Alteration of P-gp Expression after Intestinal Ischemia-reperfusion Following 16-h Fasting in Rats

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Alteration of P-glycoprotein (P-gp) expression influences the pharmacokinetics of P-gp substrates after intestinal ischemia-reperfusion (I/R). Fasting before intestinal I/R affects intestinal I/R injury. However, the effect of fasting on the alteration of P-gp expression after intestinal I/R has not been clarified. We previously reported that P-gp expression was altered after intestinal I/R following feeding. In the present study, we investigated the expression of P-gp after intestinal I/R following 16-h fasting. The mdr1a levels were decreased at 6 h in the jejunum, at 1 h in the ileum, at 6 and 24 h in the liver and at 6 h in the kidney. The mdr1b levels were decreased at 6 h in the ileum and at 1 and 6 h in the kidney. The mdr1b level in the liver was increased at 1 and 6 h. The expression of P-gp was decreased at 6 h in the jejunum, at 1, 6 and 24 h in the ileum, and at 6 h in the kidney. These alterations were different to those after intestinal I/R following feeding. In particular, the decrease in P-gp expression in the 16-h fasting I/R rats occurred at an early time during reperfusion compared with that in the feeding I/R rats. In conclusion, feeding condition affects the alteration of P-gp expression after intestinal I/R. Therefore, it is important to understand the patient's feeding condition for safe drug therapy.

Key words——intestinal ischemia-reperfusion; fasting; P-glycoprotein

INTRODUCTION

Intestinal ischemia-reperfusion (I/R) is a common clinical problem in small bowel transplantation, circulatory shock, and strangulation ileus. The intestinal mucosa is damaged structurally and functionally after intestinal I/R.[1] Additionally, intestinal mucosa damage induces local production of inflammatory cytokines.[2] Therefore, intestinal I/R damages remote organs, including the lung,[3] liver[4] and kidney,[5] as well as the intestine, and promotes multi-organ failure (MOF).[6] Injury of the intestinal mucosa is induced by production of reactive oxygen species and release of inflammatory cytokines. Both reactive oxygen species and inflammatory cytokines activate NF-κB.[7,8] Activated NF-κB induces further production of reactive oxygen species and release of inflammatory cytokines, resulting in more serious intestinal mucosa damage and MOF. It has been reported that feeding condition has an effect on intestinal I/R injury. Thirteen-hour fasting before intestinal I/R resulted in more serious intestinal I/R injury,[9] and presurgical administration of carbohydrates reduced intestinal I/R injury.[10] However, these findings were obtained at an early time (1-3 h) during reperfusion. Therefore, the effect of feeding condition before intestinal I/R injury has remained unclear. In clinical setting, feeding condition before intestinal I/R can be controlled artificially in patients undergoing surgical operations, including small bowel transplantation, but cannot be controlled artificially in patients with intestinal diseases, including circulatory shock and strangulation ileus. Therefore, determination of the effect of feeding condition before intestinal I/R is important for appropriate treatment for intestinal I/R patients.

P-glycoprotein (P-gp) belongs to the ATP-binding cassette (ABC) superfamily and is expressed in almost all tissues, including the intestine, liver and kidney, and effluxes a broad range of endogenous and xenobiotic compounds. The mammalian *mdr* gene family is composed of two members in humans (*MDR1* and *MDR3*) and three members in rodents (*mdr1a*, *mdr1b* and *mdr2*). Although these *MDR/mdr* genes correspond to P-gp, *MDR1*, *mdr1a* and *mdr1b* correspond to drug transporter, and *MDR3* and *mdr2* correspond to phospholipid transporter.[11,12] Since P-gp, which originates *MDR1*, *mdr1a* and *mdr1b*,

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effluxes many drugs, including tacrolimus, digoxin, quinidine and colchicines, P-gp expression provides useful information for understanding pharmacokinetics of many drugs. Indeed, Omae et al. reported that alteration of P-gp expression affected the pharmacokinetics of tacrolimus.[13] We previously reported that the expression of P-gp after intestinal I/R following feeding was significantly altered in the ileum and kidney.[14] Although the expression of P-gp was not altered in the liver after intestinal I/R following feeding, mdr1a and mdr1b levels were altered.[14] However, the effect of fasting on these alterations of P-gp expression after intestinal I/R has not been clarified. It has known the 16-h fasted rodent gut lumen to be empty.[15] Therefore, we chose a 16-h fasting. The aim of this study was to determine the alteration of P-gp expression in fasting I/R rats.

MATERIALS AND METHODS

Animals　　Male Wistar rats, aged 6 weeks, were obtained from Jla (Tokyo, Japan). The rats were housed for at least 1 week (until reaching 250–350 g in weight). The housing conditions were the same as those described previously.[16] The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the "Guide for the Care and Use of Laboratory Animals".

Intestinal I/R Model　　Surgical procedures were carried out as described in a previous report with some modification.[17] Rats were not fed for 16 h prior to experiments but were allowed free access to water. The animals were anesthetized with sodium pentobarbital (30 mg/kg body weight, i.p. injection). Through a midline laparotomy, each rat was subjected to 30 min of ischemia by ligating small anastomosing vessels and occluding the superior mesenteric artery (SMA). Reperfusion was induced by removing the clamp. The abdomen was sutured and not anesthetized during reperfusion. Rats were killed under surgical anesthesia at 1, 6 and 24 h after reperfusion. Tissues were harvested just before killing. Blood samples were collected at 1, 6 and 24 h during the 24-h reperfusion period.

Histological Examination　　Histological samples were sent to the Sapporo General Pathology Laboratory Co., Ltd. Briefly, samples of small intestine were harvested after reperfusion and immediately fixed in 10% buffered formalin. The fixed tissue was embedded in paraffin, sectioned to 4 μm in thickness, and stained with hematoxylin-eosin. The degree of injury was scored with grading system used by Masuko et al. as follows: 0＝normal mucosa; 1＝disruption of epithelial cells in the tips of the villi; 2＝disruption of epithelial cells in half of the villi; 3＝disruption of epithelial cells in the full length of the villi.[18]

Semi-quantitative Real-time PCR　　Total RNA was prepared from tissue homogenate using an ISO-GEN (Nippon Gene, Tokyo) and an RNase-Free DNase Set (QIAGEN). Single-strand cDNA was made from 2 μg total RNA by reverse transcription (RT) using a ReverTra Ace (TOYOBO). Semi-quantitative real-time PCR was performed using an ABI PRISM 7700 sequence detector (Applied Biosystems) with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) as per the manufacturer's protocol. PCR was performed using mdr1a-specific primers through 40 cycles of 94°C for 15 s, 50°C for 30 s and 72°C for 30 s or using mdr1b-specific primers through 40 cycles of 95°C for 15 s, 50°C for 30 s and 72°C for 30 s, in both cases after pre-incubation at 50°C for 2 min and at 95°C for 15 min, or using GAPDH-specific primers. The primers specific to rat mdr1a, 1b and GAPDH were designed on the basis of sequences in GenBankTM database (accession no.: NM_133401, NM_012623 and AF106860, respectively). The sequences of the specific primers were as follows: the sense sequence (209–228) was 5'-GCA GGT TGG CTG GAC AGA TT-3' and the antisense sequence (259–278) was 5'-GGA GCG CAA TTC CAT GGA TA-3' for rat mdr1a, the sense sequence (199–218) was 5'-CTG CTA TCA TCC ACG GAA CC-3' and the antisense sequence (317–338) was 5'-GCT GAC GGT CTG TGT ACT GTT G-3' for rat mdr1b, and the sense sequence (1034–1053) was 5'-ATG GGA AGC TGG TCA TCA AC-3' and the antisense sequence (1235–1254) was 5'-GTG GTT CAC ACC CAT CAC AA-3' for rat GAPDH. The PCR products were normalized to amplified GAPDH, which was the internal reference gene (housekeeping gene). Standard curves were prepared for each target and housekeeping gene. The standard curve was established between the threshold cycles (Ct) and the $\log_{10}$ (copy numbers) by using Applied Biosystems sequence detection system software, version 1.9.1. The software calculates the relative amount of the target gene and the housekeeping gene based on the Ct.

Western blot analysis　　The following samples

were used for Western blot analysis: intestinal brush-border-membrane vesicles (BBMVs), liver crude membrane and renal BBMVs. These samples were prepared as described previously.[19,20] The protein concentrations of these samples in clear supernatant were determined by the method of Lowry et al. with bovine serum albumin as a standard.[21] Each sample was denatured at 100℃ for 3 min in a loading buffer containing 50 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% BPB and 3.6 M urea and separated on 4.5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred electrophoretically onto nitrocellulose membranes (Trans-Blot; BIO-RAD) at 15 V for 90 min. The membranes were blocked with PBS containing 0.05% Tween 20 (PBS/T) and 10% non-fat dry milk for 1 h at room temperature. After being washed with PBS/T, the membranes were incubated overnight at room temperature with a mouse monoclonal antibody to Mdr1 (Santa Cruz Biotechnology) (diluted 1 : 500) and washed three times with PBS/T for 10 min each time. The membranes were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) at a dilution of 1 : 2000 and washed three times with PBS/T for 10 min each time. The bands were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham).

Determination of Cytokine Concentrations Serum cytokines were determined using ELISA kits, which were purchased from BioSorce. The assays were performed according to the manufacturer's instructions.

Oxidative Stress Assessment The amount of lipid peroxide in each organ was determined as that of malondialdehyde (MDA) by the method of Ohkawa et al. with some modification.[22] Thiobarbituric acid (TBA) solution consisted of 2.6 mM TBA, 918 mM trichloroacetic acid, 0.3 mM HCl, and 1.8 mM 2, 6-di-tert-butyl-4-metylphenol (BHT) in 22% ethanol. The reaction mixture contained 0.2 ml of tissue homogenate, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution (pH 3.5), and 1.5 ml of TBA solution. The mixture was heated at 95℃ for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of n-butanol were added, and the mixture was shaken vigorously. After centrifugation at 3000 $\times$ g for 10 min, the absorbance of the organic layer (upper layer) was measured at 535 nm with 1, 1, 3, 3-tetraethoxypropane (TEP) as a standard.

Data Analysis Data obtain from all experiments were analyzed by ANOVA followed by the post-hoc Scheffe test. All statistical analyses were performed using Origin 6.0 J and StatView 5.0 J. A p value less than 0.05 was considered a significant difference.

RESULTS

Histopathology of Small Intestine The histopathological features of the small intestine at different post reperfusion times are shown in Figs. 1 and 2. Upper villous cells were sloughed by I/R injury, and the most severe damage to the intestinal mucosa was observed at 1 h after intestinal I/R following 16-h fasting. The height of villi was almost half of that of sham-operated rats. At 6 h after intestinal I/R following 16-h fasting, almost all of the epithelia were improved but the width of villi was lower than that of sham-operated rats. The mucosal histological features had normalized by 24 h after intestinal I/R following 16-h fasting. The height and width of villi were not different compared with sham-operated rats.

Expression of Mdr1a and Mdr1b mRNA Mdr1a mRNA levels were decreased at 6 h in the jejunum and at 1 h in the ileum after intestinal I/R. The mRNA levels of mdr1a were decreased at 6 and 24 h in the liver and at 6 h in the kidney after intestinal I/R (Fig. 3). Mdr1b mRNA level was decreased at 6 h after intestinal I/R in the ileum but was not significantly altered at any time in the jejunum. The mRNA levels of mdr1b were increased at 1 and 6 h in the liver and decreased at 1 and 6 h in the kidney after intestinal I/R (Fig. 4).

Expression of P-gp We investigated whether P-gp expression correlated with mdr1a or mdr1b expression. Figure 5 shows the expression of P-gp protein after intestinal I/R. P-gp protein level was decreased at 6 h in the jejunum. This result was associated with the level of mdr1a. Although the mRNA level of mdr1a was decreased at 1 h and that of mdr1b was decreased at 6 h in the ileum, P-gp expression in the ileum was decreased at 1, 6 and 24 h after intestinal I/R compared with that in sham-operated rats. P-gp protein was not significantly altered at any time in the liver. This result also did not correspond to levels of mdr1a and mdr1b mRNA. In the kidney, P-gp protein level was significantly decreased 6 h after intesti-
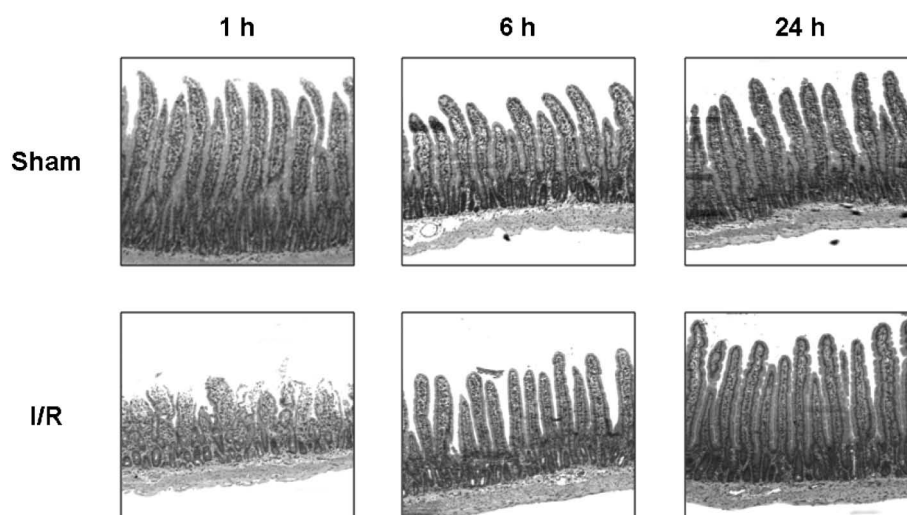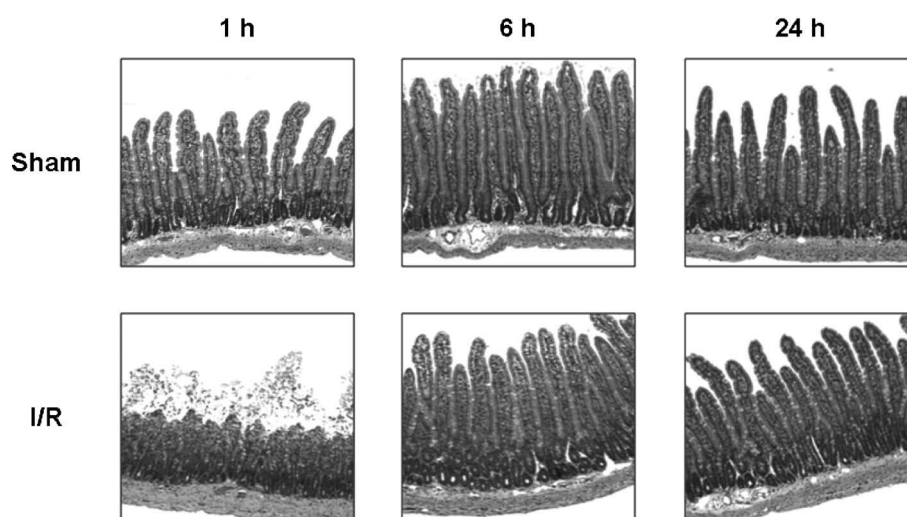
Fig. 1. Histopathology of the Jejunum after Intestinal I/R Following 16-h Fasting

Alteration of the villous structure after intestinal I/R following 16-h fasting was evaluated by hematoxylin-eosin staining using 4-μm-thick small intestine sections. Each image was taken at $\times$100 magnification.

Supplemental Fig. 1. Histopathology of the Jejunum after Intestinal I/R Following Feeding

nal I/R and corresponded to the level of mdr1a and mdr1b.

Serum Concentrations of Inflammatory Cytokines

P-gp expression can be downregulated by inflammatory cytokines.[23] We previously reported that the decrease in P-gp expression after intestinal I/R might be partly caused by IL-6.[14] Inflammatory cytokines were quantified after intestinal I/R following 16-h fasting. The serum level of IL-6 was increased at 1, 6 and 24 h after intestinal I/R but was not statistically different at 1 and 24 h (Fig. 6). Only trace amounts of serum IL-1β and TNF-α were detected at any time (data not shown). These results suggested that systemic inflammation was induced by IL-6 after intesti-

nal I/R.

Lipid Peroxidation in the Organs   P-gp expression can be also affected by oxidative stress, as well as inflammatory cytokines.[24] To investigate whether oxidative stress after intestinal I/R was affected by fasting, the MDA levels in the organs were quantified after intestinal I/R following 16-h fasting. The MDA levels in I/R rats were higher at 6 h in the jejunum and ileum after intestinal I/R than those in sham-operated rats. The MDA levels in I/R rats were higher at 6 h in the liver and at 24 h in the kidney. In feeding I/R rats, the MDA levels were higher at 6 h in the jejunum, liver and kidney and at 1-6 h in the ileum (Supplemental Fig. 3). These results indicated that
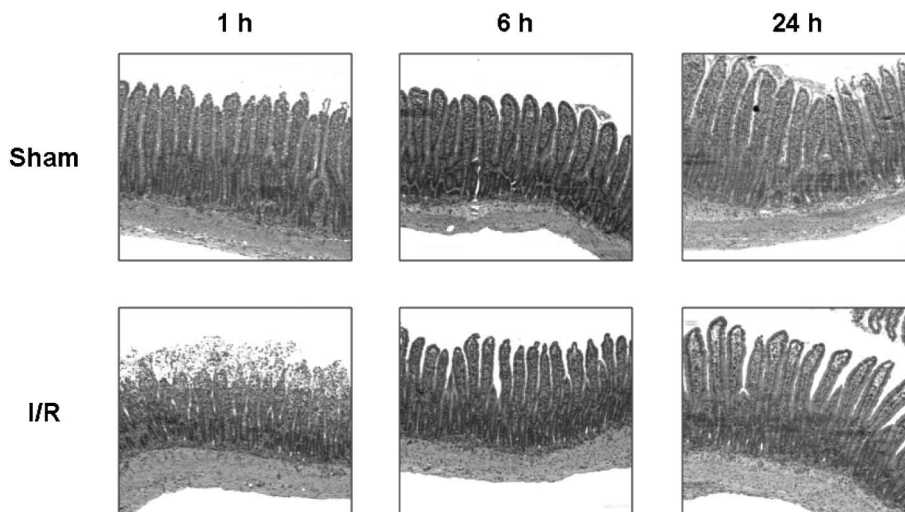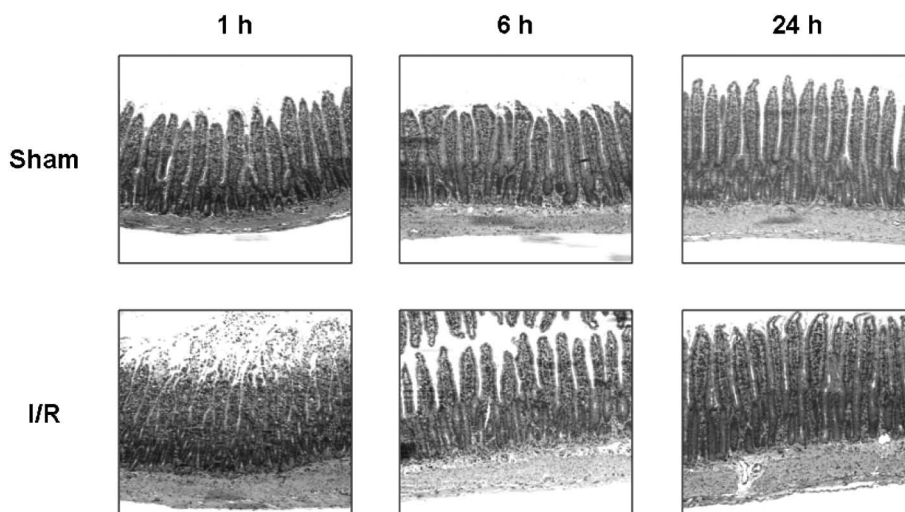
Fig. 2.   Histopathology of the Ileum after Intestinal I/R Following 16-h Fasting
    Alteration of the villous structure after intestinal I/R following 16-h fasting was evaluated by hematoxylin-eosin staining using 4-μm-thick small intestine sections. Each image was taken at ×100 magnification.

Supplemental Fig. 2.   Histopathology of the Ileum after Intestinal I/R Following Feeding

16-h fasting before intestinal I/R affected the period of oxidative stress after intestinal I/R.

DISCUSSION

In the present study, we assessed P-gp expression after intestinal I/R following 16-h fasting. The expression of P-gp was decreased in the jejunum, ileum and kidney. We previously reported that P-gp expression after intestinal I/R following feeding was decreased in the ileum and kidney.[14] Comparison of these findings shows two different points. First, the decrease in P-gp expression in the 16-h fasting I/R rats (Fig. 5) occurred at an early time during reperfusion compared with that in the feeding I/R rats

(previous report).[14] This difference might be caused by the effect of 16-h fasting on intestinal I/R injury. The release of IL-6 after intestinal I/R following 16-h fasting (Fig. 6) occurred at an early time and was maintained for a long time compared with that following feeding (previous report).[14] In contrast, the increases in MDA level in the ileum and kidney after intestinal I/R following 16-h fasting (Fig. 7) occurred at a later time than that following feeding (Supplemental Fig. 3). Because the increase in IL-6 occurred at an early time during reperfusion in 16-h fasting I/R rats, as well as the decrease in P-gp expression, the difference in the alteration of P-gp expression between 16-h fasting I/R rats and feeding I/

Table 1.   Time Courses of Mucosal Injury Scores, Villus Heights and Villus Widths after Intestinal I/R Following 16-h Fasting

		1 h	6 h	24 h
Jejunum	Mucosal injury score	2	0	0
	Villus height	0.44±0.06**	1.02±0.07	1.08±0.11
	Villus width	1.08±0.17	0.82±0.13**	1.02±0.19
Ileum	Mucosal injury score	2	0	0
	Villus height	0.62±0.04**	1.00±0.07	0.93±0.04
	Villus width	0.90±0.15	0.72±0.09*	1.01±0.16

Data show ratio of villus height or width to that of sham-operated rats. * $p<0.05$ significantly different from sham. ** $p<0.01$.

Supplemental Table 1.   Time Courses of Mucosal Injury Scores, Villus Heights and Villus Widths after Intestinal I/R Following Feeding

		1 h	6 h	24 h
Jejunum	Mucosal injury score	2	0	0
	Villus height	0.60±0.04**	0.95±0.07	1.00±0.06
	Villus width	0.91±0.08	0.80±0.09**	1.01±0.13
Ileum	Mucosal injury score	1	0	0
	Villus height	0.90±0.06**	1.10±0.07	1.01±0.06
	Villus width	0.88±0.13	0.94±0.15	1.06±0.24

Data show ratio of villus height or width to that of sham-operated rats. ** $p<0.01$ significantly different from sham.
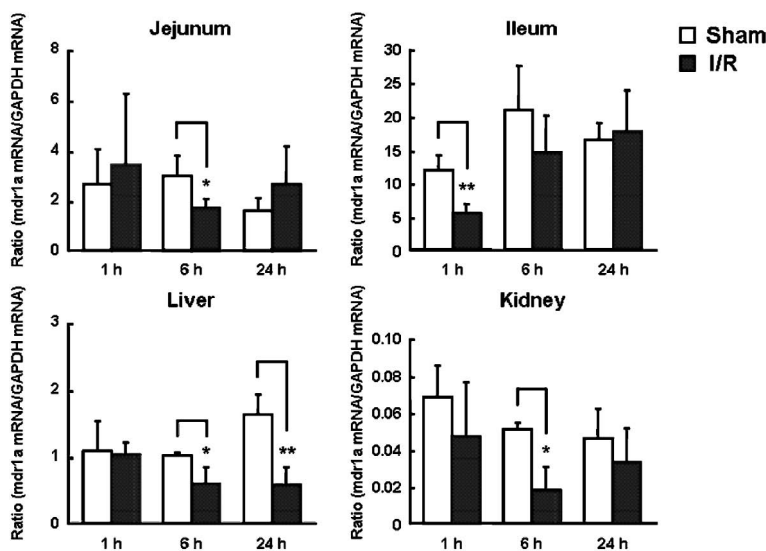

Fig. 3.   Time Course of Mdr1a mRNA Level after Intestinal I/R Following 16-h Fasting

Each column represents the mean and S.D. of 3–5 measurements. *$p<0.05$ significantly different from sham. **$p<0.01$.

R rats might be caused by the increase in IL-6. Indeed, Hartmann et al. reported that P-gp expressions of mice which were injected i.p. with IL-6 were significantly decreased.[25] This finding confirm our hypothesis that the difference in the alteration of P-gp expression between 16-h fasting I/R rats and feeding I

/R rats might be caused by the increase in IL-6.

Second, the alteration of mdr1b level was characteristic in the 16-h fasting I/R rats. In the ileum, mdr1b level was significantly decreased at 6 h in 16-h fasting I/R rats （Fig. 4） but was not altered at any time in feeding I/R rats (previous report).[14] In the
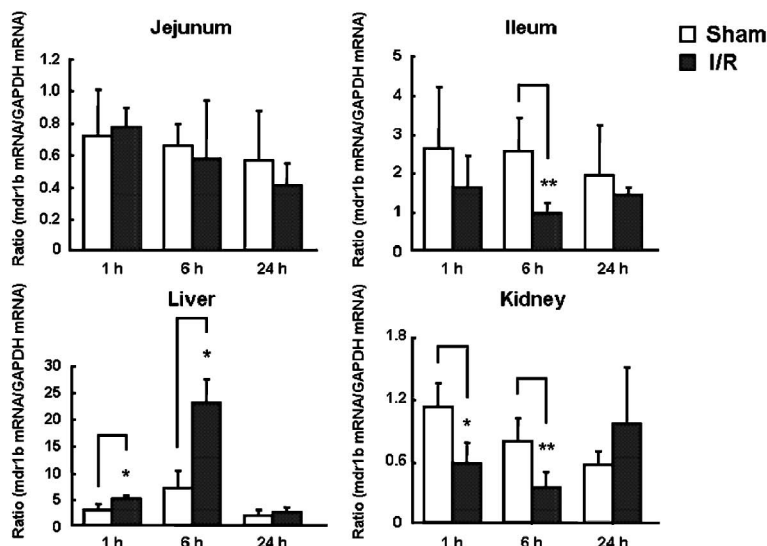
Fig. 4.   Time Course of Mdr1b mRNA Level after Intestinal I/R Following 16-h Fasting

Each column represents the mean and S.D. of 3–5 measurements. $*p < 0.05$ significantly different from sham. $**p < 0.01$.
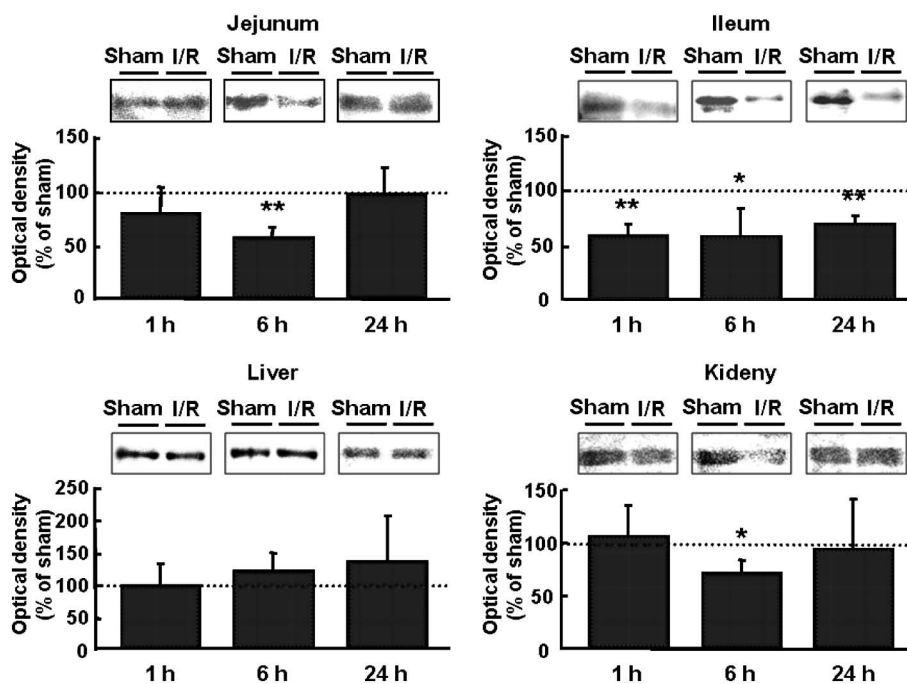

Fig. 5.   Time Course of P-gp Protein Expression in the Jejunum, Ileum, Liver and Kidney after Intestinal I/R Following 16-h Fasting

Western blot analysis using jejunal BBMVs, ileal BBMVs, crude membrane of the liver and renal BBMVs at 1, 6 and 24 h after I/R. Upper data shown are typical results from three independent experiments. Lower data represent percentage of P-gp expression level to the control. Each column represents the mean and S.D. of 3 measurements. $*p < 0.05$ significantly different from sham. $**p < 0.01$.

kidney, mdr1b level was also decreased at 1 and 6 h in 16-h fasting I/R rats (Fig. 4) but was not altered at any time in feeding I/R rats (previous report).[14] These findings indicated that mdr1b level contributed to the decrease in P-gp expression in 16-h fasting I/R rats. Unfortunately, we were not able to elucidate the effect of fasting before intestinal I/R in humans. It is

necessary for understanding the effect of fasting on human I/R patients to clarify the mechanism of the decreasing mdr1b level in 16-h fasting I/R rats. Therefore, further detailed investigation of the mechanism by which mdr1b is regulated in 16-h fasting I/R rats is needed. If this mechanism regulates human MDR1, fasting before intestinal I/R may affect

P-gp expression in intestinal I/R patients.

The alteration of P-gp expression after intestinal I/R did not necessarily correspond to the alteration of mdr1a or mdr1b level. In the ileum at 24 h after intestinal I/R, mdr1a and mdr1b level were not altered, but P-gp expression was decreased. Moreover, P-gp expression was not altered in the liver at any time after intestinal I/R though mdr1a level was decreased and mdr1b level was increased. As is the case with our results, several researchers reported the discrepancy between P-gp expression and mdr genes.[26,27] Inflammatory cytokines are known to be one of the inducer of this discrepancy.[28] Furthermore, it has known that

P-gp expression is regulated post-transcriptionally.[29] These findings suggest that intestinal I/R was affected the post-transcriptional regulation mechanism of P-gp.

Mdr1a and mdr1b levels were also altered after the sham operation (Figs. 3 and 4). In particular, mdr1a level in the ileum was significantly increased by sham-operation (1 h sham operation vs. 6 h sham-operation or 24 h sham-operation; $p < 0.05$). We did not investigate the factors leading the alterations of mdr genes by sham-operation, but it is possible that laparotomy may have contributed to these alterations. Indeed, several workers reported that laparotomy induced the production of stress hormone, particularly catecholamine and adrenocorticotropin (ACTH).[30,31] Moreover, Ogawa et al. reported that laparotomy affected some of gene and protein expression, such as serine dehydrogenase.[32] These findings suggest that laparotomy may affect the alteration of mdr genes by sham operation.

It has been reported that feeding condition before intestinal I/R affects severity of intestinal I/R injury.[9,10] The intestinal I/R injury of 16-h fasted I/R rats was more severe than that of feeding I/R rats (Figs. 1 and 2, Table 1, Supplemental Figs. 1 and 2, Supplemental Table 1). However, the severities of inflammation and lipid peroxidation had almost no effect on the severity of intestinal I/R. Maximum serum concentration of IL-6 was not significantly different between 16-h fasting I/R rats (Fig. 6) and feeding I/R rats (previous report).[14] Maximum MDA levels
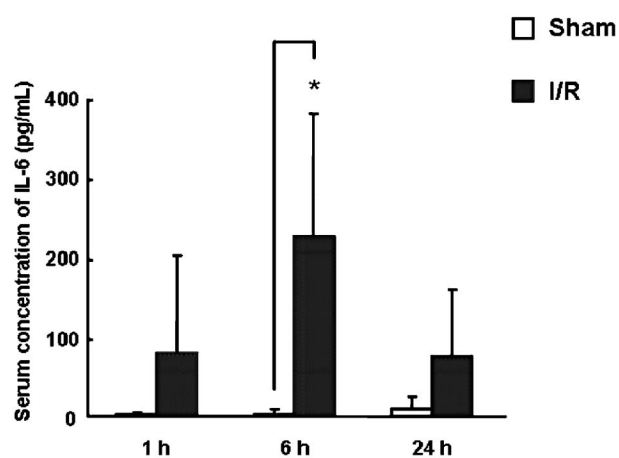

Fig. 6.   Serum Concentrations of Inflammatory Cytokines after Intestinal I/R Following 16-h Fasting

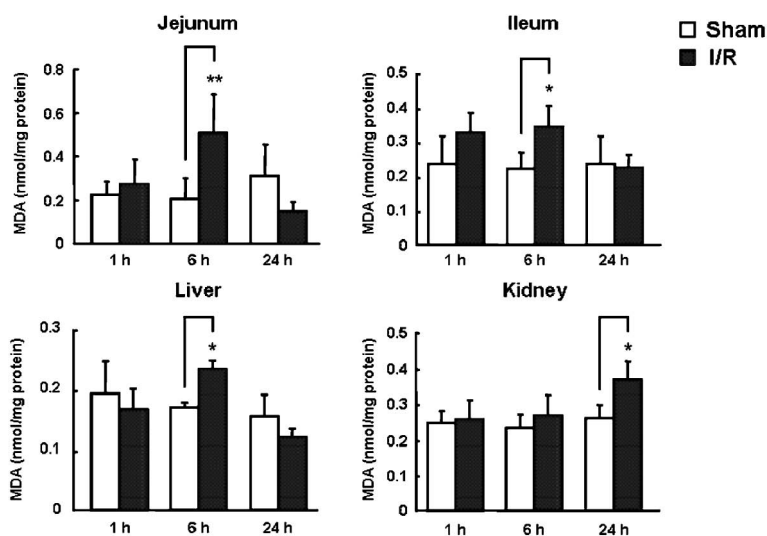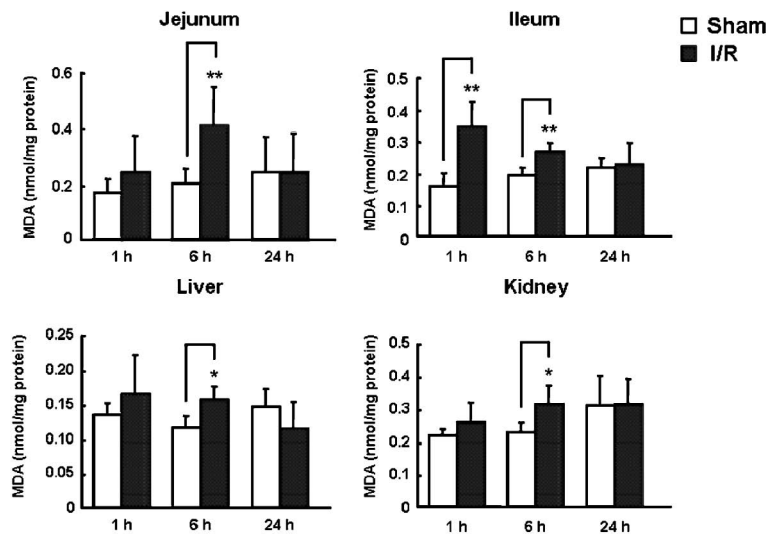Each column represents the mean and S.D. of 4–5 measurements. *$p < 0.05$ significantly different from sham.

Fig. 7.   MDA Levels in Organs after Intestinal I/R Following 16-h Fasting

Each column represents the mean and S.D. of 3–6 measurements. *$p < 0.05$ significantly different from sham. **$p < 0.01$.

Supplemental Fig. 3.   MDA Levels in Organs after Intestinal I/R Following Feeding
Each column represents the mean and S.D. of 3–6 measurements. $^*p < 0.05$ significantly different from sham. $^{**}p < 0.01$.

in the organs were also not significantly different between 16-h fasting I/R rats (Fig. 7) and feeding I/R rats (Supplemental Fig. 3). Van Hoorn *et al.* had also reported that feeding condition had no effect on MDA level in the intestine. It has been reported that flavonoids, ascorbic acid, glutamine and carbohydrate prevent intestinal I/R injury.[10,33–36] We have also been reported that antioxidants (lutein, bamboo grass, ferulic acid and rutin) prevent intestinal I/R injury.[37–40] Some of these nutrients (flavonoids, ascorbic acid, lutein bamboo grass, ferulic acid and rutin) had protective effect on increase in MDA level after intestinal I/R. It is important to reveal the protective effects of antioxidants on intestinal I/R injury that intestinal I/R injury must be assessed by several marker including histopathology, MDA level and inflammatory cytokines until recovery from injury must be assessed.

In conclusion, 16-h fasting before intestinal I/R affects the alteration of P-gp expression in the intestine, liver and kidney. The decrease in P-gp expression in the 16-h fasting I/R rats occurred at an early time during reperfusion. Therefore, it is important to understand the patient's feeding conditon for safe drug therapy.

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