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Research Paper

Effect of intestinal ischaemia/reperfusion on P-glycoprotein-mediated ileal excretion of rhodamine 123 in the rat

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Abstract

Objectives We have shown that ischaemia/reperfusion in the small intestine at an early phase, such as 1 h after reperfusion, induced not only functional changes in the membrane, such as P-glycoprotein (P-gp) dysfunction, but also decreased expression of P-gp protein and mdr1a mRNA. In the present study we examined whether intestinal ischaemia/reperfusion modifies the P-gp-mediated ileal excretion transport system in rats beyond 1 h after reperfusion.

Methods To evaluate the contribution of P-gp-mediated transport to the ileal excretion of rhodamine 123, we used Western blotting to measure the expression of P-gp protein levels isolated from the ileum at different reperfusion times after 60 min of ischaemia. We also measured the expression of inducible nitric oxide synthase (iNOS) mRNA using real-time RT-PCR.

Key findings Ileal excretion of rhodamine 123 decreased at 3 h after reperfusion and had recovered at 24 h. Changes in villi structure at 3 h and its recovery at 24 h were also observed. Verapamil, a competitive inhibitor of P-gp, significantly inhibited ileal clearance of rhodamine 123 to the lumen at 24 h after reperfusion, suggesting that P-gp was working at this time. These results suggest that intestinal ischaemia/reperfusion-induced decrease in P-gp-mediated ileal excretion of rhodamine 123 was probably due to impaired P-gp-mediated transport. Levels of P-gp protein and iNOS mRNA in the ileum decreased 3 h after ischaemia/reperfusion and returned to control levels after 24 h.

Conclusions These findings suggest that intestinal ischaemia/reperfusion markedly decreases P-gp-mediated ileal excretion of rhodamine 123, probably by decreasing the expression of P-gp protein, which is likely to be due to increased lipid peroxidation via iNOS.

Keywords ileal clearance; iNOS; intestinal ischaemia/reperfusion; P-glycoprotein-mediated transport

Introduction

Intestinal ischaemia injury is an important clinical problem in several disorders,[1] leading to depletion of cellular energy[2] and accumulation of toxic metabolites, resulting in cell damage and death. Reperfusion exacerbates ischaemia-induced mucosal injury via the synthesis of reactive oxygen species,[3] which is connected to neutrophil infiltration and release of inflammatory mediators.[4] Intestinal mucosal lesions after ischaemia and reperfusion (I/R) injury include: loss of activity in brush-border enzymes,[5] cellular death (necrosis and apoptosis)[6] and increased intestinal permeability.[7] I/R injury of the intestine and other organs has also been related to decreased drug metabolism.[8]

The importance of intestinal drug absorption, transport and metabolism for drug bioavailability after oral administration has been documented for many compounds.[9] Changes in drug bioavailability due to pathological conditions, mainly inflammatory reactions, are responsible for some adverse events of drugs.[10]

Although the development of intestinal mucosal injury after I/R has been studied intensively, most studies have focused on the relatively late phase. We have already established an in-vivo system for assessing early I/R injury using rat intestine, in which

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lipid peroxidation caused by hanging arteries and veins acts as an injury trigger.[10] Using this model, we showed that reperfusion 60 min after ischaemia for 60 min induced the dysfunction of intestinal P-glycoprotein (P-gp).[11] In particular, we have quantified P-gp dysfunction using rhodamine 123 as a substrate and verapamil as an inhibitor.

The aim of the present study was to clarify in detail whether intestinal I/R modifies the P-gp-mediated ileal excretion transport system in rats over a long period. Rhodamine 123 was chosen as the model drug since this compound is primarily excreted into the bile and urine unchanged.[12] In order to evaluate the contribution of P-gp-mediated transport to the ileal excretion of rhodamine 123, we used Western blotting to measure the expression of P-gp protein isolated from the ileum at different reperfusion times after ischaemia for 60 min. We also measured the expression of inducible nitric oxide (NO) synthase (iNOS) mRNA, which is a trigger of oxidative stress, using real-time RT-PCR.

Materials and Methods

Materials

Rhodamine 123 and verapamil were purchased from Sigma (St Louis, MO, USA). All other reagents were of analytical grade or better.

Animals and experimental design

Male Wistar/ST rats (8 weeks old) were purchased from Japan SLC Ltd (Shizuoka, Japan). All animal experiments were performed according to the guidelines of Tokyo University of Pharmacy and Life Sciences. The animals were fasted for 18 h before starting the experiment. Water was available ad libitum while fasting. Rats were anaesthetised with pentobarbital sodium (50 mg/kg) and the right jugular vein cannulated with polyethylene tubing for rhodamine 123 administration and blood collection. All experiments were performed under pentobarbital sodium anaesthesia. Body temperature was maintained at 37°C with a heat lamp. The superior mesenteric artery and vein were occluded by hanging using surgical sutures (Natsume No.3) connected to a spring balance for 60 min (ischaemia condition), as described previously,[11] after which the sutures were cut to allow reperfusion. Based on our previous paper,[11] the hanging forces of blood vessels of 50 g and 100 g were used during the ischaemia. The control (sham-operated) group was subjected to the same surgical intervention but without occlusion of the superior mesenteric artery and vein.

For rhodamine 123 clearance experiments, untreated control rats and rats subjected to ischaemia for 1 h with 50 g and 100 g loads were reperfused. Ileal excretion of rhodamine 123 was investigated at different times after reperfusion.

Blood samples (300 μl) were taken before and 1.5, 3, 5, 10, 20, 30 and 60 min after administration and centrifuged for 5 min at 12 000 rpm. Samples of the supernatants (100 μl) were diluted with purified water in order to make an adequate volume for assay.

Excretion to ileal perfusate from blood

A cannula (silicone tubing, Kaneka Medix Co. Osaka, Japan) was placed in the jugular vein for drug administration and blood sampling. The lumen of the ileum (7 cm length) was flushed with saline prewarmed to 37°C, and the proximal end of the lumen catheterised with an in-flow glass cannula, which was connected to the perfusion system. The distal end of the ileum was catheterised with an out-flow glass cannula to collect intestinal effluent serially. Single-pass perfusion of Krebs–Heinslett bicarbonate buffer into the ileal lumen was started at a rate of 1 ml/min. Rhodamine 123 dissolved in saline was injected into the jugular vein cannula as a 2.8 mg/kg bolus, followed by the same volume of saline. Samples of intestinal effluent were collected for 10-min periods from 0 to 60 min. Excretion of rhodamine 123 from blood to the ileal lumen was expressed as total amount excreted in 60 min. The ileal luminal excretion clearance (CL_{lumen}) was calculated by dividing the excretion rate by the plasma concentration in the middle of the collection period. The effects of intestinal I/R on ileal excretion of rhodamine 123 were determined 3 h and 24 h after reperfusion. The pharmacokinetic parameters at these time points were calculated from the data from 3 to 4 h and from 24 to 25 h after reperfusion, respectively.

Evaluation of P-glycoprotein mediated transport

To elucidate the effects of verapamil (0.06 mg/kg) on the ileal excretion of rhodamine 123 (0.01 mg/kg), verapamil and rhodamine 123 were administered intravenously at the same time. Blood and ileal perfusate were sampled as described above.

Estimation of relative P-glycoprotein activity

The relative activities of P-gp were obtained from $(P_{app} - P_{pass}) / P_{pass}$, where P_{pass} and P_{app} are the ileal excretion clearance of rhodamine 123 in the presence and absence of an inhibitor, respectively,[11] which in this study was verapamil.

Western blotting

An in-situ ileal loop (60 cm length) was isolated in each rat at different times (0, 1, 3, 6, 12 and 24 h) after reperfusion, and brush-border membrane fractions were prepared from the ileal loop using magnesium chloride precipitation.[11–13] Protein concentration was determined using a Micro BCA protein assay reagent kit. The levels of P-gp protein in the brush-border membrane fraction were evaluated by Western blotting using C219 monoclonal antibody (Alexis, San Diego, USA) for P-gp, as reported previously.[14]

Haematoxylin and eosin staining

Intestinal biopsies were fixed in 4% formalin (Wako Pure Chemical Industries, Ltd, Tokyo, Japan) in neutral buffer for 16 h and then embedded in paraffin. Deparaffinised sections (7 μm thick) were stained with haematoxylin and eosin (Wako Pure Chemical Industries) and analysed by light microscopy.

Preparation of RNA and cDNA synthesis

Total RNA was isolated from ileal specimens using Trizol reagents (Invitrogen, Paisley, UK) according to the

manufacturer's instructions. Complementary DNA (cDNA) was prepared from total RNA using GeneAmp9600 (Applied Biosystems, Foster City, CA, USA) for real-time PCR according to the manufacturer's instructions. The two-step reaction mixture contained 2 μg RNA, 100 ng random hexamers, 0.5 mmol/l dNTP mix (dATP, dCTP, dGTP, dTTP), 10 mmol/l Tris-HCl (pH 8.4), 25 mmol/l KCl, 5 mmol/l $MgCl_2$, 10 mmol/l DTT and 10 units RNaseOUT recombinant ribonuclease inhibitor.

Analysis of intestinal iNOS gene expression

To perform real-time RT-PCR, 96-well reaction plates with optical adhesive covers and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used. Assay On-demand gene expression products were purchased for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and iNOS (Table 1) (Sigma-Aldrich, Tokyo, Japan). Reverse transcription was performed for 1 μg RNA using a cDNA high-capacity archive kit (Applied Biosystems) and random hexamers as primers. Quantitative PCR was performed on an SDS 7000 system using a Universal MasterMix (Applied Biosystems). The PCR conditions were 10 min at 90°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. All assays were pre-designed RNA-specific (spanning exon–exon junctions) TaqMan gene expression assays from Applied Biosystems (Table 1).

Statistical analysis

All results are expressed as the mean ± SE. Statistical significances between groups were analysed using Tukey's test; $P < 0.05$ was considered significant.

Results

Effects of ischaemia/reperfusion on ileal excretion of rhodamine 123 to lumen from blood

The ileal excretion clearance (CL_{lumen}) of rhodamine 123 was decreased 3 h after reperfusion compared with the control group, and recovered to the control level at 24 h (Figure 1). The decrease in rhodamine 123 excretion in the ileum 3 h after reperfusion did not depend on the load during ischaemia (Figure 1).

Effects of ischaemia/reperfusion on P-glycoprotein expression in ileal epithelial cells

P-gp expression in the ileal brush-border membrane fraction 1 was significantly lower than the control value 3 h after reperfusion and depended on the load during ischaemia. The

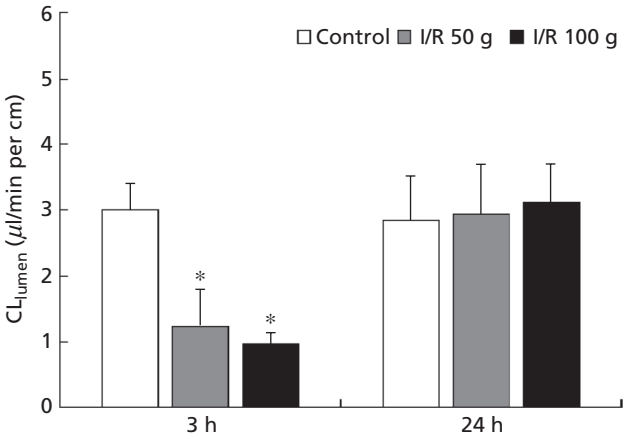


Figure 1 Ileal excretion of rhodamine 123 after ischaemia/reperfusion. Effects of ischaemia/reperfusion (I/R) on ileal luminal excretion clearance (CL_{lumen}) of rhodamine 123 3 h and 24 h after reperfusion. Values are means ± SE (n = 6–9). *$P < 0.05$ vs control group.

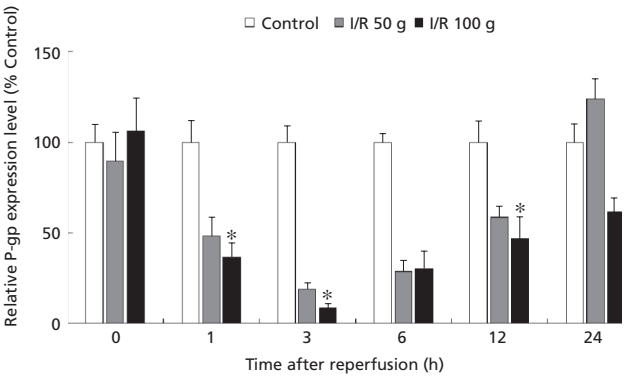


Figure 2 P-glycoprotein expression in ileal epithelilal cells after ischaemia/reperfusion. Changes in the level of P-glycoprotein (P-gp) protein in the ileal epithelial brush-border membrane fraction caused by ischaemia/reperfusion (I/R). The expression level was normalized by the total protein level. Data are means ± SE (n = 3–5). *$P < 0.05$ vs control group.

level in the 50 g load group returned to the control level 24 h after reperfusion (Figure 2) whereas recovery in the 100 g load group was incomplete (Figure 2).

Histological changes of mucosal epithelium

As shown in Figures 3a–c, 3 h after reperfusion, the ileal shape in the 50 g load group was severely distorted, and the villi in the 100 g load group were shortened compared with

Table 1 Sequences of primers used for real-time RT-PCR

Gene	Sequence (5′–3′)	Amplicon size (bp)	GeneBank ID
GAPDH	Forward TGAGGTGACCGCATCTTCTTG Reverse TGGTAACCAGGCGTCCGATA	102	NM-017008
iNOS	Forward CTGGGTGAAAGCGGTGTTCT Reverse CCGACTTCCTTGTCTCAGTAGCA	101	S-71597

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase.

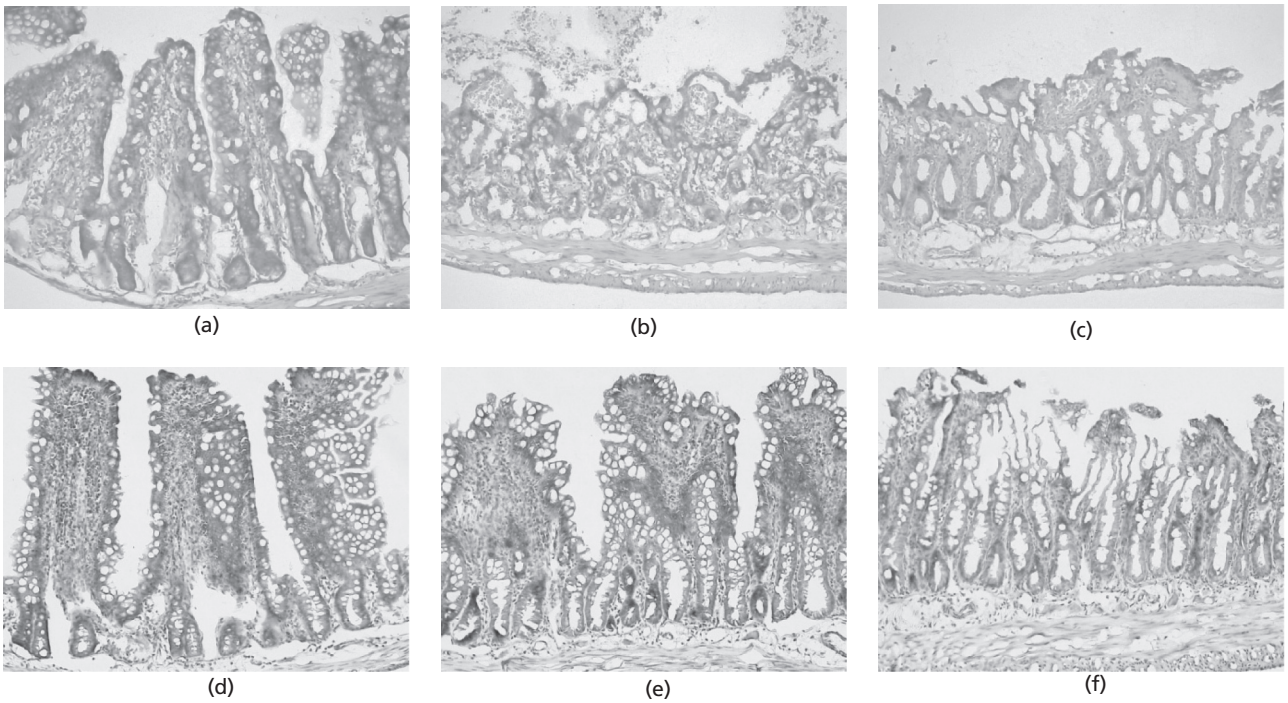


Figure 3 Histological changes of mucosal epithelium induced by intestinal ischaemia/reperfusion. Micrographs a–c were obtained 3 h after reperfusion and d–f 24 h after reperfusion; a and d are from the control (sham-operated) group; b and e from the 50 g load group; c and f from the 100 g load group. Frozen sections were stained with haematoxylin and eosin. Magnification ×400.

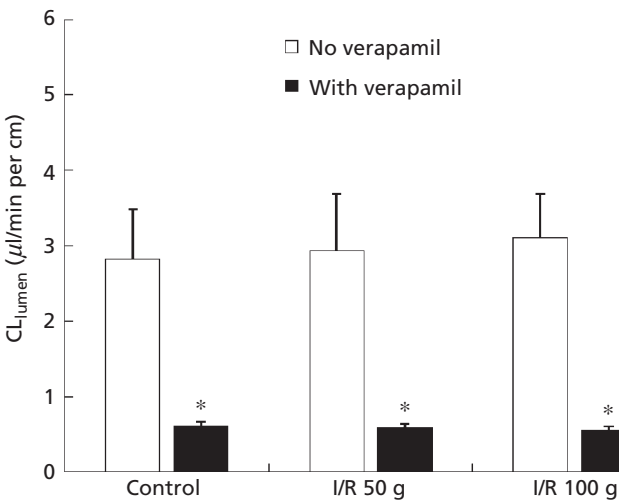
the control group. The sharpness and height of villi in the 50 g load group recovered to control levels 24 h after reperfusion (Figure 3d–f) and the 100 g load group showed a tendency towards recovery.

Recovery of ileal P-glycoprotein-mediated transport using verapamil

Ileal P-gp-mediated rhodamine 123 transport was examined using verapamil and evaluated by the recovery of P-gp function 24 h after reperfusion. No marked change in CL_{lumen} of rhodamine 123 was observed in the 50 g and 100 g load groups compared with the control conditions (Figure 4). Verapamil significantly inhibited CL_{lumen} of rhodamine 123 in both groups in the same manner as the control group (Figure 4). These results corresponded well with the relative activity of P-gp (Figure 4), indicating that ileal P-gp-mediated excretion of rhodamine 123 returned to control levels 24 h after reperfusion.

Expression of iNOS and nNOS in ileal tissues

Levels of iNOS and neuronal NOS (nNOS) mRNA were measured at different times after reperfusion. The time course in the expression of iNOS and nNOS as a mediator after intestinal I/R is shown in Figure 5. iNOS mRNA in the ileum had increased in a load-dependent manner by 3 h after reperfusion and returned to the control level 24 h after reperfusion (Figure 5). nNOS mRNA was not expressed 3 or 24 h after reperfusion (data not shown).



Relative activity of ileal P-gp	
Control	3.80
I/R 50 g	4.06
I/R 100 g	4.79

Figure 4 Effects of verapamil on ileal excretion of rhodamine 123 after ischaemia/reperfusion. Inhibitory effects of verapamil on ileal luminal excretion clearance (CL_{lumen}) of rhodamine 123 in the intestinal ischaemia/reperfusion (I/R) groups 24 h after reperfusion. Data are means ± SE (n = 5–9). *P < 0.05 vs absence of verapamil.

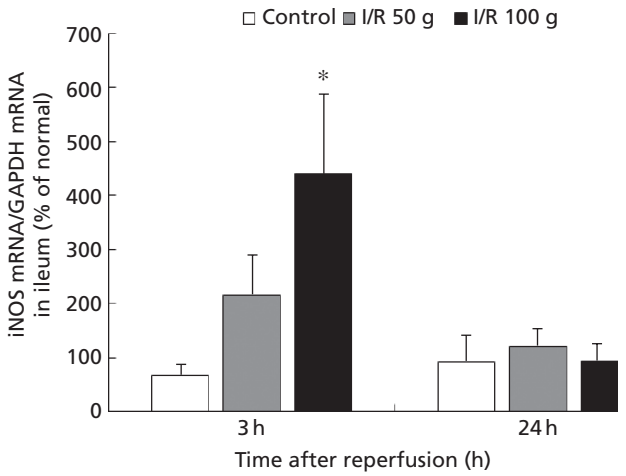


Figure 5 Expression of inducible nitric oxide synthase in ileum after ischaemia/reperfusion. Levels of inducible nitric oxide synthase (iNOS) mRNA in ileum tissues 3 h and 24 h after reperfusion. Data are means ± SE (n = 6). *P < 0.05 vs control group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; I/R, ischaemia/reperfusion.

Discussion

We have previously reported a change in ileal P-gp function 1 h after reperfusion.[11] To investigate whether the active luminal excretion of rhodamine 123 is mediated mainly by P-gp, the effects of a well-known P-gp substrate, verapamil, were investigated in control conditions.[11] Verapamil had a marked inhibitory effect on both absorption and excretion under control conditions, but not 1 h after reperfusion, indicating that intestinal I/R induces the dysfunction of ileal P-gp.[11] In the present study, ileal excretion clearance of rhodamine 123 decreased 3 h after reperfusion and recovered to the control level 24 h after reperfusion.

We have already reported a significant decrease in P-gp expression after I/R depending on the extent of load during ischaemia *in vitro* using Caco-2 cell monolayers[15] and in an in-vivo rat model.[11] Furthermore, the extent of histological changes in intestinal villi due to intestinal I/R (Figure 3) corresponded well with the change in P-gp function and P-gp protein level shown in Figures 1 and 2.

In the control (sham-operated) rats, CL_{lumen} of rhodamine 123 at 24 h after reperfusion decreased significantly in the presence of verapamil compared with its absence (Figure 4), indicating that P-gp was expressed and functional at this time point (Figures 2 and 4). In the intestinal I/R groups, verapamil also significantly inhibited CL_{lumen} of rhodamine 123 24 h after reperfusion, indicating that the function of P-gp which mediated ileal CL_{lumen} of rhodamine 123 24 h after reperfusion returned to control levels (Figure 4).

The relative role of P-gp in permeability can be evaluated from absorptive and secretory quotients,[16] which are presented along with the mucosal to serosal and serosal to mucosal flux ratios in our previous paper.[11] For this in-vivo study using verapamil and rhodamine 123, a decrease in the relative P-gp activity caused by I/R could also be demonstrated quantitatively.[11] As shown in Figure 4, no marked difference in relative activity at 24 h after reperfusion was observed in either the 50 g or 100 g load groups compared with the control group, indicating that P-gp was functional in ileum epithelial cells 24 h after reperfusion.

Previously, we examined the permeability properties of our in-vitro I/R model of a human intestinal cell line, Caco-2, with several compounds, such as P-gp substrates verapamil, ciclosporin and rhodamine 123, and the decreased permeability of rhodamine 123 mediated by P-gp was dependent on lipid peroxidation.[15] We have also shown that the decreased P-gp function observed in previous in-vitro and in-vivo studies corresponded with the production of thiobarbituric acid reactive substance as an indicator of lipid peroxidation.[11,15] It is well known that the increase in lipid peroxidation is caused by iNOS, including superoxide anions, hydrogen peroxide, hydroxyl radical etc., and that iNOS might play an important role in changes in the transporter-mediated ileal excretion system induced by intestinal I/R. Ogura *et al.* reported that the increases in interleukin 6 but not tumour necrosis factor α induced decreases in the level of mdr1a mRNA in the ileum but not in the jejunum;[17] however, the roles of these cytokines and mediators such as NO and platelet-activating factor in the physiological function of drug transporters remain unclear. We therefore focused on the role of iNOS as a representative participating in NO synthesis that induced decreases in the CL_{lumen} of rhodamine 123. The level of iNOS in the ileum increased 3 h after reperfusion and recovered to the control level after 24 h (Figure 5). This suggests that iNOS is involved in the decreased P-gp-mediated luminary transport of rhodamine 123.

The effects of intestinal I/R on the expression of mRNA of other ABC transporters located in the ileum remain unclear, although it has been reported that intestinal I/R might induce the down-regulation of mdr1a mRNA in the ileum, but not that of mrp2, mrp3 or mdr1b 6 h after reperfusion, and that of the protein level of both P-gp and mrp2 in the jejunum and ileum, respectively.[17] Therefore, we attempted to investigate in detail the effect of intestinal I/R on the expression of P-gp protein in the ileum up to 24 h (Figure 2). Western blot analysis showed that the expression of P-gp protein decreased up to 3 h after reperfusion and thereafter increased stepwise up to 24 h (Figure 2). These observations correspond well with the recovery pattern in CL_{lumen} of rhodamine 123 3 h and 24 h after reperfusion (Figure 1). We also showed that the increase in iNOS mRNA (Figure 5) induced the decrease in CL_{lumen} of rhodamine 123 (Figure 1) and the decrease in level of P-gp protein (Figure 2) in the ileum.

It also has been suggested that are several endogenous modulators of P-gp in human plasma.[18] It is therefore possible that intestinal I/R-induced decreases in P-gp-mediated ileal excretion of rhodamine 123 were related to the production of endogenous P-gp substrates in the rats' plasma by intestinal I/R.[18]

Conclusions

The present study was the first to use in-vivo clearance experiments and Western blot analysis to show that intestinal I/R decreases P-gp-mediated luminal excretion of rhodamine

123 by reducing the level of P-gp protein. The results suggest that iNOS is at least one component associated with intestinal I/R-induced decrease in the luminal excretion of rhodamine 123. Further studies are needed to clarify the role of other factors in reducing the ileal excretion of rhodamine 123 and the P-gp protein level in intestinal I/R.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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