Differential Effect of Acute Hepatic Failure on *in Vivo* and *in Vitro* P-Glycoprotein Functions in the Intestine

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Purpose. The expression and function of P-glycoprotein (P-gp) in the intestine in carbon tetrachloride-induced acute hepatic failure (AHF) were evaluated in rats.

Methods. The expression of P-gp, *in vivo* absorption and exsorption of P-gp substrates (digoxin and rhodamine 123), and *in vitro* efflux transport of these P-gp substrates were studied in the absence and presence of a P-gp inhibitor (verapamil or cyclosporin A) using the distal region of small intestine of control and AHF rats.

Results. Western blot analysis revealed that intestinal P-gp expression level remained unchanged, or rather increased, in AHF. The *in vivo* intestinal P-gp function was significantly lower in AHF, as evaluated by the absorption and exsorption of P-gp substrates. In contrast, *in vitro* P-gp function was significantly higher in AHF, as evaluated by the efflux transport of P-gp substrates across the everted intestine. Collectively, the intestinal P-gp function was differently affected by AHF between *in vivo* and *in vitro* conditions.

Conclusions. The *in vivo* intestinal P-gp function was suppressed in AHF, which could not be predicted from *in vitro* functional studies nor from P-gp expression level. The discrepancy between *in vivo* and *in vitro* results may be explained by the presence of endogenous P-gp inhibitors in the plasma of AHF rats.

KEY WORDS: acute hepatic failure; P-glycoprotein; intestine; function; expression.

INTRODUCTION

P-Glycoprotein (P-gp), an ATP-dependent efflux pump, transports many structurally and pharmacologically unrelated hydrophobic compounds, including anticancer agents, immunosuppressants, steroid hormones, calcium channel blockers, ß-blockers, cardiac glycosides, and so on. P-gp serves as a first line absorption barrier by limiting the influx and as a secretory detoxifying mechanism by facilitating the efflux of P-gp substrates from blood to the intestinal lumen (1,2). Lown *et al.* (3) analyzed the oral bioavailability of cyclosporin A (CsA), a P-gp substrate, in kidney transplant recipients pharmacokinetically, and found that the intestinal P-gp, rather than intestinal cytochrome P450 (CYP) 3A, plays the dominant role in the first-pass elimination of CsA and an interpatient variation of its bioavailability. Masuda *et al.* (4) reported that the ratio of trough concentration of tacrolimus, a P-gp substrate, against its oral dose correlates well with the intestinal mRNA expression level of P-gp, but not of CYP3A4, in a small bowel transplant recipient. In addition, Westphal *et al.* (5) found a good correlation between the systemic clearance of talinolol, a P-gp substrate without appreciable metabolic disposition, given intravenously and P-gp expression in biopsy specimens of gut mucosa in humans. Based on these findings, the intestinal P-gp has been recognized to have a determinant role in pharmacokinetics and pharmacodynamics of certain P-gp-related drugs.

So far, various factors have been shown to modulate the expression and function of P-gp. For example, many substrates of the CYP3A subfamily are also substrates or inhibitors of P-gp, and they modulate the oral bioavailability of other P-gp/CYP3A-related drugs when administered concomitantly (6–8). The coinduction of intestinal P-gp and CYP3A by many pharmaceuticals (including rifampicin) during pharmacotherapy is also a clinically important and intractable problem in controlling and predicting the oral bioavailability of P-gp/CYP3A-related drugs (5,9). In addition, Veau *et al.* (10) reported a decrease in the intestinal efflux transport of rhodamine 123 (Rho123), a P-gp substrate (11), in everted gut sacs of partial (7/8) nephrectomy rats, though the intestinal P-gp protein or mRNA levels remained unchanged. Okabe *et al.* (12) reported an increase in bioavailability of tacrolimus in cisplatin-induced renal failure rats. We also studied the effects of diseased states, such as glycerol-induced acute renal failure (ARF) and carbon tetrachloride (CCl_4)-induced acute hepatic failure (AHF), on the expression and function of P-gp in the liver, kidney, and brain in rats (13–15). In those studies, P-gp levels in the target injury organ (the kidney in ARF and the liver in AHF models) were found to be increased significantly, while others remained unchanged or increased only slightly. In contrast, P-gp functions *in vivo* in these tissues were all markedly suppressed, as evaluated by biliary and renal secretory clearances and brain distribution of Rho123.

In the present study, we further evaluated the effect of CCl_4-induced AHF on the intestinal P-gp function and expression in rats. CCl_4 is thought to induce necrotic cell death and apoptosis of hepatocytes, probably due to the interaction of radical products of CCl_4 (trichloromethyl free radical) with membrane structures and the generation of lipid peroxides (16,17). Such hepatic failure affects the pharmacokinetics and pharmacodynamics of various drugs (18,19). However, the effect of AHF on intestinal function, especially on P-gp-mediated transport, is not known. Therefore, in the present study, the expression and function of intestinal P-gp in AHF were evaluated under in-vivo and in-vitro conditions, using digoxin and Rho123 as P-gp substrates (11,20).

MATERIALS AND METHODS

Materials

CCl_4 was obtained from Hayashi Pure Chemical Ind. Ltd. (Osaka, Japan). Rho123 was obtained from Acros Organics (Geel, Belgium). Digoxin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). CsA was kindly supplied by Novartis (Tokyo, Japan). Verapamil hydrochloride was purchased from Nacalai Tesque (Kyoto, Japan). A monoclonal antibody for P-gp, C219, was from Signet Laboratories,

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Inc. (Dedham, MA, USA), and a secondary antibody, peroxidase-labeled affinity-purified antibody to mouse IgG (H + L), was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD, USA). All chemicals used were of the highest purity available.

Animal Treatment

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. AHF was induced in male Wistar rats (252 ± 7 g) by intraperitoneal injection of a mixture of CCl_4 and olive oil (50%, v/v) at a dose of 1.25 or 2.0 mL/kg. These rats were used for experiments 24 h after CCl_4 injection. Induction of AHF in rats was confirmed by measuring plasma activity of glutamic pyruvic transaminase (GPT) with the GPT-UV Test Wako (Wako Pure Chemicals, Osaka, Japan). Plasma GPT activities were 7.2 ± 0.4 IU/L (mean ± SEM) after olive oil alone (control rats), 154 ± 40.1 IU/L after 1.25 mL/kg of CCl_4-oliver oil (1:1, v/v) mixture, and 576 ± 231 IU/L after 2.0 mL/kg of CCl_4-oliver oil mixture (n = 5-6). Each rat given CCl_4 or olive oil was fasted overnight with free access to water before the experiments.

Determination of P-gp Levels in Crude Intestinal Membrane Fractions

The intestinal epithelial cells were collected from the lower half of the small intestine of control and AHF rats according to the reported method (21), with a small modification as reported previously (8). The amount of P-gp in the crude intestinal membrane fractions was evaluated by Western blot analysis after SDS-polyacrylamide gel electrophoresis (PAGE) in the same manner as described previously, using a monoclonal antibody for P-gp, C219, and peroxidase-labeled affinity-purified antibody to mouse IgG as the secondary antibody (8).

In Vivo Intestinal Absorption of Digoxin

The absorption of digoxin from the distal region of small intestine (20 cm) in the absence or presence of 300 μM verapamil, a P-gp inhibitor, was measured in an *in situ* recirculating perfusion system in control and AHF rats. The initial concentration of digoxin in perfusate (10 mL of Dulbecco's-phosphate buffered saline (D-PBS) containing 25 mM glucose) was 3 nM and the perfusion rate was 1 mL/min. The absorption rate of digoxin was estimated from the amount of digoxin remaining in perfusate (the product of perfusate concentration times volume of perfusate).

In Vivo Intestinal Exsorption of Digoxin and Rho123 Under Steady State

The *in vivo* intestinal exsorption clearances of digoxin and Rho123 under steady states in the absence or presence of a P-gp inhibitor (verapamil or CsA) were measured as reported previously (7). Briefly, a steady-state plasma concentration (Cpss) of digoxin or Rho123 was settled by a bolus injection (12.4 μg (15.87 nmol)/kg for digoxin, 4.36 μmol/kg for Rho123), followed by constant infusion at a rate of 0.48 μg (0.61 nmol)/h for digoxin or 200 nmol/h for Rho123. Both ends of the distal region of the small intestine were catheterized with silicon cannulae to make a 20-cm long loop, and D-PBS containing 25 mM glucose (pH 7.4) was perfused by single perfusion at a rate of 0.5 mL/min for digoxin or 1 mL/min for Rho123. After Cpss of digoxin or Rho123 was achieved (145 min for digoxin and 55 min for Rho123), the intestinal effluent and blood were collected several times at 10-min intervals as a control phase. Blood was collected via a cannula inserted at the femoral artery at each midpoint of intestinal effluent collection. After the control-phase clearance study, CsA dissolved in 50% ethanol was injected intravenously at a dose of 10 mg/kg, or the intestinal perfusate was changed to the buffer containing verapamil (300 μM), to inhibit the intestinal P-gp function. The intestinal effluent and blood were further collected periodically as an inhibition phase after a perfusion for 30 min for stabilization. Intestinal exsorption clearance (CLexp, mL/min/20-cm long intestine) was estimated by dividing the exsorption rate from blood to the intestinal lumen (the product of drug concentration in the perfusate times perfusion rate) with Cpss of the drug. P-gp-mediated CLexp was estimated by subtracting CLexp in the inhibition phase from CLexp in the control phase.

In Vitro Transport of Digoxin and Rho123 across Everted Intestine

In vitro transport studies of digoxin and Rho123 across everted intestine (a 10-cm long sac prepared from the distal region of small intestine) were conducted as reported (7). Digoxin and Rho123 were dissolved at a concentration of 0.5 and 5 μM, respectively, in pH 7.4 isotonic D-PBS containing 25 mM glucose. The solution (1 mL) was introduced into the everted sac (serosal side), which was prewarmed to 37°C and preoxygenated with 5% CO_2/95% O_2 for 15 min, in 40 mL of pH 7.4 D-PBS. During bubbling with a CO_2/O_2 mixture gas, the transport of digoxin or Rho123 from serosal to mucosal surfaces across the intestine was measured by sampling the mucosal medium periodically. In some experiments, verapamil was added to the incubation medium (mucosal side) at a concentration of 300 μM to estimate the P-gp-mediated transport of these compounds.

Analysis

The concentrations of digoxin in blood, intestinal perfusate, or the incubation medium of the in-vitro transport study were analyzed by a fluorescence polarization immunoassay (FPIA, TDX^R, Dainabot Co., Ltd., Tokyo, Japan). The concentration of Rho123 was determined by HPLC equipped with a fluorometric detector as reported previously (7). Proteins were determined by the method of Bradford (22) using γ-globulin as the standard. Differences among group mean values were assessed by Student *t* test. A p value of less than 0.05 was considered statistically significant.

RESULTS

Western Blot Analysis for P-gp Levels in the Intestine

Representative immunoblots for P-gp in the crude intestinal epithelial membrane fractions collected from control and AHF rats are shown in Fig. 1. The averaged P-gp level in

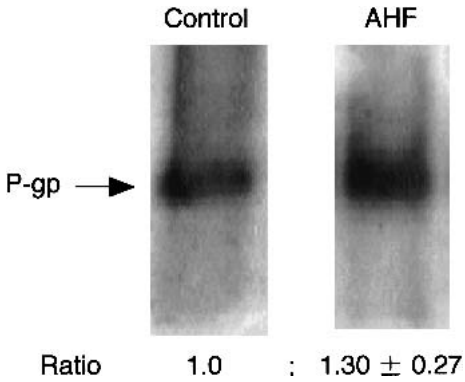

Fig. 1. Western blot analysis with a monoclonal antibody for P-gp (C219) of crude epithelial membrane fractions in the lower intestine obtained from control and acute hepatic failure (AHF) rats. AHF was induced by an intraperitoneal injection of CCl_4 dissolved in olive oil (50% v/v, 2.0 mL/kg), and control rats were injected with olive oil (2.0 mL/kg) alone. These rats were examined 24 h after injection. The ratio represents the relative staining intensity for P-gp (n = 5).

AHF rats was slightly, but not significantly, higher than that of control rats.

In Vivo Intestinal Absorption of Digoxin

The *in vivo* P-gp function in the intestine was evaluated by periodically measuring the disappearance rate of digoxin from the in-situ intestinal perfusate. The disappearance rate, or absorption rate, of digoxin in normal (untreated) rats increased greatly in the presence of the P-gp inhibitor verapamil, indicating that the intestinal absorption of digoxin is limited by P-gp (Fig. 2A). As compared with that of control (olive oil treated) rats, the absorption rate of digoxin in AHF rats was approximately 3.5-fold higher (Fig. 2B). These results suggest that the intestinal P-gp is functionally suppressed in AHF rats.

In Vivo Intestinal Exsorption of Digoxin and Rho123

The intestinal P-gp function in AHF rats was further evaluated by measuring in-vivo exsorption of P-gp substrates (digoxin and Rho123) from blood to the intestinal lumen under steady state. The exsorption clearance (CLexp) of digoxin in the absence of a P-gp inhibitor in AHF rats was significantly lower than that of the control rats (Fig. 3A). Intravenous injection of CsA, a potent P-gp inhibitor, further decreased the CLexp value of digoxin in AHF rats, as well as in control rats. The *in vivo* P-gp-mediated CLexp of digoxin in AHF rats, estimated by the difference in CLexp before and after CsA administration, was approximately half that of the control rats (Fig. 3B). Similarly, CLexp of Rho123 in the absence of a P-gp inhibitor was low in AHF rats, and the addition of verapamil, another P-gp inhibitor, further decreased CLexp, as it did in the control rats (Fig. 4A). The P-gp-mediated CLexp of Rho123 indicated that the higher dose of CCl_4 for the induction of AHF caused a greater suppression of intestinal P-gp function in vivo; 74% of control at 1.25 mL/kg and 32% of control at 2.0 mL/kg of 50% CCl_4 (Fig. 4B).

In Vitro Transport of Digoxin and Rho123 across Everted Intestine

The *in vitro* P-gp function in the intestine was evaluated by measuring the transport of P-gp substrates across everted

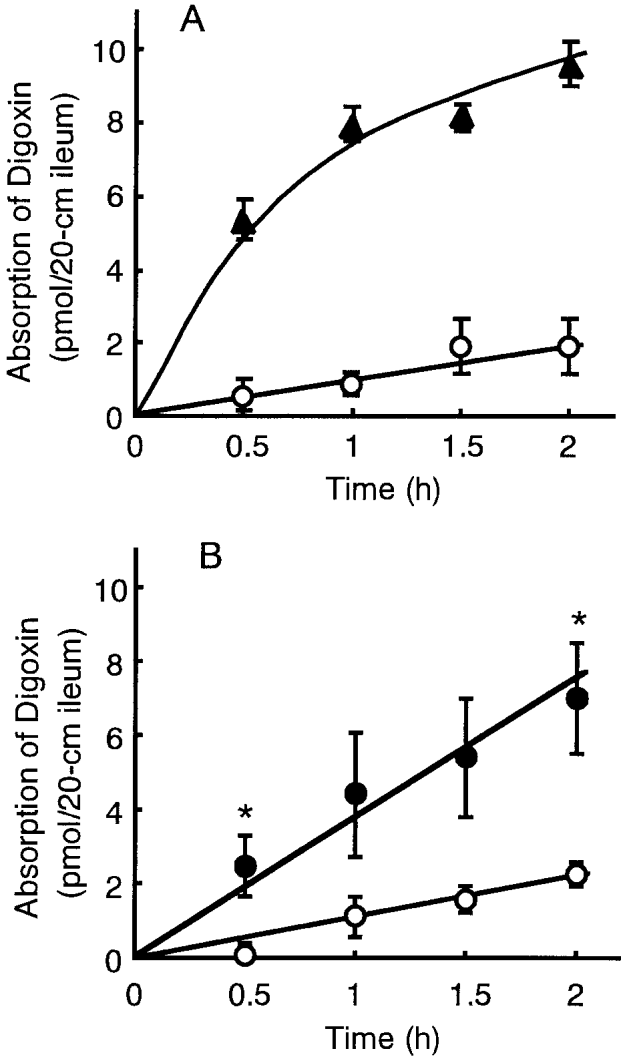

Fig. 2. *In vivo* absorption of digoxin from 20-cm long intestinal loop in the absence (○) and presence (▲) of verapamil (300 μM) in normal rats (A) and comparison of intestinal absorption of digoxin between control (olive oil-treated, ○) and AHF (●) rats (B). Acute hepatic failure was induced with an intraperitoneal injection of 50% CCl_4 (2.0 mL/kg). The initial concentration of digoxin in the perfusate (10 mL) was 3 nM, and a recirculating perfusion rate was 1 mL/min. The averaged initial absorption rates of digoxin, estimated by periodically measuring the amount remaining in the perfusate, were 1.0 pmol/h/20-cm long intestine in the absence of verapamil, 8.5 in the presence of verapamil in normal rats (A), 1.1 in control rats, and 3.7 in acute hepatic failure rats (B). Each point represents the mean ± SEM (n = 5). *$p < 0.05$ compared with control rats.

intestine. AHF rats showed a slightly higher efflux transport of digoxin in the absence of verapamil, although the transport rates in the presence of verapamil were almost comparable between control and AHF rats (Fig. 5A). As a result, the in-vitro P-gp-mediated CLexp of digoxin in AHF rats was approximately 1.7-fold that of control rats (Fig. 5B). Similarly, the *in vitro* P-gp-mediated CLexp of Rho123 was approximately 1.4-fold greater in AHF than in control rats (Fig. 6A and B). Thus, under the *in vitro* condition, the intestinal P-gp function of AHF rats was increased rather than suppressed, in contrast to the *in vivo* condition.

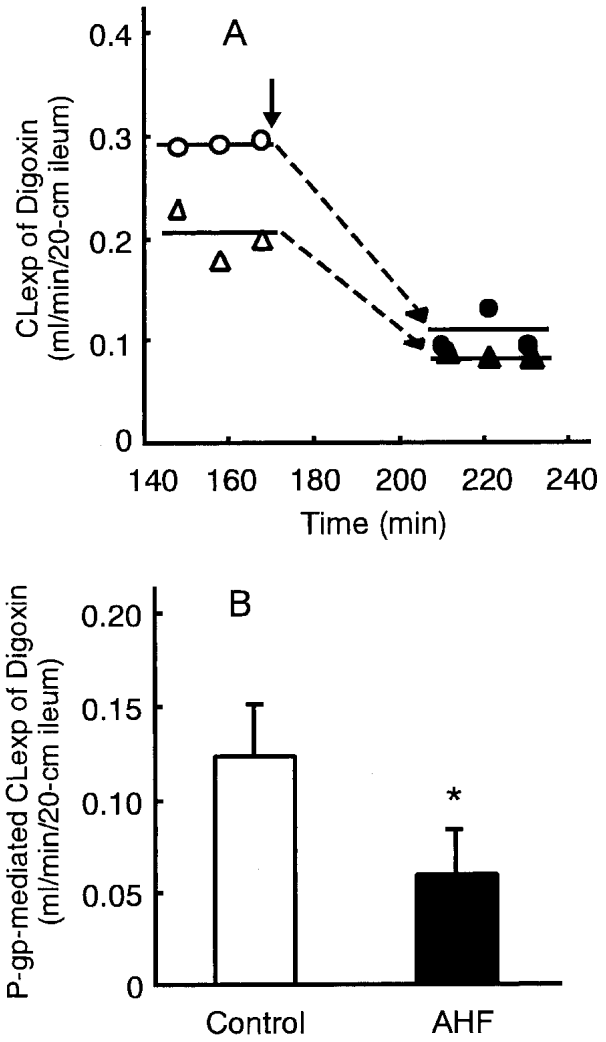

Fig. 3. *In vivo* efflux transport of digoxin from blood to a 20 cm-long intestinal lumen before (○, △) and after (●, ▲) administration of cyclosporin A (CsA) under a steady-state plasma concentration in control (○, ●) and AHF (△, ▲) rats (A; a typical result) and estimated *in vivo* P-gp-mediated exsorption clearance (Clexp) of digoxin in control and AHF rats (B). AHF was induced with intraperitoneal injection of 50% CCl_4 (2.0 mL/kg). CsA was administered intravenously at a dose of 10 mg/kg after the control-phase study (175 min after the initiation of constant rate infusion of digoxin; arrow). P-gp-mediated CLexp of digoxin denotes the difference in CLexp values in the absence or presence of CsA. Each value in (B) represents the mean ± SEM (n = 3). *$p < 0.05$ compared with control rats.

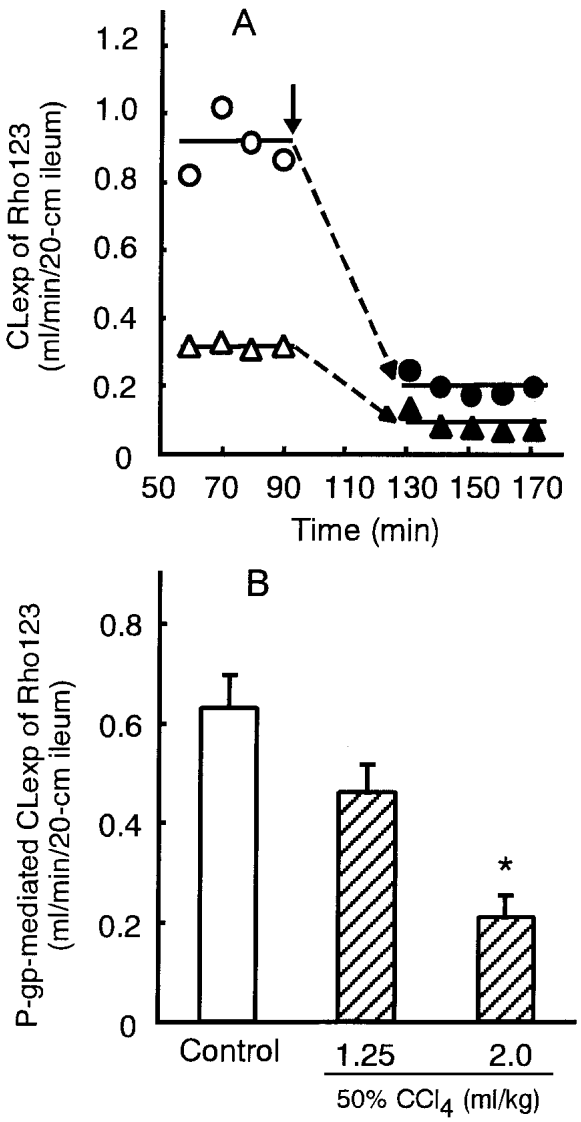

Fig. 4. *In vivo* efflux transport of Rho123 from blood to a 20 cm-long intestinal loop in the absence (○, △) and presence (●, ▲) of verapamil (300 μM) in the perfusate in control (○, ●) and AHF (△, ▲) rats (A; a typical result), and estimated *in vivo* P-gp-mediated CLexp of Rho123 in control and AHF rats (B). AHF was induced with an intraperitoneal injection of 50% CCl_4 (2.0 mL/kg for (A), 1.25 or 2.0 mL/kg for (B)). The intestinal perfusate was changed to a buffer containing verapamil after control-phase study (95 min after the initiation of constant rate infusion of Rho123; arrow). P-gp-mediated CLexp of Rho123 denotes the difference in CLexp values in the absence or presence of verapamil. Each value in (B) represents the mean ± SEM (n = 4–5). *$p < 0.05$ compared with control rats.

DISCUSSION

In the present study, we evaluated the effect of CCl_4-induced AHF on intestinal P-gp expression and function *in vivo* and *in vitro*. As described in the introduction section, some articles have reported the modulation of intestinal membrane permeability to P-gp-related compounds in renal failure rats (10,12). However, the effect of AHF on the intestinal absorption of P-gp-related compounds has not yet been reported.

To evaluate the intestinal P-gp expression and function in AHF rats, the distal region of small intestine was used. This region has a higher P-gp mRNA expression and a higher P-gp-mediated Rho123 efflux transport than other intestinal regions (7). Digoxin and Rho123 were used as substrates of P-gp (11,20). Digoxin is a drug with a narrow therapeutic index and used for treatment of supraventricular arrhythmias and cardiac failure. Other P-gp-related drugs administered concomitantly increase the plasma level of digoxin given orally (23). Greiner *et al.* (9) reported that P-gp expression levels in enterocytes of duodenal biopsies inversely correlated with plasma AUC of oral digoxin in healthy volunteers treated with or without rifampicin, a strong P-gp inducer, and reported that intestinal P-gp is a determinant of the disposition of digoxin. On the other hand, digoxin is also known as

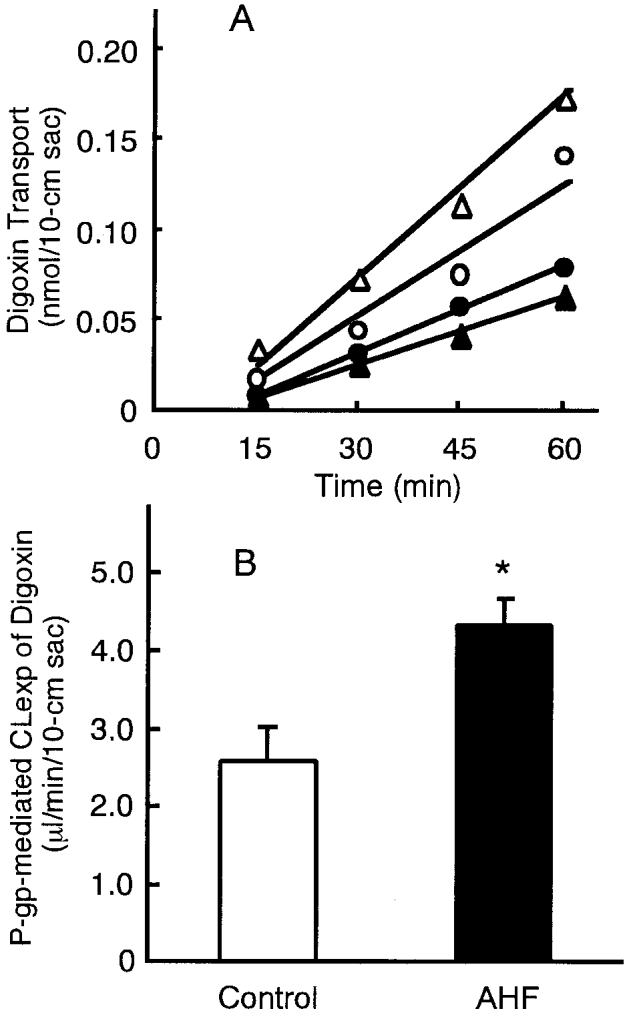

Fig. 5. *In vitro* transport of digoxin from serosal to mucosal surfaces across the everted intestine (a 10-cm long sac) of control (○, ●) and AHF (△, ▲) rats in the absence (○, △) and presence (●, ▲) of 300 μM verapamil (A; a typical result), and estimated *in vitro* P-gp-mediated CLexp of digoxin (B) for control and AHF (50% CCl_4 2.0 mL/kg) rats. *In vitro* P-gp-mediated CLexp of digoxin denotes the difference inCLexp values in the absence or presence of verapamil. Each value in (B) represents the mean ± SEM (n = 3). *p < 0.05 compared with control rats.

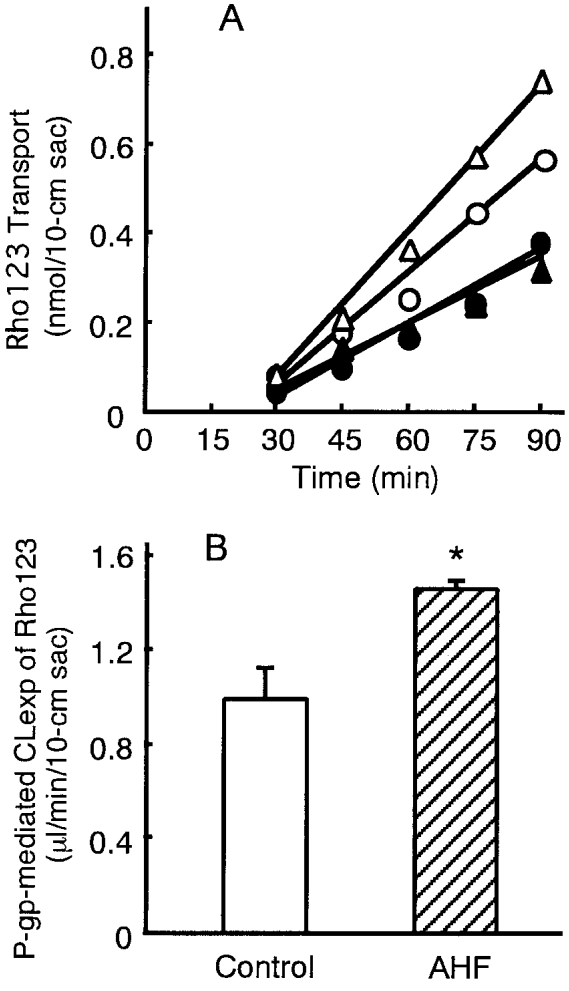

Fig. 6. *In vitro* transport of Rho123 from serosal to mucosal surfaces across the everted intestine (a10-cm long sac) of control (○, ●) and AHF (△, ▲) rats in the absence (○, △) and presence (●, ▲) of 300 μM verapamil (A; a typical result), and estimated *in vitro* P-gp-mediated CLexp of Rho123 (B) for control and AHF (50% CCl_4 2.0 mL/kg) rats. *In vitro* P-gp-mediated CLexp of Rho123 denotes the difference in CLexp values in the absence or presence of verapamil. Each value in (B) represents the mean ± S.E.M.(n = 3–4). *p < 0.05 compared with control rats.

a substrate of rat organic anion transporting polypeptide (oatp) 2 (24), and the transport into the liver and brain is mediated by this transporter (25,26). However, oatp 2 would not be involved in the intestinal transport of digoxin, because reverse transcription polymerase chain reaction analysis revealed that oatp 2 is not expressed in the small intestine (27).

In the CCl_4-induced AHF state, P-gp expression in the intestinal epithelial cells did not decrease. It increased slightly (1.3 times control on average, though it did not reach significance; Fig. 1). With respect to the effect of CCl_4 -intoxication on P-gp levels, it has been reported that levels of mdr1a and mdr1b mRNA were increased in rat liver 3 h after administration of CCl_4 and remained increased for the following 5 days (28). We also observed an approximately 1.5-fold increase in P-gp level in the liver and an approximately 1.3-fold increase in the brain of CCl_4-induced AHF rats (13). A similar tendency in P-gp expression has been observed in glycerol-induced acute renal failure (ARF) rats (14). The increase in P-gp levels, especially in the target injury organ, may be a compensation for the decreased P-gp function in diseased states.

The involvement of P-gp as the intestinal absorption barrier for digoxin was confirmed by examining the effect of verapamil, a P-gp inhibitor, in normal rats, in which the initial absorption rate of digoxin increased remarkably in the presence of verapamil (Fig. 2A). In AHF rats, the *in vivo* intestinal absorption rate of digoxin was higher (Fig. 2B) and the P-gp-mediated intestinal CLexp of digoxin was lower (Fig. 3) as compared to control rats. The *in vivo* exsorption study for Rho123 showed similar results (Fig. 4). The suppression of *in vivo* intestinal P-gp function in AHF rats is in good agreement with our previous findings, in which *in vivo* P-gp functions in the kidney and brain as well as the liver were significantly suppressed (13,15). In contrast, the in-vitro intestinal P-gp function in AHF rats was significantly greater than that in

control rats (Figs. 5 and 6), a result that differed from the in-vivo studies. The discrepancy of intestinal P-gp function between *in vitro* and *in vivo* conditions in AHF rats may be derived from the presence of biologic fluids under *in vivo* conditions. Biologic fluids contain various endogenous P-gp-related compounds, such as cortisol, corticosterone, progesterone, aldosterone, estradiol, and their metabolites as well as unidentified compounds (29,30). Their concentrations and/or compositions are thought to be altered by different physiologic states (30). We previously found that plasma collected from AHF and glycerol-induced ARF rats exhibits a greater inhibitory potency on P-gp-mediated transport of Rho123 across Caco-2 cell monolayers than that from control rats (13,15). Also, plasma concentrations of corticosterone, a potent endogenous P-gp inhibitor, were higher in these diseased rats, as compared to control rats. These findings may support the idea that endogenous P-gp modulators are involved in the suppression of intestinal P-gp function *in vivo* in these diseased states. However, further studies are needed to identify the components of endogenous P-gp inhibitors and clarify their contributions to the suppression of *in vivo* P-gp function. It is also necessary to evaluate the involvement of other factors that would affect P-gp-mediated transport, such as intestinal blood flow.

Many P-gp-related drugs including digoxin, immunosuppressants, steroid hormones, quinidine, calcium channel blockers, and ß-blockers are administered orally in clinical use, and their intestinal absorption and intestinal exsorption clearance are limited or mediated by P-gp, though the contribution of P-gp differs among drugs (1,2). Based on our previous and present studies, the *in vivo* P-gp functions in the liver, kidney, brain and intestine were found to be suppressed in the AHF state, irrespective of the unchanged or even increased P-gp levels in these organs (13). Such a systemic suppression of P-gp function in AHF would affect not only the pharmacokinetics but also the pharmacodynamics of P-gp-related drugs. Clinically, about 10 to 30% of patients who received immunosuppressants, CsA and tacrolimus, experience some form of adverse neurotoxic events. Factors that may promote the development of CsA- and tacrolimus-induced neurotoxicity are reportedly hepatic failure, elevated blood levels of these drugs, hypocholesterolemia, and so on, though the relative risk of these factors and the underlying mechanisms are not yet understood (31). Our findings would suggest that blood levels of P-gp substrates increase in hepatic failure because of the increase in intestinal absorption and decrease in intestinal exsorption due to the suppression of P-gp function in the intestine, in addition to the possible alteration of metabolic activity in the liver. Also, the distribution of these P-gp substrates into the brain may further increase in AHF because of the suppression of *in vivo* P-gp function in the brain. The clinical significance of the findings obtained from AHF rats needs to be further clarified.

In conclusion, intestinal P-gp functions under *in vivo* and *in vitro* conditions were differently affected by AHF; *in vivo* P-gp function was lower and *in vitro* P-gp function was higher in AHF rats as compared with control rats. The apparent discrepancy between *in vivo* and *in vitro* studies may be caused by the presence of endogenous P-gp inhibitors in the plasma of AHF rats in the *in vivo* condition. These results also suggest that *in vivo* P-gp function in the diseased state can not be predicted merely from the expression of protein and/or mRNA of P-gp, and the pharmacokinetics and pharmacodynamics of certain P-gp-related drugs may be modulated in AHF states, partly because of the increase in oral absorption and decrease in intestinal exsorption clearance.

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