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Membrane permeability and antipyrine absorption in a rat model of ischemic colitis

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Abstract

The aim of this study was to determine whether the duration of ischemia affects antipyrine absorption in the large intestine. This was carried out in a rat model of ischemic colitis in which ischemia and associated inflammation was induced by marginal vessel ligation. Blood flow was disrupted by positioning an o-ring around the distal rectum and ligating the marginal vessel at two locations in the hind-gut ligament artery region. Ligation was performed for 1, 2, 3, and 5 h. We assessed large intestine damage by measuring key indicators of inflammation, myeloperoxidase (MPO) activity and thiobarbituric acid reactant substrates (TBARS) in the mucosa and by histological staining with hematoxylin–eosin stain. Antipyrine membrane permeability was assessed in Ussing-type diffusion chambers, and related pharmacokinetics were calculated from antipyrine plasma concentration measurements following colon administration of the drug. Vessel ligation caused some sloughing of epithelial cells and elevated the MPO and TBARS levels. Prolonged ligation failed to affect the apparent permeability coefficient (P_{app}) of antipyrine. Prolonged ligation, however, gradually increased plasma antipyrine concentrations to near control levels. This increase was paralleled by increases in the absorption rate constant AUC and antipyrine bioavailability. Taken together, these results suggest that the absorption kinetics of antipyrine may depend on blood flow changes in the large intestine that occur with inflammation.

Keywords: Antipyrine; Colon; Ischemia; Reperfusion; MPO; TBARS

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1. Introduction

Many pathological conditions cause intestinal ischemia by compromising intestinal circulation. Recently, intestinal ischemia reperfusion was shown to trigger inflammation, ultimately causing widespread organ failure through various mediators such as IL-1b, IL-6, etc. (Chen et al., 2003). Ischemic colitis can involve a comparatively long portion of the descending colon (Williams and Wittenberg, 1975). Production of free radicals from lipid peroxidation and cell-mediated inflammation is closely related to post-ischemia reperfusion injury (Sener et al., 2001). Thiobarbituric acid and myeloperoxidase (MPO) are widely used as indices of inflammation, because their levels are proportional to the degree of tissue injury. In general, a higher incidence of ischemia occurs in the colon compared to the small intestine (Leung et al., 1992). This might occur because collateral circulation develops preferentially in the colon in response to a compromise; thus, ischemia reperfusion frequently occurs as a result of internal pressure in the colon.

The degree of membrane permeability and drug absorption by an inflamed intestinal tract generally depends on the etiology of the inflammation. In inflammatory bowel disease, membrane permeability of D-mannitol increases (Venkatraman et al., 2000). Compared to healthy subjects, patients with Crohn's disease or ulcerative colitis absorb methotrexate more readily (Moshkowitz et al., 1997), and patients with Crohn's disease also absorb ursodeoxycholic acid less readily (Fujisawa et al., 1998). Although much information is available about drug absorption in some intestinal disorders, very little information exists about drug absorption in ischemic colitis. Because blood flow changes caused by ischemia and reperfusion affect pharmacokinetics, understanding how ischemic colitis alters drug absorption in the large intestine is paramount for developing effective delivery strategies for those drugs absorbed in the large intestine. Of course, injury-related increases in intestinal membrane permeability also contribute to drug absorption.

In the present study, we selected antipyrine, a drug whose absorption is limited by blood flow (Takahashi et al., 1988), to test in our model. We investigated effects of prolonged ischemia on antipyrine membrane permeability and absorption in the large intestine in vitro and in vivo.

2. Materials and methods

2.1. Materials

QCU-1, water soluble methacrylate (Quetol-523), 2-hydoroxypropyl methacrylate and methyl methacrylate were purchased from Nisshin EM (Tokyo, Japan). Antipyrine, paraformaldehyde, Gill's hematoxylin solution no. 2, 1% eosin Y solution, hexadecyltrimethylammonium bromide (HTAB), 30% hydrogen peroxide, 3,3′-dimethoxybenzidine dihydrochloride, 2,6-di-*t*-butyl-4-methylphenol (BHT), 4,6-dihydroxy-2-mercaptopyrimidine (TBA), and 1,1,3,3-tetramethoxypropane (TMP) were obtained from Wako Pure Chemical Ind. (Osaka, Japan). BCA protein assay reagent kit was obtained from Pierce (Rockford, IL, USA), glutaraldehyde EM 25% was from Taab (Aldermaston, Berkshire, UK), and fluorescein isothiocyanate-dextran (molecular weight: 4300; FD-4) and lucifer yellow dilithium salt were obtained from Sigma (St. Louis, MO, USA) and ICN Biomedicals Inc. (Aurora, OH, USA), respectively. Other substrates were of reagent grade.

2.2. Animals, experimental protocol, and surgical procedures

Male Wistar rats weighing 250–300 g were housed in wire-bottomed cages. They were subjected to a 12 h/12 h light–dark cycle, and standard rat chow and water was provided ad libitum. Rats were divided into six experimental groups, as summarized in Table 1. After 16 h of fasting, individual rats were anesthetized with sodium pentobarbital, injected intraperitoneally (50 mg/kg body weight). The abdomen was shaved,

Table 1
Experimental protocol

Groups	Pretreatment	Surgical operation
C	None	Laparotomy
R	o-ring	Laparotomy
RV1	o-ring	Laparotomy + 1 h ligation
RV2	o-ring	Laparotomy + 2 h ligation
RV3	o-ring	Laparotomy + 3 h ligation
RV5	o-ring	Laparotomy + 5 h ligation

A 6-mm diameter o-ring was positioned around the rectum at the rectovesial pouch. Three days later, the marginal vessel of the left colon was ligated at two sites located 3 cm apart. Abbreviations: C, sham (control); R, ring; RV, ring and vessel.

cleaned, and then a 3-cm midline incision was made with a surgical knife. The rectum at the rectovesical pouch was bound by encircling it with an o-ring (6 mm diameter). Potassium benzylpenicillin G (50,000 U per rat, Meiji Seika Co., Tokyo, Japan) was injected into the intraperitoneal cavity, and the abdomen was immediately sutured shut with thread and metal clip fastenings. The rats were then returned to their cages and housed under normal conditions. Three days after surgery, the abdomen was reopened under anesthesia. The mesenteric marginal vessel of the descending colon was ligated in two places, approximately 3 cm apart (Fig. 1), and then the abdomen was immediately sutured shut as before.

After undergoing ligation for the designated times (Table 1), rats were anesthetized with diethyl ether and killed by cutting the carotid artery. Immediately, the descending colon was removed for histopathological examination, MPO and TBARS level measurements or in vitro permeability studies. Rats used for the in vivo absorption studies were similarly housed and maintained.

All animals received humane care and were treated in accordance with *Guidelines for Animal Experimentation*, Hokuriku University.

2.3. MPO measurement

We measured MPO levels in the colonic mucosal membrane according to the method of Krawisz et al. (1984). Briefly, under ice-cold conditions, epithelial mucosal cells were collected by scraping with a glass slide the mucosal lining of the excised, descending colon. The scraped mucosa were placed into a 2 ml microtube, a solution containing 0.5% HTAB in 50 mM phosphate buffer (pH 6.0) was added at a volume ten times the wet weight of the mucosa, and the mucosa were homogenized for approximately 1 min (Polytron). The resulting homogenate was freeze-thawed three times (frozen at −80 °C, then allowed to attain room temperature), then centrifuged for 15 min at 1400 × *g* at 4 °C. The homogenate (0.3 ml) was placed in a glass cuvette containing 0.6 ml of distilled water, 0.3 ml of 2 mg/ml DMB, 0.3 ml of 30% hydrogen peroxide, in 50 mM phosphate buffer (pH 6.0), mixed, and absorbance was measured after 30 and 90 s at a wavelength of 460 nm. MPO levels were calculated by subtracting the absorbance measured at these two time points; peroxidase was used as the standard.

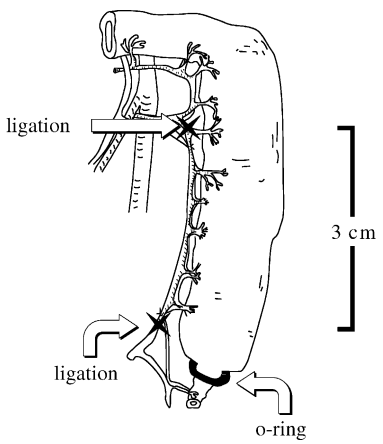

Fig. 1. Schematic drawing of rat descending colon showing positions of the o-ring and ligation sites.

2.4. TBARS measurement

The TBARS levels in the colon mucosal membrane were measured according to the method of Ohkawa et al. (1979). Briefly, the epithelial mucosa of the descending colon were scraped with a slide glass under ice-cold conditions and then put in a 2-ml microtube. Ice-cold phosphate-buffered saline (PBS), 10 times the wet weight of the mucosa, was added and the tissue homogenized for approximately 1 min (Polytron). Six hundred and fifty microliters of a 1.2% sodium dodecyl sulfate/0.8% BHT/0.8% TBA/distilled water (2:0.5:15:17, v/v), 150 μl of 20% acetate buffer solution (pH 3.5) and 200 μl of the homogenized mucosa or 5–75 μM TMP were added to a 10-ml centrifuge tube. The centrifuge tubes were left for 1 h at 4 °C and then heated for 1 h at 110 °C using a block heater (MG-2000, Tokyo Rikakikai Co., Tokyo, Japan). Next, 1.0 ml of butanol/pyridine (15:1, v/v) was added and the tubes centrifuged for 10 min at 320 × *g*, and the absorbance of the supernatant (200 μl) was measured using a multiplate spectrophotometer at a wavelength of 532 nm.

2.5. Protein assay

Protein content in the epithelial mucosa was determined with a BCA assay kit (Pierce, Rockford, IL, USA). Bovine serum albumin was used as the standard.

2.6. Histopathological assessment of ischemic descending colon

Dissected colons were cut into three 5-mm pieces with a stainless steel razor blade (FH-20, Feather, Osaka, Japan). All three pieces were placed in a fixative solution (10 ml 4% paraformaldehyde containing 1% glutaraldehyde dissolved in PBS, pH 7.4) for 4 h at room temperature and agitated constantly. After discarding the fixative, the pieces were sequentially incubated in the following solutions under constant agitation: PBS for 30 min, 50% ethanol for 30 min, 70% ethanol for 1 h, 90% ethanol for 1 h, 95% ethanol for 1 h, 100% ethanol for 1 h, 100% ethanol/HQM (1:1, v/v) for 1 h, and HQM (65% (v/v) hydroxypropyl methyl methacrylate, 10% (v/v) Quetol-523, 25% (v/v) methyl methacrylate, and 0.1% (w/v) QCU-1) for 4.5 h. All of these procedures were carried out in a total volume of 10 ml and at room temperature. Each piece of colon was placed into a flat gelatin capsule (No. 00, Lilly Co., Nisshin EM, Tokyo, Japan) filled with HQM, then cured for 20 h at 60 °C. The resulting block of embedded tissue was cut into 2-μm thick sections with a glass knife and rotary microtome (PR-50, Yamato Kohki Kogyo, Saitama, Japan). Sections were mounted on glass slides, then stained with hematoxylin and eosin. After drying, pathological changes in the stained colon sections were determined using a microscope.

2.7. In vitro membrane permeability studies

Rats were injected intraperitoneally with an anesthetic dose of sodium pentobarbital, then euthanized by bleeding the main abdominal artery. The descending colon was immediately excised with scissors, cut into ∼4 cm lengths, and flattened with the scissors. After the mucus was removed with Dulbecco's PBS, the sheet of colon was set between Ussing-type diffusion chambers (No. 3440 s, Navicyte Inc., NV, USA). PBS was used as the buffer on both serosal and mucosal sides of the chambers; either 1 mM antipyrine, 1 mM FD-4 or 4 mM lucifer yellow was included in the buffer on the mucosal side of the chamber. The total volume in each chamber was 3 ml. During the experiments, both chambers were maintained at 37 °C and bubbled with O_2/CO_2 (95:5, v/v). A 200 μl sample was taken from the receptor side after 20, 30, 40, and 50 min of incubation, filtered with a Millipore filter (LCR 13-LH, Millipore Japan, Tokyo, Japan), and then 30 μl was injected into an HPLC system for antipyrine assay, meanwhile, 150 μl was put in a microplate for FD-4 or lucifer yellow assay.

The permeation rates of antipyrine, FD-4, and lucifer yellow were expressed as an apparent permeability coefficient (P_{app}) according to the following equation:

$$P_{app} = \frac{dC}{dt}\frac{V}{C_0 A}$$

where dC/dt is the change in concentration per unit time (nmol/ml per second); V is the solution volume of the receptor side (3 ml); C_0 is the initial substrate concentration of the donor side (1 or 4 μmol/ml); and A is the apparent surface area of the descending colon sheet (1.2 cm^2).

2.8. In vivo absorption study

Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.), then 150 μl of blood was collected from the subclavian vein and used as a blank (control) sample. After administration of antipyrine, another 150 μl of blood was sampled from the subclavian vein. Plasma (60 μl) was separated from the blood by centrifugation at 1680 × g for 10 min. The samples were stored at −25 °C until analysis.

2.8.1. Intravenous administration of drug

Antipyrine (2.0 mg/ml) dissolved in PBS was administered i.v. (0.75 mg dose per kg body weight) with a 0.25 ml syringe and a needle. Blood samples (150 μl) were collected into polyethylene tubes containing small amounts of EDTA-3Na at the following times after antipyrine administration: 2, 5, 10, 20, 30, 45, 60, 90, 120, 180, and 240 min.

2.8.2. Drug administration in colon

We first opened the rat's abdomen with scissors, then removed feces from the descending colon by flushing the lobular portion of the transverse colon with PBS. The descending colon was ligated at two ends with suture, and freshly dissolved antipyrine (2.0 mg/ml in PBS) was infused into the descending colon (0.75 mg dose/kg). The abdomen was immediately closed with surgical clips. Blood samples (150 μl) were collected in polyethylene tubes containing small amounts of

EDTA-3Na at the following times after antipyrine administration: 5, 15, 30, 60, 90, 120, 180, and 240 min.

2.9. Determination of plasma antipyrine concentrations via HPLC

Plasma (60 μl) from rats in the in vivo study was collected and mixed with 20% TCA (60 μl) and PBS (50 μl) in a 1.5-ml eppendorf tube. The tubes remained at room temperature for 10 min, then were centrifuged at 2500 × *g* for 5 min. The supernatant (30 μl) was collected and antipyrine concentrations were measured by HPLC (LC-9A, Shimadzu, Kyoto, Japan). The following HPLC conditions were used: (1) column specifications, YMC-Pack Pro C18 AS-302 (ODS, 4.6 mm i.d., 150 mm length), (2) mobile phase, 250 mM acetate buffer (pH5.0)/methanol (68:32, v/v), (3) flow rate, 0.8 ml/min, (4) wavelength, 254 nm, and (5) column temperature, 40 °C.

2.10. Determination of FD-4 and lucifer yellow concentrations

FD-4 and lucifer yellow concentrations were measured with a multiplate reader (Ascent CF, Dainippon Pharmaceutical Co., Osaka, Japan) at excitation and emission wavelengths of 485 and 527 nm for FD-4 and 430 and 527 nm for lucifer yellow, respectively.

2.11. Pharmacokinetic analysis

Pharmacokinetic parameter calculations were based on the two-compartment model. The plasma concentration profile of antipyrine after i.v. administration was fit to Eq. (1) and the profile after colon administration to Eq. (2) using a non-linear least-squares program MULTI (Yamaoka et al., 1981); parameters A, B, A', B', α, β, and k_a were obtained. Antipyrine was assumed to be eliminated from the central compartment according to:

$$C_p = A\,exp(-\alpha t) + B\,exp(-\beta t) \quad (1)$$

$$C_p = A'\,exp(-\alpha(t - t_{lag})) + B'\,exp(-\beta(t - t_{lag})) - (A' + B')exp\,(-k_a(t - t_{lag})) \quad (2)$$

where C_p is the plasma concentration of antipyrine at time t or $t - t_{lag}$. The t_{lag} means a lag time. A and B are ordinate intercepts, and α and β are the corresponding first-order disposition rate constants. A' and B' were calculated as follows:

$$A' = \frac{FDk_{20}(k_{21} - \alpha)}{V_1(k_a - \alpha)(\beta - \alpha)}$$

$$B' = \frac{FDk_a(k_{21} - \beta)}{V_1(k_a - \beta)(\alpha - \beta)}.$$

where F is the fraction of dose absorbed, D is dose, k_a is the absorption rate constant, V_1 is the volume of distribution of the central compartment, and k_{20} is the elimination rate constant, and k_{21} and k_{12} are distribution rate constants. The elimination half-life ($t_{1/2}$) was calculated by dividing ln 2 by β. The area under the plasma concentration versus time curve (AUC), the total body clearance (CL_{tot}), and the volume of distribution at

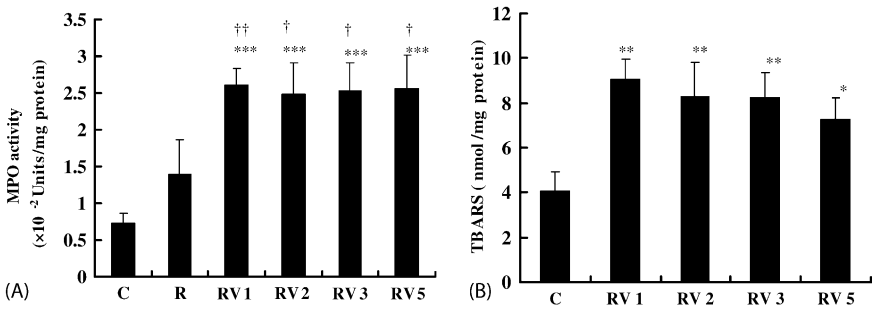

Fig. 2. MPO (A) and TBARS (B) levels in mucosal membrane of descending colon from the different conditions. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. C group; $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ vs. R group. Data are mean ± S.D. ($n = 3$–5). Experimental conditions are presented in Table 1.

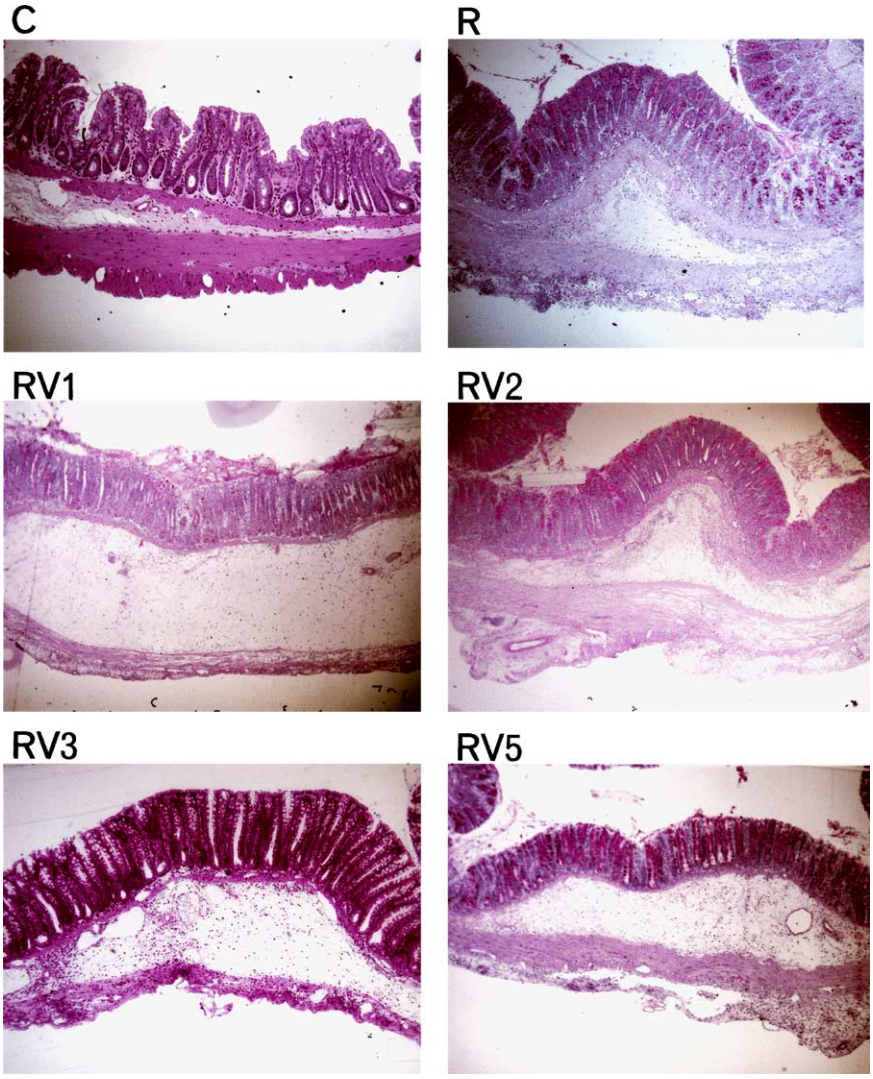

Fig. 3. Representative histological sections of the descending colon segments under the different ischemic conditions (HE stain, ×100). Experimental conditions are presented in Table 1.

steady state (V_d) were calculated by the trapezoidal rule and extrapolated to infinity, $D\alpha\beta/[(A + B)k_{21}]$, and $(1 + k_{12}/k_{21})V_1$, respectively. The bioavailability (BA) of antipyrine was calculated as the ratio of AUC after colon administration to that after i.v. administration.

Changes in the absorption rate of antipyrine after colon administration were estimated by deconvolution method (Veng-Pedersen, 1980) from plasma antipyrine concentration versus time data obtained after i.v. and colon administrations.

2.12. Statistics

All data are expressed as means ± standard deviation (S.D.). Groups of data were statistically compared using an analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons

test. Values of $P < 0.05$ were regarded as significant.

3. Results

3.1. MPO and TBARS levels during ischemia

Fig. 2 shows the changes in MPO (Fig. 2A) and TBARS (Fig. 2B) levels in the epithelial membrane of the large intestine under different ischemic conditions. The sham-operated rats (C group) had a mean MPO level of 0.73 ± 0.14 ($\times 10^{-2}$ U/mg protein). The mean MPO levels measured in the R, RV1, RV2, RV3, and RV5 groups were, respectively, 1.39 ± 0.48, 2.60 ± 0.23, 2.48 ± 0.42, 2.53 ± 0.38, and 2.56 ± 0.45 ($\times 10^{-2}$ U/mg protein). The TBARS levels in RV1, RV2, RV3, and RV5 groups were increased to 7.3–9.0 nmol/mg protein compared with 4.06 ± 0.87 nmol/mg protein in C group. Constricting the intestine with an o-ring increased MPO levels (R group). Moreover, elevated MPO and TBARS levels were maintained by ligation of the mesenteric marginal vessel after o-ring attachment (RV1–RV5 groups).

3.2. Histopathological changes in the descending colon

Fig. 3 shows the various histopathological changes we observed in the descending colon under the different ischemic conditions, as revealed by hematoxylin–eosin (HE) staining. Mucosal tissue samples from the C group appeared normal. In contrast, samples from the R and RV groups displayed hypertrophy of the subcutaneous tissues. Although we observed some sloughing of epithelial cells in groups with vessel ligation, it was no greater than that in the sham operated group.

3.3. In vitro membrane permeability

To clarify how the duration of ligation affects membrane permeability of antipyrine, the apparent permeability coefficient of antipyrine was measured during various ischemic conditions with Ussing-type diffusion chambers (Fig. 4). The P_{app} values measured in descending colons from the RV1, RV3 and RV5 groups did not recognize the significant differences compared to those measured in the C group. Because

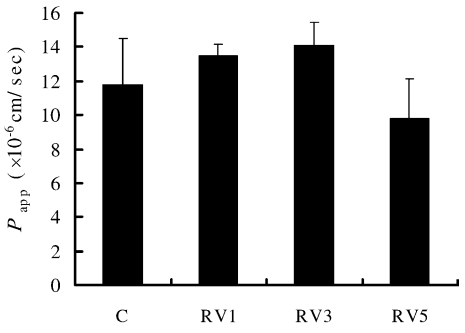

Fig. 4. Apparent permeability coefficient (P_{app}) values of antipyrine in descending colon as measured in Ussing-type diffusion chambers. Data are mean ± S.D. ($n = 4$). Experimental conditions are presented in Table 1.

antipyrine membrane permeability is extremely high, it is difficult to precisely measure the permeability increase resulting from membrane damage by comparing the antipyrine P_{app} values. To solve this problem, we investigated the membrane permeability of FD-4 (molecular weight: 4300) and lucifer yellow (molecular weight: 457.25), low membrane-permeable substrates in which the molecular weight differs, in the same manner as that done for antipyrine (Fig. 5). The C group had a FD-4 P_{app} value of 0.26 ± 0.03 ($\times 10^{-6}$ cm/s) and a lucifer yellow P_{app} value of 1.45 ± 0.07($\times 10^{-6}$ cm/s). These values gradually increased with increasing ligation times. The P_{app} values of both substrates in the RV5 group were significantly high compared with those measured in the C group.

3.4. In vivo absorption study

3.4.1. Intravenous administration study

Fig. 6 shows the time course of plasma antipyrine concentration after i.v. administration at a dose of 0.75 mg/kg. Concentrations declined in a double-exponential manner after antipyrine administration. The pharmacokinetic parameters of antipyrine were calculated using a two-compartment model and the changes in the curve. When the curves were extrapolated to time zero, plasma antipyrine concentrations in distribution and elimination phases were 0.96 ± 0.21 μg/ml and 0.76 ± 0.03 μg/ml, respectively. The slopes in the phases were $16.7 \pm 7.50\ h^{-1}$ and $0.23 \pm 0.5\ h^{-1}$, respectively. The pharmacokinetic

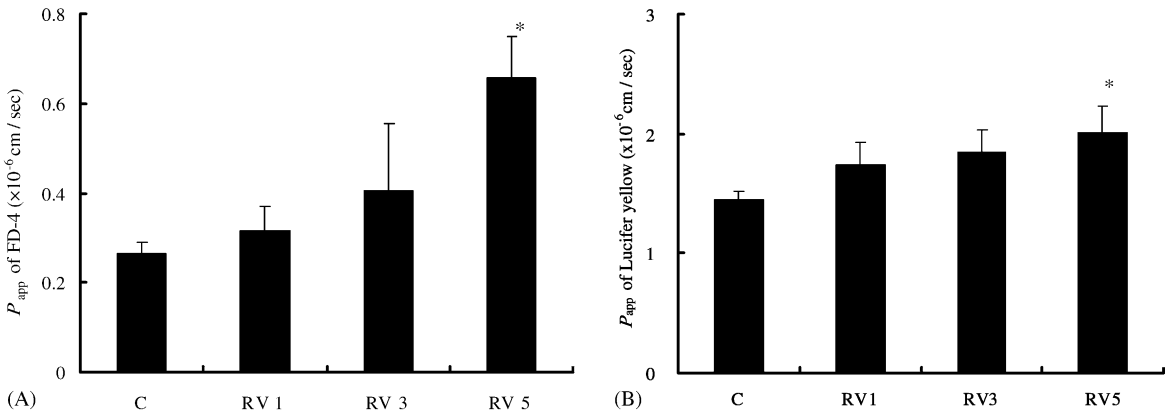

Fig. 5. Apparent permeability coefficient (P_{app}) values of FD-4 (A) and lucifer yellow (B) in descending colon as measured in Ussing-type diffusion chambers. Data are mean ± S.D. ($n = 3$). Experimental conditions are presented in Table 1.

parameters of antipyrine after i.v. administration are summarized in Table 2.

3.4.2. Descending colon administration study

Fig. 7 shows the time course of plasma antipyrine concentration after administration at a dose of 0.75 mg/kg onto the descending colon. In the C group, we detected antipyrine in the plasma 5 min after administration. The C_{max} and T_{max} were 0.84 ± 0.08 μg/ml and 15 min, respectively. Plasma antipyrine concentrations continued to decrease slightly with time. In all RV groups, however, we failed to detect antipyrine in the plasma within 30 min of administration. In RV1, RV3, and RV5 groups, we first detected plasma antipyrine 90, 60, and 30 min, respectively, after drug administration.

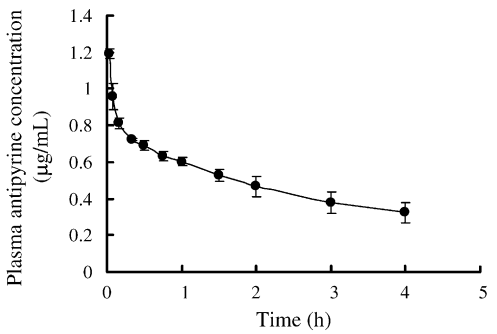

Fig. 6. Time course of plasma antipyrine concentration after intravenous administration of antipyrine (0.75 mg/kg) into normal rats. Data are mean ± S.D. ($n = 3$).

Pharmacokinetic parameters of antipyrine after colon administration are presented in Table 3. The k_a values decreased in all RV groups compared to those of the C group. These values, however, tended to shift towards the k_a of the C group as ligation times increased. V_d, AUC, CL_{tot}, and BA values also tended to approach corresponding control values (C group) as ligation times increased.

As the results showed different plasma antipyrine concentrations versus time profiles, absorption rates of antipyrine from colon were evaluated by a deconvo-

Table 2
Pharmacokinetic parameters of antipyrine after intravenous administration of antipyrine (0.75 mg/kg dose) into normal rats

Parameter	Value
A (μg/ml)	0.96 ± 0.21
α (h^{-1})	16.7 ± 7.50
B (μg/ml)	0.76 ± 0.03
β (h^{-1})	0.23 ± 0.05
k_{12} (h^{-1})	8.86 ± 4.14
k_{21} (h^{-1})	7.54 ± 3.51
k_{10} (h^{-1})	0.51 ± 0.06
$t_{1/2}$ (h)	1.38 ± 0.16
V_d (ml/kg)	949 ± 30
AUC (μg h/ml)	2.02 ± 0.15
CL_{tot} (ml/h/kg)	224 ± 48

Pharmacokinetic parameters were calculated based on the two-compartment model. Each parameter, $t_{1/2}$, V_{app}, and CL_{tot}, was calculated as follow equations: $t_{1/2} = ln\,2/k_{10}$, $V_{app} = V_1/(1 + k_{12}/k_{21})$ and $CL_{tot} = D\alpha\beta/[(A + B)k_{21}]$. AUC was calculated in the trapezoid formula. Data are mean ± S.D. ($n = 3$).

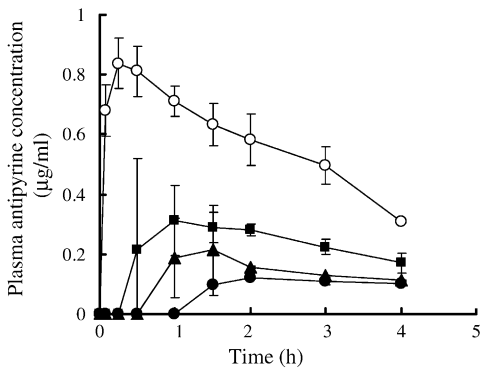

Fig. 7. Time courses of plasma antipyrine concentration after administration of antipyrine (0.75 mg/kg) onto the rat descending colon. Data are mean ± S.D. ($n = 4$). Symbols: (○) C group; (●) RV1 group; (▲) RV3 group; (■) RV5 group.

lution method using both i.v. and colon administration data. The initial absorption rate in C group reached to be 52.3 ± 6.4 mg/h. On the other hand, the initial absorption rates in RV1, RV3, and RV5 groups except for each lag time were 1.21 ± 0.04 mg/h, 1.90 ± 0.38 mg/h, and 3.21 ± 1.25 mg/h, respectively.

4. Discussion

In the large intestine, reperfusion via collateral circulation is thought to occur rapidly within the ischemic region, strongly suggesting that reperfusion injury is caused by secondary blood flow. Based on this premise, Nagahata et al. (1999) developed a rat model of ischemic colitis by attaching o-rings around the distal rectum at the rectovesical pouch and mesenteric marginal vessels of the descending colon. In the present study, we based our experimental protocol on this model, which emphasizes that ligation of the mesenteric marginal vessels is critical for initiating ischemic colitis and especially for developing collateral circulation in the large intestine. Under such conditions, reperfusion injury most likely occurs as a result of free radical generation. Indeed, the extent

Table 3
Pharmacokinetic parameters of antipyrine after administration of antipyrine (0.75 mg/kg dose) onto the descending colon of rats in the experimental and control groups

Parameter	Group			
	C	RV1	RV3	RV5
A (μg/ml)	0.36 ± 0.04	0.106 ± 0.005**	0.075 ± 0.042**	0.020 ± 0.019**
α (h^{-1})	0.64 ± 0.30	0.023 ± 0.003**	0.038 ± 0.008**	0.105 ± 0.058*
B (μg/ml)	0.56 ± 0.12	0.14 ± 0.02**	0.70 ± 0.07†††	0.41 ± 0.09†,‡
β (h^{-1})	0.09 ± 0.01	1.06 ± 0.03	2.78 ± 1.44*	0.37 ± 0.21‡
k_a (h^{-1})	17.6 ± 4.5	1.91 ± 0.06***	3.50 ± 1.11**	7.47 ± 3.95**
k_{12} (h^{-1})	0.16 ± 0.07	0.35 ± 0.09	0.80 ± 0.11***,††	0.001 ± 0.001‡‡‡
k_{21} (h^{-1})	0.45 ± 0.23	0.62 ± 0.01	0.81 ± 0.45	0.11 ± 0.05‡
k_{10} (h^{-1})	0.14 ± 0.02	0.040 ± 0.004	0.13 ± 0.04	0.36 ± 0.19†
$t_{1/2}$ (h)	5.05 ± 0.67	17.3 ± 1.62***	5.62 ± 1.79†††	2.26 ± 1.03†††
V_d (ml/kg)	816 ± 78	2695 ± 74.1***	1538 ± 617	1636 ± 639
AUC (μg h/ml)	2.30 ± 0.36	0.25 ± 0.07***	0.42 ± 0.10***	0.75 ± 0.24***
CL_{colon} (ml/h/kg)	329 ± 55	3184 ± 1101***	1874 ± 527*	1062 ± 332
BA (%)	105 ± 18	12.5 ± 3.6***	20.8 ± 5.1***	37.4 ± 11.7***

Pharmacokinetic parameters were calculated based on the two-compartment model. CL_{colon} was calculated by D/AUC. Data are mean ± S.D. ($n = 4$).

* $P < 0.05$ vs. C group.
** $P < 0.01$ vs. C group.
*** $P < 0.001$ vs. C group.
† $P < 0.05$ vs. RV1 group.
†† $P < 0.01$ vs. RV1 group.
††† $P < 0.001$ vs. RV1 group.
‡ $P < 0.05$ vs. RV3 group.
‡‡‡ $P < 0.001$ vs. RV3 group.

of inflammation is typically gauged by peroxidase activity and acute inflammation (Grisham et al., 1986; Granger, 1988; Zimmerman et al., 1990). Further supporting this, increases in free radicals have also been measured in the large intestine during ligation of the mesenteric marginal vessel (no treatment of reperfusion process) (Ichihara et al., 1994).

MPO levels are closely related to the histopathological damage observed in the intestinal mucosa after experimentally induced ischemic injury (McConnico et al., 1999; Musemeche et al., 1995). Similarly, TBARS are known as an index of lipid peroxidation in intestinal mucosa (Bhaskar et al., 1995; Nalini et al., 1993; Janero, 1990). Because one of the mechanisms by which free radicals damage the cells is through peroxidation of membrane lipids. Therefore, to determine whether prolonged ischemia (e.g., duration of ligation) contributes to tissue damage, we measured MPO and TBARS levels in the epithelial mucosa of the descending colon. We found that MPO and TBARS levels rose regardless of the duration of the mesenteric marginal vessel ligation. These results were supported by similar reports. Namely, MPO level of intestinal mucosal membrane showed the maximum value at 2 h after reperfusion in cats, and then the MPO level was decreased after 3 h of reperfusion (Grisham et al., 1986). They indicated the possibility that the metabolism of MPO did not advance, and the MPO production decreased from the studies using allopurinol and SOD. The changes in TBARS levels in colonic mucosa after vascular ligation were maximum at 3 h and then decreased with an increase of ligation period in rats (Nagahata et al., 1999). By the estimation from resembled phenomena in these reports, it was guessed that the MPO and TBARS levels in our studies did not increase in time dependence.

Upon histopathological assessment of the descending colon with HE stain, we failed to detect histopathological differences, such as evident sloughing or edema of the epithelial membrane, in any of the RV groups. Ligation for 1 h failed to exacerbate existing mucosal injury. Some have hypothesized that intestinal tissue MPO levels reflect granulocyte infiltration into the intestine (McConnico et al., 1999). Consistent with this hypothesis is our observation that granulocytes flooded the descending colon following ligation. Taken together, our MPO, TBARS levels and observations with HE staining suggest that epithelial mucosa damage was not affected by the ligation duration (1–5 h).

Drug absorption in the intestinal tract depends mainly on membrane permeability and blood flow rates within the epithelial mucosa. Intestinal absorption of antipyrine strongly depends on blood flow but not on epithelial membrane permeability (Takahashi et al., 1988). In our in vitro membrane permeability study, we observed that mucosal damage resulting from 1 to 5 h of vessel ligation barely affected the antipyrine P_{app} value when compared control conditions. In fact, we failed to observe any remarkable differences in the P_{app} values among all groups. Thus, in the large intestine, absorption levels and antipyrine pharmacokinetics may reflect blood flow status during ischemia. In addition, some report that reperfusion induces mucosal barrier disruption (Chen et al., 2003; Payne and Kubes, 1993). It is difficult, however, to assess accurately ligation-related mucosal damage by measuring the P_{app} of antipyrine. Thus, we assessed mucosal barrier disruption during increasing periods of ischemia by examining the membrane permeability of FD-4 and lucifer yellow in which the molecular weight differs. Membrane permeability of both substrates gradually increased with longer ligation periods, suggesting that mucosal barrier integrity decreased during prolonged ischemic conditions. The elevation in permeability of FD-4 and lucifer yellow is consistent with the report that intestinal permeability of hydrophilic drugs increases with inflammation (Venkatraman et al., 2000). In terms of antipyrine permeability in which high P_{app} values were measured (approximately 20–60 times of FD-4 P_{app} values), these findings indicate that the effect of mucosal damage on the membrane permeability of antipyrine can be disregarded.

In considering the processes of blood flow restriction via ischemia and blood flow enhancement via reperfusion in the large intestine, it is assumed that antipyrine pharmacokinetics will also be affected by ischemic status. In fact, ligation duration affected antipyrine pharmacokinetics. With shorter ligation times, antipyrine appeared in the plasma 60–90 min after administration, whereas with longer ligation times, antipyrine appeared 30 min after administration, suggesting that antipyrine was absorbed via collateral circulation of the ischemic region. As the reason why plasma concentration of antipyrine after the lag time rapidly heightened, the antipyrine administered in colon leaked out and then the leaked antipyrine might be absorbed from the peritoneal cavity. However, the

kinetic patterns of antipyrine in RV5 group did not change in the absence and presence of the treatment which lays a dried cotton in the colon circumference in order to prevent the leak of antipyrine to peritoneal cavity (data not shown). Therefore, the contribution of intraperitoneal absorption to the pharmacokinetics of antipyrine can be disregarded. This conclusion is reasonable since collateral veins can form in the large intestine (Leung et al., 1992). Taken together, our findings suggest that, in the colon, reperfusion follows ischemia. In agreement with this hypothesis is our observation that changes in antipyrine absorption kinetics, specifically the absorption rate constant k_a, depends on blood flow in the intestine. It was reported that the blood flow in rat colon lowered at about 40% of control group under the ischemic condition, and then the blood flow was recovered to 65% of the control at 1 h after reperfusion (Idei et al., 1995). Furthermore, the recovery rate of blood flow in rat jejunum was closely related to ischemic period, and the recovery rate using a laser doppler flowmeter was the highest when the ischemic period was set to 30 min (Obata et al., 1995). Based on these reports, the retarded appearance of antipyrine in blood is guessed with that it depended on the degree of recovery of the blood flow in colon. Therefore, it seems that the blood flow in the group of 1 h ligation (RV1) was the lowest among all RV groups. However, we found no correlation between other rate constants, such as k_{12}, k_{21}, and k_{10}, and ligation period, indicating that, after antipyrine enters systemic circulation, its transfer between compartments is not directly related to ligation period. On the other hand, decreases in AUC and BA values closely paralleled changes in k_a values, suggesting that antipyrine absorption during reperfusion was limited by the prolonged ligation period. It seems to be the result which the initial absorption rate of antipyrine calculated from these parameters clearly reflects.

In conclusion, ligation periods of 1–5 h failed to promote epithelial membrane damage. Interestingly, our intestinal absorption experiments showed that prolonged ligation tended to improve antipyrine absorption from the intestine. This change in antipyrine absorption was defined by certain pharmacokinetic parameters (k_a, AUC, and BA). This, along with our finding that ligation period did not affect the P_{app} value of antipyrine, emphasizes the benefit of having a firm understanding of antipyrine pharmacokinetics in any particular model of ischemia, since such parameters can be used to assess blood flow status at an inflammation site. Antipyrine pharmacokinetics, therefore, can be used as an index to evaluate drug delivery efficacy in this intestinal ischemia reperfusion model.

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