

Changes in Function and Expression Level of Multidrug Resistance-Associated Protein during Intestinal Ischemia/Reperfusion

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Abstract: Ischemic bowel disease is induced by poor circulation of blood, and thus oxygen and nutrition necessary for the small intestine are not supplied enough. As a result, such condition causes inflammation and ulcers, such as ischemic colitis and acute mesenteric arterial occlusion. We previously reported changes in function and expression level of P-glycoprotein (P-gp) which is a typical and important ABC transporter in the small intestine, liver and kidney during intestinal ischemia/reperfusion (I/R). In this study, we examined I/R-induced changes in multidrug resistance-associated proteins (Mrps) which is also an important ABC transporter like P-gp. The superior mesenteric vein and artery in rat were occluded by hanging itself using surgical-sutures connected with the spring balance for 60 min, followed by a reperfusion. The excretion clearances to jejunal lumen (CL_{jejunum}), bile (CL_{bile}) and urine (CL_{urine}) of 5(6)-carboxyfluorescein (5-CF) diacetate (5-CFDA), a Mrp substrate, were determined after i.v. administration of 5-CFDA. We also investigated changes in the protein and mRNA expression levels of Mrp2 during intestinal I/R. It was shown that the expression behavior of Mrp2 is different depending on each organ during intestinal I/R. For the function of Mrp, during early term of reperfusion, the total clearance (CL_{tot}) of 5-CFDA (5-CF) was significantly decreased by intestinal ischemia compared with the control condition. However, the behavior of excretion clearance was differ depending on each organ. In longer term of reperfusion, CL_{tot} of 5-CFDA (5-CF) of intestinal I/R condition was converged to the control level until 24 hr after reperfusion. Intestinal I/R change the function and expression level of intestinal Mrp2 as well as hepatic and renal Mrp2. Therefore, when performing drug therapy to the ischemic bowel disease, the caution is necessary for the dosing of Mrp2 substrates.

Keywords: Intestinal ischemia/reperfusion, multidrug resistance-associated proteins, ABC transporter, 5(6)-carboxyfluorescein diacetate, jejunum.

INTRODUCTION

Ischemic intestinal injury is an important clinical problem in several disorders [1]. Intestinal ischemia leads to depletion of cellular energy [2] and accumulation of toxic metabolites, resulting in cell damage and death. Reperfusion exacerbates ischemia-induced mucosal injury via the synthesis of reactive oxygen species (ROS) [3], which is connected to neutrophil infiltration and the release of inflammatory reaction mediators [4]. Intestinal mucosal lesions after ischemia/reperfusion (I/R) injury include: loss of activity in brush border enzyme [5], cellular death (necrosis and apoptosis) [6] and increase of intestinal permeability [7]. I/R injury of the intestine and other organs have also been related to a decrease in drug metabolism activity [8]. As a result of such dysfunction,

it can be connected multi-organ failure (MOF) and induces the opportunity of death [9].

Multidrug resistance-associated protein families (Mrps) and P-glycoprotein (P-gp) belong to the ATP-binding cassette (ABC) transporter superfamily. These transporters are expressed in almost all tissues, including brain, small intestine, large intestine, liver and kidney, and contribute to efflux a lot of endogenous compound and drugs. Especially, MRPs transport many conjugated drugs such as glucuronides and sulfates. Therefore, the increase or decrease of these transporters may lead to alter the absorption, disposition and excretion of a number of clinically important drugs.

Because small intestine, liver and kidney excrete many substrate included drugs, dysfunction of these organs (tissues) by injury such as ischemic injury result in alteration of drug disposition. Indeed it has been reported that disposition of some drugs is changed after intestinal I/R [10, 11]. Moreover, we previously also reported that the changes in function and expression level of P-gp in small intestine, liver and kidney during intestinal ischemia/reperfusion using our intestinal I/R model which was occluded both the superior mesenteric artery and vein [12-14]. Then we speculated that Mrp2 as well as P-gp can be affected by intestinal I/R, resulting in alteration of the disposition of substrates of Mrp2.

From such background, in this study we examined changes in function and expression level of Mrp2 using our original intestinal I/R model.

MATERIALS AND METHODS

Materials

5(6)-Carboxyfluorescein diacetate (5-CFDA) was purchased from Sigma Aldrich Co. Ltd. (Tokyo, Japan). All other reagents were of analytical grade or better.

Animals

Male Wistar rats (8 weeks old) were purchased from Tokyo Laboratory Animals Science Co. Ltd. (Tokyo, Japan). All animal experiments were performed according to the guidelines of Tokyo University of Pharmacy and Life Sciences. The animals were fasted for 16-18 hr before starting the experiment. Water was freely available while fasting. We have already reported the *in vivo* intestinal I/R model established using a spring balance and surgical sutures [10-12]. Briefly, the

superior mesenteric artery and vein in rats were occluded by hanging using surgical-sutures (Shirakawa, Tokyo, Japan) connected to a spring balance for 60 min (ischemia condition), followed by reperfusion by cutting the sutures (reperfusion condition). The hanging force on the blood vessel during ischemia was 100g (Intestinal I/R). "I/R 100g" corresponding intestinal I/R. All experiments were performed under anesthesia with Somnopentyl® (pentobarbital sodium, 50 mg/kg), and the body temperature was maintained at 37°C with a heat lamp.

Preparation of RNA and cDNA synthesis

Total RNA was isolated from jejunal, hepatic and renal specimens using TRIzol reagents (Invitrogen Co. Ltd., 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 of RNA, 100 ng of random hexamers, 0.5 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 10 mM Tris-HCL (pH 8.4), 25 mM KCl, 5 mM MgCl₂, 10 mM DTT, and 10 units of RNaseOUT recombinant ribonuclease inhibitor.

Analysis of gene expression level in intestine by quantitative real time reverse transcription-polymerase chain reaction (RT-PCR)

To perform the real-time RT-PCR, 96-well reaction plates with optical adhesive covers and ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) were used. Assay-on-Demand Gene Expression Products were purchased for the Mrp2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1).

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

Gene	Sequence (5'-3')		Amplicon Size	GenBank ID
Mrp2	Forward	CTGGTGGATAGCGCCAATG	113 bp	NM-012833
	Reverse	AGGATCGATGAGGTCACCATG		
GAPDH	Forward	TGAGGTGACCGCATCTTCTTG	102 bp	NM-017008
	Reverse	TGGTAACCAGGCGTCCGATA		

Reverse transcription was performed for 1 µg of RNA using a cDNA High Capacity Archive kit (Applied Biosystems, Foster City, CA, USA) and random hexamers as primers. Quantitative PCR was performed on an ABI PRISM 7000 system from Applied Biosystems using a SYBR® qPCR Mix. The PCR conditions were 10 min at 90°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. All assays were RNA-specific (spanning exon-exon junctions) pre-designed SYBR green Gene Expression Assays from Applied Biosystems.

Western blotting

The luminal contents were thoroughly washed out with sufficient amount of ice-cold saline. The mucosal surface was scraped off with a slide glass. The mucosa

collected was homogenized in a buffer containing 0.05 mg/mL phenylmethylsulfonyl fluoride, 300 mM mannitol, 12 mM Tris, and 5 mM EGTA (pH 7.1) with a tissue homogenizer. Protein was determined by the method using Micro BCA Protein Assay Reagent Kit (Thermo Fisher Scientific). Equal amounts of protein samples were separated by SDS-PAGE and transferred onto nitrocellulose-membranes. After blocking with 5% non-fat milk for 1 hr at room temperature, the membranes were incubated with primary antibodies specific for Mrp2 (Zymed Laboratories Inc., South San Francisco, USA) overnight at 4 °C. After 3 times washes with PBS-T, the membranes were incubated with peroxidase-conjugated secondary antibodies (1:5000, GE Healthcare) for 1 hr at room temperature. The bands were visualized using western blotting

detection reagent (ECL plus; GE Healthcare). Band intensities were quantified using LAS-3000 (FUJIFILM Holdings Corporation).

Excretion experiments to jejunal perfusate, bile and urine from blood using the in situ single pass perfusion method

Cannulation (silicone tubing, Silascon®; Kaneka Medix Co.) was placed in the jugular vein for administration and sampling. Also, jejunum (7 cm length) was washed with pre-warmed saline (37°C), and the proximal end of the lumen was catheterized with an in-flow glass cannula, which was connected to the perfusion system. The distal end of the jejunum was also catheterized with an out-flow glass cannula to collect intestinal effluent serially. Single-pass perfusion of Krebs-Henselite bicarbonate buffer (KHBB) solution into the jejunal lumen was started at a rate of 1 mL/min. On the other hand, the bile duct and urinary bladder were also cannulated for bile and urine collection, respectively. Bile and urine were collected in preweighed tubes at 20 min intervals for 60 min throughout the experiment. 5-CFDA (5 µM, 1 mL) was administered by i.v. injection via the cannula inserted in the jugular vein. Intestinal effluent samples were collected every 10 min. Excretion of 5-CFDA from blood to jejunal lumen was expressed as total excreted amounts for 60 min. The jejunal luminal excretion clearance (CL_{jejunal}) was calculated by dividing the excretion rate by the plasma concentration in the middle of the collection period of jejunal excretion. Plasma

samples were obtained by centrifugation of the blood samples at 3,000 x g for 10 min. The volume of bile and urine samples was measured gravimetrically. The effects of intestinal I/R on jejunal, biliary and urinary excretion of 5-CFDA were examined 0 to 1 hr, 3 to 4 hr and 24 to 25 hr after reperfusion.

STATISTICAL ANALYSIS

All results are expressed as the mean ± standard error (S.E.). Statistical significances between groups were analyzed using Student's t-test; P < 0.05 was considered significant.

RESULTS AND DISCUSSION

Changes in mRNA Expression Level of Mrp2 in Jejunum, Liver and Kidney

In this study, we focused on MRP2 which is a particularly important transporter in clinical situation. The mRNA expression level of Mrp2 in jejunum was significantly decreased by intestinal I/R (Fig-1a). Moreover, the level did not recover to control level by 24 hr after reperfusion in intestinal I/R group. In the liver, the mRNA expression level of Mrp2 was significantly increased by intestinal ischemia, but that increase was not observed 3 hr after reperfusion. Conversely, the significantly decrease of Mrp2 mRNA was observed after 12 and 24 hours from the start of reperfusion (Fig-1b). On the other hand, Mrp2 mRNA level in kidney continued to decrease during intestinal I/R for at least until after 24 hours from the start of reperfusion (Fig-1c).

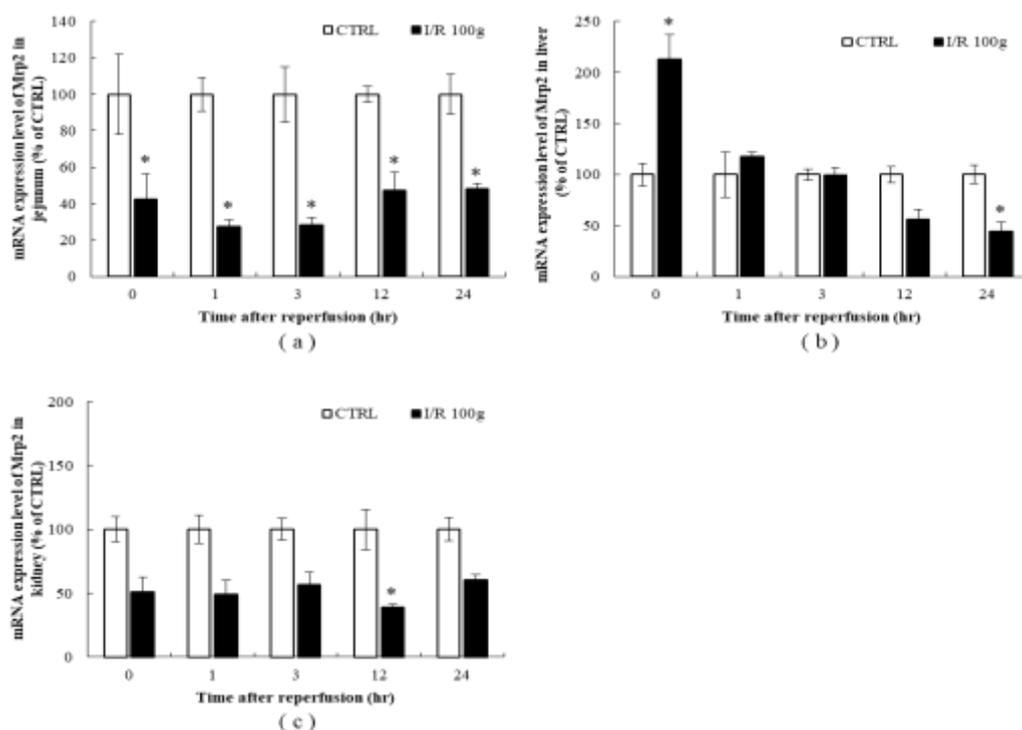


Fig-1: Effect of intestinal I/R on mRNA expression level of Mrp2 in jejunum (a), liver (b) and kidney (c). Column of white and black show the data of CTRL and intestinal I/R respectively. *P<0.05 compared with control condition of each reperfusion time, Data represent means and S.E. (n = 6 for each condition)

Similar to the change of *mdr-1a* (P-gp) mRNA which we have already reported [13-15], regularity was not observed in the change in mRNA expression of *Mrp2* in jejunum, liver and kidney by intestinal I/R. About gene expression regulation, Kimura *et al.* considered that the recovery rates of various transporters from the disorder states are in accordance with the orders of the role in the recovery process of each transporter [16]. Because the same pattern was not observed this study, our results did not correspond to their suggestion. So further investigation was necessary to clarify the mechanism of transporter regulation system which controls the expression level of transporters

Changes in protein expression level of *Mrp2* in jejunum, liver and kidney

The relative protein expression level of *Mrp2* in jejunum was significantly decreased by intestinal I/R, but they recovered to the control levels by 12 hr after reperfusion (Fig-2a). The relative protein expression level of *Mrp2* in liver tended to be also decreased by intestinal I/R, and its decrease continued until 12 hr after reperfusion. However, the level returned to the control level by 24 hr after reperfusion (Fig.-2b). On the other hand, in kidney, its expression level was significantly increased by intestinal I/R at 3 hr after reperfusion (Fig-2c).

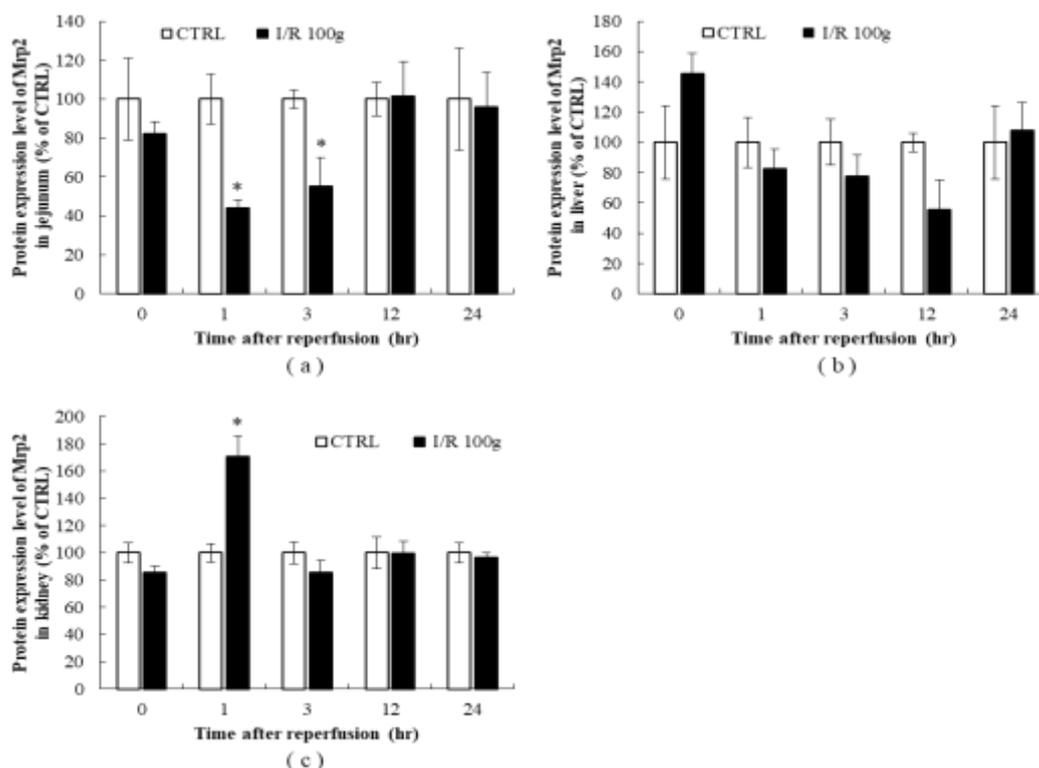


Fig-2: Effect of intestinal I/R on protein expression level of *Mrp2* in jejunum (a), liver (b) and kidney (c). Column of white and black show the data of CTRL and intestinal I/R respectively. *P<0.05 compared with control condition of each reperfusion time, Data represent means and S.E. (n = 4 for each condition)

It was shown that change in relative protein expression level of *Mrp2* by intestinal I/R was different in each organ. Therefore, it could be suggested that the mechanism of injury by intestinal I/R in each organ is different. The behavior of *Mrp2* protein expression level did not correspond to that of P-gp in each organ [13-15]. Therefore, even with the same ABC-transporter group, it was suggested that the regulatory mechanism of each ABC-transporter is different under intestinal I/R condition.

In this study, the correlation of expression level behavior between mRNA and protein of *Mrp2* was not observed (Fig-1 and Fig-2). It was reported that the behavior of protein expression of *Mrp2* and *Mrp3* demonstrated the same pattern in rat hepatocytes during

liver regeneration after 90% hepatectomy in rats [17], but not in the gene expression levels [16]. In that reports, the reason why correlation was not observed by expression behavior of gene and protein was not discussed. Moreover, it was reported that the correlation of mRNA and protein expression was not observed during disease condition [18].

On the other hand, Ogura *et al.* and Terada *et al.* previously reported the changes in the expression level of transporters including *Mrp2* in multiple organs by intestinal I/R. Their intestinal I/R model were created by occluding only superior mesenteric artery (SMA) and the ischemic time were performed 15 and 30 min [19, 20]. Our results were different from the results reported by Ogura *et al.*. Because, our intestinal I/R

model was created by occluding SMA and superior mesenteric vein (SMV), these intestinal I/R model were different. At the case of small intestinal transplantation, small intestine of both donor and recipient is exposed to both SMA and SMV ischemic condition. Our intestinal I/R model do not assume the transplant intestinal tract, but assume intestines that are left in the living body.

Therefore, it was suggested that the occluding of SMV has the effect on the expression of transporters. Since the expression behavior of the transporters varied depending on changes in the ischemic site (SMA, SMV) and changes in ischemic time (15, 30 or 60 min), more detailed examination is necessary to clarify the mechanism of transporters expression during intestinal I/R.

Changes in total clearance (CL_{tot}) and organ (tissue) excretion clearance ($CL_{jejunum}$, CL_{bile} , CL_{urine}) of 5-CFDA by intestinal I/R

During 0 to 1 hr after reperfusion, CL_{tot} of 5-CFDA (5-CF), a Mrp substrate [21], was significantly decreased by intestinal I/R (Fig-3a). The degree of the decrease became small during 3 to 4 hr and 24 to 25 hr reperfusion, and significance was disappeared. Since reperfusion time of 3 to 4 hr condition has a long anesthesia time, CL_{tot} of CTRL was lower than other condition. However, the difference between CTRL and I/R of CL_{tot} at each time is smaller in time dependent manner of reperfusion (Fig-3b). It can be thought that the intestinal I/R injury was almost recovered by 24 hr after reperfusion. However, correlation of function and expression amount of Mrp2 was not obtained.

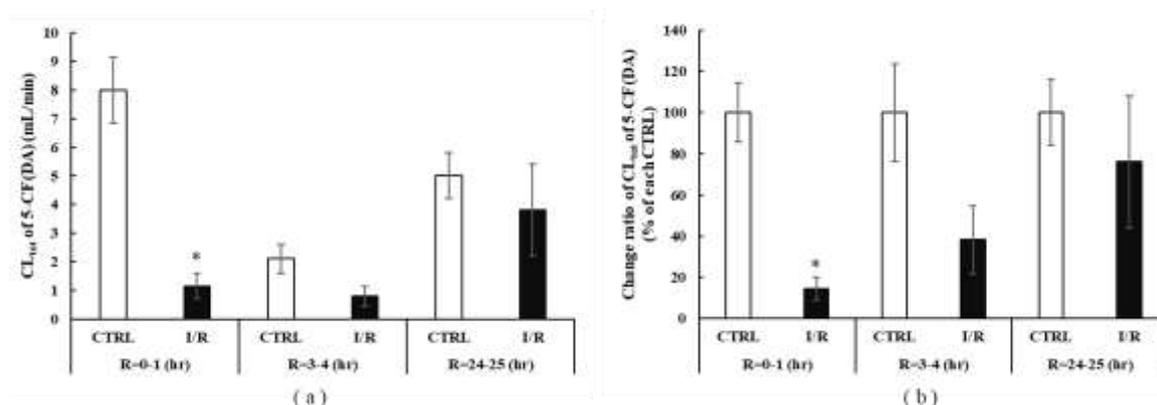


Fig-3: CL_{tot} of 5-CFDA (5-CF) during intestinal ischemia/reperfusion after i.v. administration of 5-CFDA (a) and their ratio (b). Reperfusion time is 0 to 1, 3 to 4 and 24 to 25 hr, respectively. Column of white and black show the data of CTRL and intestinal I/R respectively. *P<0.05 compared with control condition of each reperfusion time, Data represent means and S.E. (n = 4-8 for each condition)

On the other hand, $CL_{jejunum}$ was significantly increased by intestinal I/R (Fig. 4a). In early phase of reperfusion, intestinal barrier function was significantly decreased, and tight junction was opened [22]. Because 5-CFDA (5-CF) can be passed paracellular route, it was thought that 5-CFDA is not suitable for use as substrate for Mrp during TJ opening.

In liver and kidney, CL_{bile} and CL_{urine} of 5-CFDA (5-CF) were also changed by intestinal I/R (Fig. 4b and C), but the correlation of function and expression was not observed as well as small intestine. In contrast to small intestine, changes of liver and kidney continue at least until 24 hr after reperfusion (longer phase).

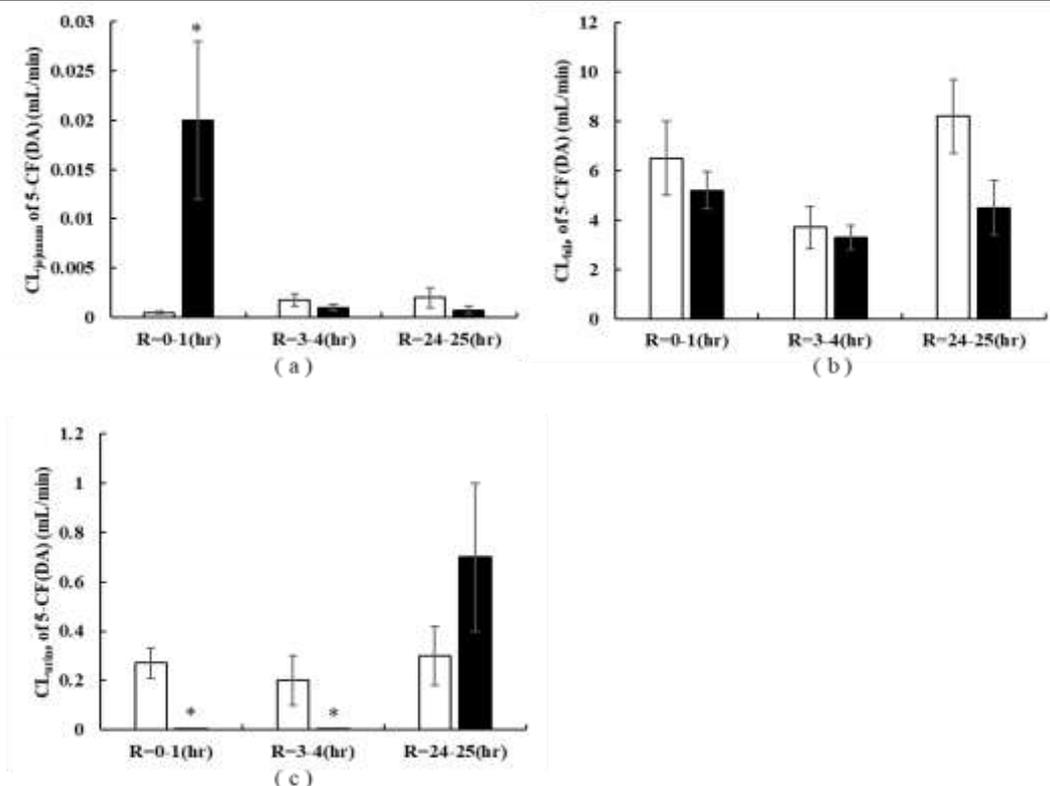


Fig-4: $CL_{jejunum}$ (a), CL_{bile} (b) and CL_{urine} (c) of 5-CFDA (as 5-CF) during intestinal ischemia/reperfusion after i.v. administration of 5-CFDA. Reperfusion time is 0 to 1, 3 to 4 and 24 to 25 hr, respectively. Column of white and black show the data of CTRL and intestinal I/R, respectively. * $P < 0.05$ compared with control condition of each reperfusion time, Data represent means and S.E. (n = 4-8 for each condition)

CONCLUSION

It was observed that the behavior of Mrp2 mRNA changed by intestinal I/R was different between the small intestine and liver and kidney. Therefore, it can be suggested that the injury mechanism in each organ was different. A correlation was not observed between the expression behavior of mRNA and protein level of Mrp2 during intestinal I/R. It was shown that the disposition of 5-CFDA (5-CF) which is a Mrp substrate was changed during intestinal I/R. In the drug therapy to an ischemic bowel disease patient, the dosing of Mrp substrate is necessary to be careful.

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CONFLICT OF INTEREST (COI)

The authors declare no conflict of interest.

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