNP-SG across the everted intestine after application of CDNB to the serosal side was measured by sampling the mucosal medium periodically for 120 min. In an inhibition study, probenecid (1 mM) or ciclosporin (100 μM) was added as a typical inhibitor to the mucosal medium (pH 6.0 PBS containing 4% DMSO) at an appropriate final concentration.

Intestinal DNP-SG efflux after application of CDNB in SD rats and EHBR in the in-situ perfusion study

Rats were fasted overnight, anaesthetized with pentobarbital (30 mg kg^{-1}, i.p. injection) and affixed supine on a surface kept at 37°C to maintain the body temperature at approximately 36°C. In-situ intestinal perfusion was carried out to estimate the efflux (secretion) of DNP-SG from enterocytes into the intestinal perfusate after application of CDNB in SD rats and EHBR. In this study, jejunum (a 20-cm long segment from 5 cm below the bile duct opening) and ileum (a 20-cm long segment from the ileocaecum) were used to elucidate the regional difference in intestinal MRP2 function in SD rats and EHBR. Each intestinal segment was perfused with 20 mL pH 6.5 PBS containing 4% DMSO and 20 μM CDNB in a re-circulating perfusion manner at a rate of 3 mL min^{-1}. GSH was added to stabilize DNP-SG in intestinal perfusate, and DMSO was used to increase the solubility of CDNB. The intestinal perfusate was sampled periodically to determine the concentrations of CDNB and DNP-SG.

Effect of bilirubin treatment on DNP-SG efflux in the in-situ jejunum loop of SD rats

Concentrations of conjugated and unconjugated bilirubin in plasma after intravenous administration of bilirubin were measured with time in SD rats. Bilirubin (8.6 or 85.5 μmol kg^{-1}) was administered intravenously via a

cannula (polyethylene tubing, PE-50) inserted at a femoral vein. Blood was sampled periodically via a cannula (PE-50) inserted at a femoral artery. Blood samples (0.2 mL) were taken at 1, 3, 5, 10, 30 60 and 90 min after the intravenous injection.

The effect of bilirubin treatment on DNP-SG efflux after application of CDNB was evaluated using the in-situ jejunum loop method. Briefly, anaesthetized SD rats were cannulated with polyethylene tubing (PE-50) at a femoral vein, and probenecid (175.2 μmol kg^{-1}) or bilirubin (8.6 or 85.5 μmol kg^{-1}) was administered intravenously via the cannula. CDNB (5 μmol kg^{-1}) was administered into the closed jejunum loop 10 min after administration of an inhibitor. At the end of the study (60 min), intestinal solution was collected. The mucosal membrane at the jejunum segment was collected by scraping with a cover glass to determine the concentrations of CDNB and DNP-SG.

Metabolism study of CDNB and DNP-SG in intestinal mucosa in-vitro

Intestinal mucosa was collected by scraping with a cover glass and was homogenized in a 9-fold volume of ice-cold pH 7.4 Dulbecco's phosphate buffered saline (D-PBS (in mM): 1.5 KH_2PO_4, 8 Na_2HPO_4, 137 NaCl, 3 KCl, 5 glucose, 1 $CaCl_2$, 0.5 $MgCl_2$) by means of a glass–Teflon Potter homogenizer (1000 rev min^{-1}, 20 strokes). An S9 fraction (supernatant of 9000 g) of intestinal mucosa was prepared by centrifuging the 10% homogenate at 9000 g for 20 min, and the supernatant was diluted with D-PBS to make a final concentration of protein at 2 mg mL^{-1} for DNP-SG metabolism and at 0.1 mg mL^{-1} for CDNB metabolism. All the procedures were performed at 4°C. DNP-SG was dissolved at a concentration of 200 μM in pH 7.4 D-PBS with or without 2.28 mM GSH. CDNB was dissolved at a concentration of 2 mM in pH 7.4 D-PBS containing 2% DMSO and 2.28 mM GSH. The metabolism reaction was initiated by mixing 0.5 mL S9 fraction and 0.5 mL of a drug solution, both of which were pre-warmed at 37°C in a shaking waterbath for 5 min. The enzyme reaction for DNP-SG was conducted in a range from 2.5 to 30 min and for CDNB from 3 to 60 s. In examining the effect of MRP-related compounds on CDNB metabolism, a mixture of 4 mM CDNB and 4.56 mM GSH (0.25 mL), a test solution containing 4 mM probenecid, 400 μM ciclosporin, 400 μM 17β-estradiol, or 400 μM bilirubin (0.25 mL), and S9 fraction for CDNB (0.5 mL) were mixed (final concentrations: CDNB, 1 mM; GSH, 1.14 mM; probenecid, 1 mM; ciclosporin, 100 μM; 17β-estradiol, 100 μM; bilirubin, 100 μM). The reaction was terminated by adding ice-cold 10% PCA (1 mL). The suspension was centrifuged at 3000 rev min^{-1} for 10 min, and the supernatants were subjected to high-performance liquid chromatography (HPLC) to determine the concentration of CDNB and/or DNP-SG.

Analysis

Blood samples were centrifuged to obtain plasma samples, then 50 μL pH 7.4 D-PBS and 50 μL 20% PCA were added to each 100-μL plasma sample. Intestinal perfusate was diluted with an equal volume of 10% PCA. Intestinal mucosa was homogenized in a 9-fold volume of 4% PCA. All these biological samples were kept on ice for at least 30 min, and centrifuged at 3000 g for 10 min. Concentrations of CDNB and DNP-SG in the supernatants of various biological samples were determined by HPLC using a column of Mightysil RP-18 (Kanto Kagaku, Tokyo, Japan). Mobile phases used were a mixture of acetonitrile and 1% acetic acid (15:85, v/v) for DNP-SG, and a mixture of acetonitrile and 1% acetic acid (35:65, v/v) for CDNB at a flow rate of 1 mL min^{-1}. Detection was made at wavelengths of 365 nm for DNP-SG and 305 nm for CDNB, respectively. The concentration of protein in S9 fraction was measured by the Lowry method (Lowry et al 1951) using bovine serum albumin as the standard. The concentrations of bilirubin and conjugated bilirubin in various biological samples were measured with a commercially available analytical kit (Bilirubin BII-Test Wako, Wako Pure Chemicals, Osaka, Japan).

Differences among group mean values were assessed by the Kruskal–Wallis test followed by post-hoc test (Dunn's test) or Student's t-test. A difference of $P < 0.05$ was considered statistically significant.

Results

Intestinal DNP-SG efflux after intestinal application of CDNB in SD rats and EHBR in the in-situ perfusion study

The MRP2 function in jejunum and ileum of SD rats was evaluated by measuring DNP-SG efflux into the lumen after intestinal application of CDNB. For comparison, EHBR lacking MRP2 was also used. As shown in Figure 1, the disappearance rates of CDNB from the intestinal perfusate, or influx rates of CDNB into enterocytes, were of the same magnitude between the jejunum and ileum in SD rats and EHBR. The amount of CDNB remaining in the intestinal perfusate at 90 min after application was $4.6 \pm 1.8\%$ dose in jejunum and $6.9 \pm 1.9\%$ in ileum in SD rats, and $8.9 \pm 1.1\%$ in jejunum and $9.3 \pm 1.1\%$ in ileum in EHBR. The efflux of DNP-SG into the intestinal perfusate occurred rapidly after the initiation of intestinal perfusion of CDNB (Figure 2). In SD rats, the intestinal efflux of DNP-SG in jejunum was significantly greater than in the ileum: $47.7 \pm 4.6\%$ of dose in jejunum and $26.8 \pm 1.0\%$ in ileum at 90 min ($P < 0.05$). In contrast, in EHBR, the intestinal efflux of DNP-SG was of the same magnitude between the jejunum and ileum and the value was comparable with that of ileum of SD rats: $26.0 \pm 0.7\%$ of dose in jejunum and $21.9 \pm 1.8\%$ in ileum of EHBR. These data suggested that functional MRP2 was poor in the ileum of SD rats.

Effect of bilirubin on mucosal efflux of DNP-SG in the in-vitro everted sac

Everted jejunum and ileum sacs of SD rats were used to examine the effects of bilirubin, probenecid, ciclosporin

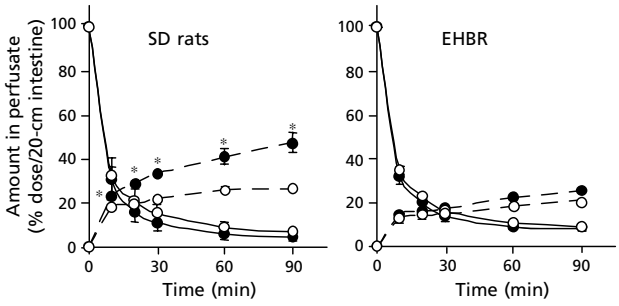

Figure 1 Influx of CDNB into enterocytes and efflux of DNP-SG from enterocytes into intestinal lumen during a re-circulation of CDNB in jejunum (solid circle) and ileum (open circle) of SD rats and EHBR. Solid line denotes CDNB and dotted line DNP-SG. The initial concentration of CDNB was 20 μM and perfusion was made at a rate of 3.0 mL min^{-1}. Each value represents the mean ± s.e. of three determinations. *$P < 0.05$ compared with the value for ileum.

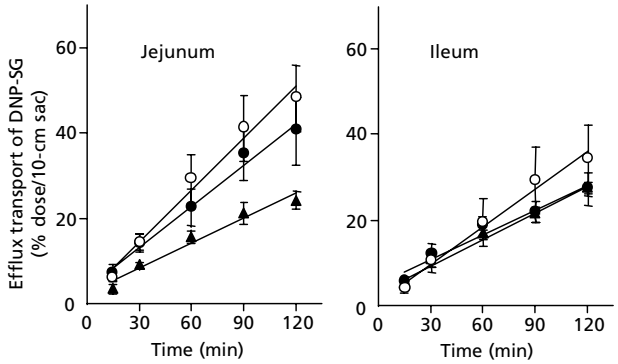

Figure 2 Effect of bilirubin on DNP-SG efflux after application of CDNB in everted jejunum and everted ileum of SD rats. CDNB 50 nmol was applied to serosal side of the 10-cm long everted sac. Open circles, control; closed circles, 10 μM bilirubin; and closed triangles, 100 μM bilirubin. Each value represents the mean ± s.e. of three determinations.

and 17β-estradiol on mucosal efflux of DNP-SG after application of CDNB to the serosal side. CDNB was taken up by the sac rapidly and was metabolized to DNP-SG. The efflux of DNP-SG to the mucosal side followed in a zero-order rate fashion with no lag time in either jejunum or ileum, and the efflux rate (slope) in jejunum was approximately 1.5-fold compared with ileum. The efflux rates of DNP-SG in the absence and presence of bilirubin in everted jejunum and ileum were as follows: jejunum, 0.41 ± 0.07% dose min^{-1} for control, 0.32 ± 0.06 in the presence of 10 μM bilirubin, and 0.20 ± 0.01 in the presence of 100 μM bilirubin; ileum, 0.29 ± 0.07 for control, 0.20 ± 0.02 in the presence of 10 μM bilirubin, and 0.19 ± 0.02 in the presence of 100 μM bilirubin. Though the difference was not of a significant level, 100 μM bilirubin suppressed DNP-SG efflux transport by approximately 50% in jejunum. Inhibitory

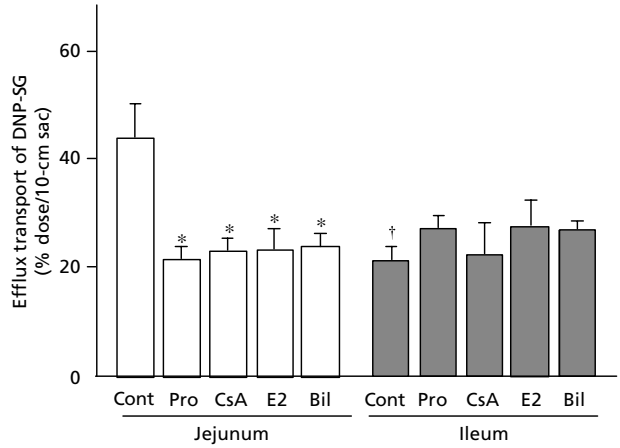

Figure 3 Effect of MRP-related compounds on DNP-SG efflux 120 min after application of CDNB in everted jejunum and ileum of SD rats. CDNB 50 nmol was applied to serosal side of the 10-cm long everted sac. Cont, control; Pro, 1 mM probenecid; CsA, 100 μM ciclosporin; E2, 100 μM 17β-estradiol; Bil, 100 μM bilirubin. Each value represents the mean ± s.e. of three determinations. *†$P < 0.05$ compared with the value for control (jejunum).

potencies of these MRP2-related compounds on DNP-SG efflux are summarized in Figure 3. All compounds examined significantly suppressed the mucosal efflux of DNP-SG in jejunum, but not in ileum. In jejunum, bilirubin and 17β-estradiol at a concentration of 100 μM, as well as 1 mM probenecid and 100 μM ciclosporin, suppressed the DNP-SG efflux to the same level in ileum. The suppressive effects of bilirubin and 17β-estradiol would be due to the formation of their glucuronide conjugates in the intestinal membrane. In this study, conjugated bilirubin was detected in the incubation medium at a concentration of approximately 5 μM at the end of the transport study (120 min).

Effect of bilirubin treatment on intestinal efflux of DNP-SG in the in-situ loop method

The plasma levels of conjugated and unconjugated bilirubin in plasma after intravenous administration were dependent on the dose of bilirubin (8.6 or 85.5 μmol kg^{-1}) in SD rats (Figure 4). Conjugated bilirubin was kept at an almost constant level after 10 min post-administration (approximately 8 μM at a dose of 8.6 μmol kg^{-1} and 104 μM at a dose of 85.5 μmol kg^{-1}).

The effect of intravenous bilirubin and probenecid on intestinal DNP-SG efflux was examined in the jejunum loop of SD rats. The amounts of CDNB and DNP-SG recovered in the loop 60 min after the application of CDNB are summarized in Figure 5A. Treatment with 85.5 μmol kg^{-1} bilirubin intravenously decreased the intestinal DNP-SG efflux markedly, as did 175.2 μmol kg^{-1} probenecid intravenously (Figure 5A). Treatment with probenecid (175.2 μmol kg^{-1}) or bilirubin (85.5 μmol kg^{-1}) significantly increased the accumulation of DNP-SG in the mucosal membrane of jejunum (Figure 5B).

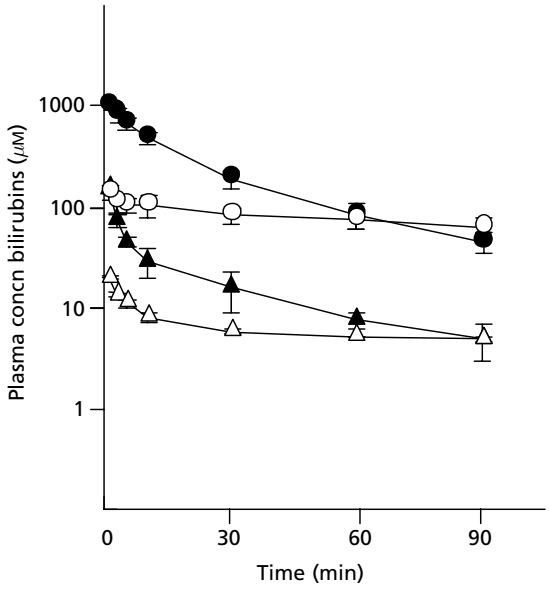

Figure 4 Concentrations of conjugated and unconjugated bilirubin in plasma after intravenous administration of bilirubin at a dose of 8.6 and 85.5 μmol kg^{-1} in SD rats. Open and solid circles represent the plasma conjugated and unconjugated bilirubin concentrations, respectively, after administration at a dose of 85.5 μmol kg^{-1}. Open and solid triangles represent the plasma conjugated and unconjugated bilirubin concentrations, respectively, after administration at a dose of 8.6 μmol kg^{-1}. Each value represents the mean ± s.e. of three determinations.

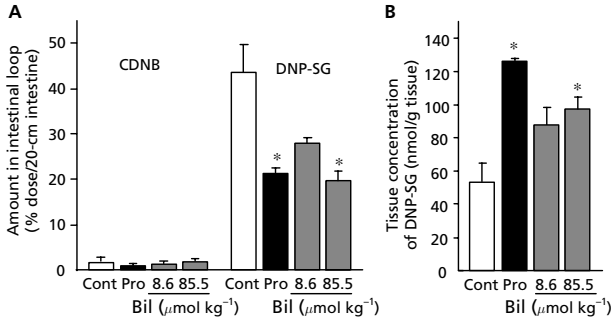

Figure 5 Effect of intravenous administration of probenecid and bilirubin on CDNB and DNP-SG amounts recovered in jejunum loop (A) and accumulation of DNP-SG in jejunum mucosa (B) 60 min after application of CDNB in SD rats. Probenecid (Pro) was administered at a dose of 175.2 μmol kg^{-1} and bilirubin (Bil) was administered at a dose of 8.6 or 85.5 μmol kg^{-1}. Each value represents the mean ± s.e. of three determinations. *$P < 0.05$ compared with the value for control.

Glutathione S-transferase activity in intestinal mucosa and effect of MRP-related compounds in-vitro

Glutathione S-transferase (GST) activity in jejunal and ileal mucosa in the absence or presence of a MRP-related compound was evaluated by measuring the DNP-SG formation from CDNB. The GST activity in jejunal mucosa was slightly higher compared with ileal mucosa ((85 ± 11 and 59 ± 8 μmol min^{-1} (mg protein)$^{-1}$, respectively), though no significant difference was detected between them. Probenecid (1 mM), ciclosporin (100 μM), 17β-estradiol (100 μM), or bilirubin (100 μM) exerted no effect on in-vitro GST activity.

Discussion

Previous data from Western blot and/or Northern blot analyses revealed the expression and localization of the MRP family in the intestine of rats. Expression of MRP1, located on the basolateral membranes, in the small intestine is low and is limited to undifferentiated enterocytes at the base of crypts (Peng et al 1999; Cherrington et al 2002). MRP3 is located on the basolateral membranes and is expressed at high levels in the ileum and colon (Kool et al 1999; Rost et al 2002). In contrast to MRP1 and 3, MRP2 is concentrated on brush-border membranes at the tip of the villus, with the highest expression in the proximal intestine, with little MRP2 protein in the ileum (Mottino et al 2001). Also, the distribution pattern of phase II conjugating enzymes, UDP-glucuronosyltransferase and glutathione S-transferase, is reportedly similar to that of MRP2 in rat small intestine (Pinkus et al 1977; Mottino et al 2001; Chan et al 2004).

We compared the MRP2 function between jejunum and ileum of SD rats and EHBR by the in-situ perfusion method. We evaluated the effect of bilirubin treatment on MRP2 function by in-vitro everted sac and in-situ perfusion methods in SD rats. In these studies, GSH at a physiological concentration (1.14 mM) was added to the incubation medium and intestinal perfusate. DNP-SG is degraded to 2,4-dinitrophenyl-S-cysteinylglycine (DNP-CG), 2,4-dinitrophenyl-S-cysteine (DNP-Cys), and then to 2,4-dinitrophenyl-N-acetylcysteine (DNP-NAc) in biological samples (Hinchman et al 1991; Gotoh et al 2000). Acivicin, an inhibitor for γ-glutamyltransferase, has been used to stabilize DNP-SG in in-vivo and in-vitro samples in previous studies (Oude Elferink et al 1993; Gotoh et al 2000; Hirohashi et al 2000; Mottino et al 2001). In this study, GSH was found to be effective in inhibiting DNP-SG degradation in biological samples, possibly as a competitive inhibitor for γ-glutamyltransferase-mediated metabolism. For example, the degradation rate of DNP-SG (100 μM) in the S9 fraction of intestinal mucosa was 0.43% min^{-1} (mg protein)$^{-1}$, and it decreased to 0.05% min^{-1} (mg protein)$^{-1}$ in the presence of 1.14 mM GSH. Oxidized glutathione (GSSG) is reportedly transported by MRP2 (Nishida et al 1992; Stieger et al 2000). In contrast, GSH is not transported by rat MRP2 (Akerboom et al 1991), though it may be a substrate of human MRP2 (Paulusma et al 1999). By adding GSH to the incubation medium or intestinal perfusate, the degradation of DNP-SG was almost completely inhibited. In this study, the collaborating interplay between conjugating enzymes and MRP2 was clearly demonstrated (Figure 1). The intestinal DNP-SG efflux in jejunum, where MRP2 is

abundantly expressed, was significantly higher than ileum in SD rats (Figure 1). The efflux of DNP-SG in the ileum of SD rats was to the same extent as that in jejunum and ileum of EHBR, indicating that functional MRP2 was absent in the ileum of SD rats. The marked regional difference in DNP-SG efflux was not ascribed to the difference in GST activity, because GST activity was not significantly different between jejunum and ileum of SD rats. Although MRP2 expression in the intestine was not evaluated in this study, the marked regional difference in MRP2 function was in good agreement with the reported regional difference in MRP2 expression (Mottino et al 2001). The negligible MRP2 function in the ileum of SD rats was observed also in the in-vitro everted sac study, where MRP2 inhibitors such as probenecid and ciclosporin showed no effect on DNP-SG efflux (Kamisako et al 1999).

We evaluated the effect of endogenous MRP-related compounds, bilirubin and 17β-estradiol, on intestinal MRP2 function in SD rats. Bilirubin and 17β-estradiol are metabolized to their glucuronide conjugates in-vivo, and they are substrates of MRP2 (Keppler & Konig 2000). Under various diseased states such as hepatoma and cholestasis, the concentration of unconjugated/conjugated bilirubin in plasma reaches to more than 100 μM, though this is not the case for unconjugated/conjugated 17β-estradiol (Frank et al 1990; Toh et al 1999; Feng et al 2003; Ben-Ari et al 2004). Previously, we studied the expression and function of P-gp in rats with acute renal failure and acute hepatic failure (Huang et al 2000, 2001; Murakami et al 2002). In that study, the expression of P-gp at the target injury sites, the kidney in renal failure rats and the liver in hepatic failure rats, was significantly increased as compared with normal rats. However, the in-vivo function of P-gp was low due to the alteration in blood concentrations and/or composition of endogenous P-gp inhibitors. Similarly, it was speculated that the high conjugated bilirubin level in plasma could affect MRP function systemically. In rats with hyperbilirubinaemia, the expression of protein and mRNA of MRP2 and MRP3 were greatly modulated (Tanaka et al 2002). Thus, in this study, normal rats were treated with bilirubin to evaluate the effect of hyperbilirubinaemia itself on intestinal MRP2 function.

Treatment with 100 μM bilirubin suppressed the mucosal efflux of DNP-SG in jejunum significantly, but not in ileum, in the in-vitro everted sac study (Figure 3). In that study, the concentration of conjugated bilirubin in transport medium at 120 min was approximately 5 μM. It has been reported that the Michaelis constant (Km value) of bilirubin monoglucuronide for MRP2 is 0.8 μM, and that of bilirubin bisglucuronide is 0.5 μM (Jedlitschky et al 1997; Kamisako et al 1999). Therefore, the inhibitory effect of bilirubin treatment was probably due to the formation of conjugated bilirubin in the intestinal cells. The significant inhibition of intestinal MRP2 function was observed also after intravenous administration of 86.5 μmol kg^{-1} bilirubin, where plasma concentrations of conjugated and unconjugated bilirubin were higher than 70 μM throughout the study (Figure 5A). The concentrations of conjugated and total bilirubin in EHBR plasma were 59 and 89 μM, respectively. Also, as described already, conjugated and/or unconjugated hyperbilirubinaemia are frequently observed under various diseased states such as obstructive jaundice caused by cancer (Toh et al 1999; Feng et al 2003; Ben-Ari et al 2004). The high concentration of conjugated bilirubin may suppress MRP function and modulate the pharmacokinetics of MRP substrates.

In this study, intestinal MRP2 function was evaluated pharmacokinetically by measuring the efflux transport of DNP-SG after application of CDNB, which was completely and rapidly metabolized to DNP-SG in the intestinal epithelial cells. However, DNP-SG used in this study was just a model substrate for MRP2. Therefore, further studies are necessary to clarify the role or contribution of MRP2 in the pharmacokinetics of clinically available MRP2 substrate drugs and alterations in their pharmacokinetics under hyperbilirubinaemic conditions.

In conclusion, we analysed the effect of bilirubin treatment on intestinal MRP2 function by measuring the pharmacokinetics of DNP-SG in rats. Treatment with bilirubin resulted in high concentrations of conjugated/unconjugated bilirubin in the plasma, and significantly suppressed the MRP2 function in the intestine. These results suggested that diseased states accompanied with hyperbilirubinaemia could modulate the intestinal absorption, or oral bioavailability, of MRP2 substrates by suppressing MRP2 function at the proximal intestinal region.

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