

**Prediction of human intestinal absorption of the prodrug temocapril by *in situ*
single-pass perfusion using rat intestine with modified hydrolase activity**

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Abbreviations: ACE, angiotensin-converting enzyme; AUC, area under the curve; BNPP, bis-*p*-nitrophenyl phosphate; BSA, bovine serum albumin; CES, carboxylesterase; CL, clearance; DFP, diisopropyl fluorophosphonate; DMSO, dimethyl sulfoxide; FD-4, fluorescein isothiocyanate dextran 4000; HPLC, high performance liquid chromatography.

Abstract

Intestinal absorption of temocapril, a prodrug of temocaprilat, was evaluated in an *in situ* rat jejunal perfusion model under various conditions of luminal pH and in the presence and absence of carboxylesterase-mediated hydrolysis. Temocapril was more easily taken up by mucosal cells at a luminal pH of 5.4 than at pH 6.4 or 7.4, and was extensively hydrolyzed to temocaprilat in mucosal cells. The hydrolysis was limited by the intrinsic clearance and the influx rate at luminal perfusate pHs of 5.4 and 7.4, respectively. Temocaprilat, derived from temocapril, was transported into both mesenteric vein and jejunal lumen according to pH partition theory. The net absorption of both temocapril and temocaprilat was highest at a luminal perfusate pH of 5.4. When both the luminal and venous fluid were at pH 7.4, temocaprilat was transported about three-fold faster into the lumen than into the vein, due presumably to the greater surface area of the brush border membrane because of the presence of microvilli. Under carboxylesterase-inhibited conditions, the hydrolysis of temocapril was inhibited by only 50%. It is postulated that serine esterases located on the membranes of the epithelial cells were responsible for the residual hydrolysis. We have confirmed that temocapril is most easily absorbed in the proximal intestine after meals, due to prolongation of the gastric emptying time, the lower intraluminal pH caused by secretion of bile acid and the interaction between serine esterases and the digesta.

Introduction

Prodrug modification is a major factor in drug design that can improve, delay, prolong, control or target the action of the parent drug (Ettmayer *et al.*, 2004; Testa, 2004). The prodrug, itself an inactive compound, is activated by enzymes in the body, to allow the pharmacological effect of the parent drug to be delivered at the target sites. The majority of prodrugs are ester derivatives, involving modification of carboxyl and hydroxyl groups in the parent drugs, because of the ubiquitous expression of hydrolase, which is required for their activation, in many organs (Imai, 2006; Imai and Hosokawa, 2010). Most prodrugs used in clinical practice are developed to improve the oral bioavailability of the parent drug, e.g., valacyclovir and prulifloxacin (Liederer and Borchardt, 2006). These prodrugs are more easily taken up into the intestine and liver due to their membrane permeability, which depends on their lipophilicity, and are mostly activated by first-pass metabolism before entering the systemic circulation.

Previously, we have reported the extensive hydrolysis of an ester prodrug in the intestine in an *in situ* rat jejunal single-pass perfusion experiment (Masaki *et al.*, 2006). In that report, isovaleryl propranolol was easily taken up into rat jejunal mucosal cells by passive diffusion and completely hydrolyzed at a rate limited by its membrane permeability. Both the propranolol and isovaleric acid derived from isovaleryl propranolol were present at their highest concentrations in epithelial cells, compared with blood vessels and the intestinal lumen. Propranolol (pKa 9.44) and isovaleric acid (pKa 4.77) were predominantly transported into the lumen and vein, respectively, by passive diffusion according to pH partition theory. These results suggested that successful oral prodrugs are stable in the intestine and rapidly hydrolyzed in the liver, and that extensive intestinal hydrolysis would be able to provide adequate bioavailability for an acidic but

not a basic parent drug.

In the present study, we examined the possibility of increasing the bioavailability of a prodrug that is hydrolyzed to an acidic parent drug in the intestinal mucosa. Temocapril, an angiotensin-converting enzyme (ACE) inhibitor, was selected as a model prodrug. The parent drug of temocapril is temocaprilat, a dicarboxylic acid compound. It has been reported that the oral bioavailability of temocapril is 81% and 65% in rat and man, respectively (Koike *et al.*, 1992; Püchler *et al.*, 1998). The absorption of temocapril could be achieved by improving its membrane permeability in the intestinal mucosa. We have previously reported that carboxylesterase (CES) is one of the major enzymes involved in the hydrolysis of temocapril in the intestine and liver (Imai *et al.*, 2005). In man, temocapril is hardly hydrolyzed by human CES2 isozyme (hCE2), which is expressed in the intestine, while it is rapidly hydrolyzed by human CES1 isozyme (hCE1), the major CES isozyme in the liver (Imai *et al.*, 2006); therefore, it may be expected that temocapril is absorbed in the intestine as an intact prodrug and is then rapidly hydrolyzed to temocaprilat in the liver. In contrast, rat intestine contains more than three CES2 isozymes (Sanghani *et al.*, 2002; Furihata *et al.*, 2005) and shows more extensive hydrolytic activity than human intestine (Taketani *et al.*, 2007).

In this study, the absorption and hydrolysis of temocapril was evaluated in an *in situ* rat jejunal single-pass perfusion model, under various conditions of luminal pH, to clarify the direction of efflux of temocaprilat produced in epithelial cells. The relative areas of the brush border membrane and the plasma membrane were determined by comparing the efflux rates of temocaprilat from mucosal cells into the intestinal lumen and blood vessels. In order to predict the human intestinal absorption of temocapril, rat jejunum perfusion experiments were performed in the absence of CES-mediated hydrolysis, following

treatment with a specific CES inhibitor. Finally, we examined the contribution of serine proteases other than CES, in the intestinal hydrolysis of temocapril.

Materials and Methods

Materials

Temocapril and temocaprilat were kindly provided by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA, fraction V), and fluorescein isothiocyanate dextran 4000 (FD-4) were purchased from Sigma Aldrich (St. Louis, MO, USA). HEPES and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Bis-*p*-nitrophenyl phosphate (BNPP) and pentobarbital sodium salt were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were of analytical grade.

Animals

Male Wistar rats (230–280 g, 8 weeks of age) were housed in an air-conditioned room with free access to commercial chow and tap water. Animals were fasted for 18–24 h before the experiments.

***In situ* intestinal single-pass perfusion**

The perfusion study was carried out according to a previously described method (Masaki *et al.*, 2006). Briefly, rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium salt dissolved in physiological saline (0.5 ml). A segment of about 10 cm of upper jejunum was isolated, and both ends of the jejunal loop cannulated with teflon tubing (3 mm i.d., 5 mm o.d.) after flushing out the intestinal contents with warmed physiological saline. The superior mesenteric artery and the portal vein were cannulated with polyethylene tubes (PE10 and PE50, respectively) to enable perfusion of the blood

vessels. The cannulated intestinal segment was isolated and immersed in Krebs–Henseleit bicarbonate buffer containing 3% BSA and 10 mM D-glucose (pH 7.4), warmed to 37°C.

Krebs–Henseleit bicarbonate buffer was used as the vascular perfusate at a flow rate of 2.5 ml/min. The jejunal loop was perfused with phosphate buffer adjusted to pH 5.4, 6.4 or 7.4 at a flow rate of 0.2 ml/min. The osmolality of the luminal perfusate containing temocapril or temocaprilat (100 µM) was adjusted to 290 mOsm before the addition of FD-4 (0.1 mg/ml), a nonabsorbable marker, to enable monitoring of the luminal volume. The perfusates from the luminal segment and vascular outflow were collected at appropriate points. A CES-inhibited condition was achieved by pre-perfusion with BNPP (400 µM) for 40 min, in accordance with previously reported methodology (Masaki *et al.*, 2007). The chemical degradation of temocapril was negligible during the perfusion experiment.

For the determination of temocapril and temocaprilat concentrations, luminal perfusate samples (0.5 ml) were diluted with equal volumes of acetonitrile and shaken. A sample of the supernatant (180 µl) was added to 3.4 M phosphoric acid (20 µl) and injected onto an HPLC column. FD-4 levels (0.1 mg/ml) in the luminal samples were determined using a fluorescence spectrometer (F-4500, Hitachi High-Technologies Co., Tokyo, Japan). Samples (5 ml) of vascular perfusate were treated with 0.67 M phosphoric acid (0.5 ml) and extracted with ethyl acetate (20 ml). The organic phase was separated and evaporated. The resulting residue was re-dissolved in 180 µl of a solution of acetonitrile–water (1:2 v/v) with 3.4 M phosphoric acid (20 µl) and injected onto an HPLC column. After the perfusion period, the intestinal segment was cut open and washed with ice-cold phosphate buffer (pH 7.4). A portion of the intestinal mucosa

(350–400 mg) was immediately stripped and homogenized in a solvent (6 ml) consisting of acetonitrile–methanol (1:2 v/v). After centrifugation at 1500 g for 10 min, the supernatant (4 ml) was evaporated. An HPLC sample was made from the residue, using the same method as for the vascular perfusate sample.

Preparation of homogenate and S9 fraction of intestinal mucosa

Rats were anesthetized with ether and sacrificed by exsanguination from the external jugular vein. The duodenum and jejunum were removed and washed with ice-cold 1.15% KCl. The mucosa was stripped, minced and homogenized with three volumes of 50 mM HEPES buffer (pH 7.4) containing 1.15% KCl using a Potter–Elvehjem Teflon pestle under ice-cold conditions. The homogenates were centrifuged at 9000 g for 20 min at 4°C to obtain the supernatant (S9 fraction). Protein contents were determined by the method described by Bradford (1976) using BSA as the standard. The homogenates and S9 fractions were stored at –80 °C until use.

Hydrolysis of temocapril in the rat duodenal and jejunal mucosa

The duodenal and jejunal S9 fractions and homogenates were diluted with 50 mM HEPES buffer (pH 7.4) to the required concentrations. The S9 solutions (200 µl) were pre-incubated at 37°C for 5 min, and the reaction started by adding 2 µl temocapril (final concentration 10–500 µM) dissolved in DMSO. DMSO inhibits hydrolase activity at higher concentrations, but has no effect at 1%. The concentration of DMSO was therefore maintained at 1%. After 20 min incubation, reactions were terminated by adding 200 µl ice-cold acetonitrile. The reaction mixture was centrifuged at 7200 g for 3 min. Phosphoric acid (40 µl, 2.9 M) was added to a 200 µl sample of the supernatant, and the

resultant mixture analyzed by HPLC. Hydrolytic activity was evaluated by measuring the formation of temocaprilat. Kinetic parameters, K_m and V_{max} , were calculated by fitting the data to the Michaelis–Menten equation by nonlinear least-squares analysis, using the MULTI program (Yamaoka *et al.*, 1981).

The hydrolase activities of intestinal homogenate and S9 fractions were inhibited using BNPP, a specific CES inhibitor, as well as the serine protease inhibitors diisopropyl fluorophosphonate (DFP) and paraoxon, by pre-incubation for 5 min prior to the addition of temocapril. The degree of inhibition was calculated as a percentage of control activity.

HPLC analysis

Temocapril and temocaprilat concentrations were determined by HPLC. The HPLC system (Jasco International Co., Ltd., Tokyo, Japan) consisted of a pump (PU-980), an UV detector (UV-970), an autosampler (AS-950), a column oven (CO-965), and ChromNAV data application apparatus (version 1.5). An aliquot of the sample was injected onto a Mightysil RP-18 column (5 μ m, 250 \times 4.6 mm i.d.; Cica-Merck Co., Inc., Tokyo, Japan) and eluted at a flow rate of 0.8 ml/min with methanol (solvent A) and 10 mM phosphoric acid (solvent B) according to the following gradient schedule: 40% solvent A for the first 20 min, a linear gradient from 40 to 55% solvent A over the next 2 min, 55% solvent A for 15 min, a linear gradient from 55 to 70% solvent A over the next 3 min, and 70% solvent A for 5 min. The temperature of the column was maintained at 40°C. The elution times of temocaprilat and temocapril were 23.1 and 41.0 min, respectively. UV detection was performed at 258 nm, and the detection limits for both temocapril and temocaprilat were 10 pmol.

Data analysis

Figure 1 shows the absorption parameters in the *in situ* perfusion experiment, calculated as normalized values for 10 cm intestine, as described in a previous report (Masaki *et al.*, 2006). The rates of appearance of temocapril (v_2) and temocaprilat (v_4) in the mesenteric vein were calculated by $v_2 = Q_b \times C_{b, \text{temocapril}}$ and $v_4 = Q_b \times C_{b, \text{temocaprilat}}$, respectively, where Q_b is the flow rate of vascular perfusion and C_b is the concentration in the mesenteric vein. The rate of disappearance (v_1) of administered compound and the rate of appearance (v_3) of temocaprilat in the intestinal lumen were calculated by:

$$v_1 = Q_l \times (C_{in} - C_{out}), \text{ and } v_3 = Q_l \times C_{l, \text{temocaprilat}},$$

where Q_l is the flow rate of intestinal perfusion, C_{in} and C_{out} are the concentrations of administered compound at the entrance and exit of the jejunal segment, respectively, and $C_{l, \text{temocaprilat}}$ is the concentration of temocaprilat at the exit of the jejunal segment.

The apparent absorption clearance into the mesenteric vein (CL_{abs}) was calculated by:

$$CL_{abs} = Q_b \times AUC_b / AUC_l = \text{absorbed amount} / AUC_l,$$

where AUC_b and AUC_l are the areas under the curve of the administered compound in the mesenteric vein and the intestinal lumen, respectively, at steady state. The degradation clearance of temocapril to temocaprilat in the jejunal mucosa (CL_{deg}) was calculated by:

$$CL_{deg} = Q_l \times AUC_{l, \text{temocaprilat}} / AUC_{l, \text{temocapril}} + Q_b \times AUC_{b, \text{temocaprilat}} / AUC_{l, \text{temocapril}} = \\ \text{amount of temocaprilat} / AUC_{l, \text{temocapril}},$$

where $AUC_{l, \text{temocaprilat}}$ and $AUC_{l, \text{temocapril}}$ are the areas under the curve of temocaprilat and temocapril in the intestinal lumen, respectively. It was assumed that the concentration of administered compound in the intestinal loop decreases according to first-order kinetics when calculating the AUC in the intestinal lumen. $AUC_{b, \text{temocaprilat}}$ is the area under the curve of temocaprilat in the mesenteric vein.

The permeability rate constant (P_{eff}) was calculated by:

$$P_{\text{eff}} \text{ (cm/min)} = Q_1 \times (1 - C_{\text{out}} / C_{\text{in}}) / 2\pi RL,$$

where R is the radius of the segment, assumed to be 0.178 cm (Yamashita *et al.*, 1997), and L is the length of the segment (i.e., 10 cm).

Statistical analysis

Results are expressed as the mean \pm S.D. Data were analyzed using Student's *t*-test with $p < 0.05$ as the minimal level of significance.

Results

Absorption of temocaprilat by rat jejunal single-pass perfusion

When 100 μM temocaprilat was perfused in the rat jejunal lumen at pH 6.4, steady state was achieved after 10 minutes. The rate of appearance of temocaprilat in the blood vessel was 0.193 nmol/min; the concentration of temocaprilat in the luminal perfusate was nearly constant during the experiment. The absorption clearance (CL_{abs}) was calculated as 1.87 $\mu\text{l}/\text{min}$, as shown in Table 1.

Absorption of temocapril by rat jejunal single-pass perfusion

Temocapril (100 μM) was perfused into the jejunal lumen at pH 6.4. The results are shown in Fig. 2 and Table 1. Steady state was achieved after 70 minutes. The rate of disappearance of temocapril from the jejunal lumen (v_1) was 3.89 nmol/min, and the effective permeability coefficient (P_{eff}) was 3.43×10^{-3} cm/min; however, the rate of appearance of intact temocapril in the mesenteric vein (v_2) was 0.147 nmol/min, only 3.8% of the rate of disappearance in the jejunum lumen. Therefore, temocapril molecules taken up into jejunal mucosa were mostly hydrolyzed to temocaprilat in the cell and temocaprilat was then transported into both the jejunal lumen and the mesenteric vein. The rate of appearance of temocaprilat in the blood vessel (v_4) and in the jejunal lumen (v_3) was 1.22 nmol/min and 2.25 nmol/min, respectively, being about 31% and 58% of the disappearance rate of temocapril in the jejunum lumen, respectively (Fig. 2). CL_{deg} was calculated as 35.4 $\mu\text{l}/\text{min}$; this value is 22-fold greater than the CL_{abs} of temocapril (1.59 $\mu\text{l}/\text{min}$). The ratio of CL_{deg} to the sum of CL_{deg} and CL_{abs} is 0.957, indicating nearly complete hydrolysis of temocapril in the mucosal membrane.

Effect of the pH of the luminal perfusates on the absorption of temocapril

The absorption and hydrolysis of temocapril and the efflux of its hydrolysate, temocaprilat, from the mucosal cell were examined in intestinal fluid with the pH adjusted to 5.4, 6.4 or 7.4. The results are shown in Fig. 3 and Table 1. The values of v_1 and P_{eff} were increased at pH 5.4, but were nearly identical at pH 6.4 and pH 7.4, due to nonionic fractions of temocapril with pI values of 3.69 (nonionic form: 85.8% at pH 5.4, 98.4% at pH 6.4 and 99.8% at pH 7.4; Vistoli *et al.*, 2009). The greater influx of temocapril into the mucosal cells at a luminal perfusate pH of 5.4 resulted in higher intracellular concentrations of temocapril at this pH compared with pH 6.4 or 7.4 (Table 1); therefore the highest rate of appearance of temocapril in the blood vessel (v_2) was observed at a luminal fluid pH of 5.4. CL_{deg} increased with increasing pH, and the hydrolysis fraction calculated from $CL_{\text{deg}} / (CL_{\text{deg}} + CL_{\text{abs,temocapril}})$ was smallest at pH 5.4. The intracellular concentration of temocaprilat was about two-fold higher at pH 5.4 than at pH 6.4 and 7.4, and the rate of appearance of temocaprilat (v_4) in the blood vessel was 1.6–2.2-fold faster at pH 5.4 than at pH 6.4 or 7.4. In contrast, the rate of appearance of temocaprilat in the jejunal lumen (v_3) was slowest at pH 5.4.

Interestingly, when the luminal and venous pH were both 7.4, the rate of transport of temocaprilat into the lumen was about three-fold faster than that into the blood, as shown in Fig. 3. Under the same pH conditions in the vascular and luminal perfusates, temocaprilat permeates through brush border membrane and plasma membrane according to the characteristics of each membrane (e.g., surface area and expression of efflux transporters). It seems likely that one reason for the greater permeability of temocaprilat into the lumen is the larger surface area of the brush border membrane, due

to the presence of microvilli.

Hydrolysis of temocapril in rat intestinal S9

Having demonstrated that temocapril was well absorbed in the proximal segment of the intestine, where the luminal pH is around 5, the hydrolysis of temocapril was also evaluated in rat duodenal and jejunal S9. K_m and V_{max} values and the intrinsic hydrolytic clearance, calculated as the V_{max}/K_m , are listed in Table 2. The enzyme kinetic parameters were nearly the same in duodenal and jejunal S9. Hydrolysis in the duodenal and jejunal S9 was inhibited by 89% and 88%, respectively, following the addition of 1 mM BNPP. These data indicate that CES is predominantly responsible for the intestinal hydrolysis of temocapril in the rat.

Intestinal absorption of temocapril by rat jejunal single-pass perfusion in the absence of CES-mediated hydrolysis

It has been reported that temocapril is hardly hydrolyzed to temocaprilat by human intestinal CES (Takai *et al.*, 1997; Imai *et al.*, 2005). Since temocapril is rapidly hydrolyzed by rat intestinal CES, human intestinal absorption of temocapril cannot be predicted by rat single-pass perfusion experiments; therefore, this single-pass perfusion experiment was performed under inhibition of CES-mediated hydrolysis using BNPP, according to previously reported methodology (Masaki *et al.*, 2007). Masaki and co-workers (2007) proposed a methodology whereby rat intestinal CES was inhibited by BNPP; BNPP irreversibly inhibits all CES isozymes with K_i values of 44.9 ± 4.95 nM in rat jejunal S9, but has no effect on other hydrolases such as aminopeptidase. Masaki *et al.* also established that nearly complete inhibition of CES is obtained by pre-perfusion with

BNPP (400 μ M for 40 min). More recently, Ohura *et al.* (2010) have reported that BNPP affects neither active transport, e.g., *p*-glycoprotein, peptide and organic anion transporters, nor passive diffusion in Caco-2 cell monolayers.

The perfusion experiment under inhibition of CES-mediated hydrolysis was performed at pH 5.4 and 6.4, and the results are presented in Table 3. Under CES-inhibited conditions, the disappearance rate of temocapril from jejunal lumen (v_1) is about 60% of control values at both pH 5.4 and 6.4 (2.98 nmol/min vs 4.82 nmol/min at pH 5.4; 2.44 nmol/min vs 3.89 nmol/min at pH 6.4). P_{eff} was also decreased to 2.99×10^{-3} and 2.32×10^{-3} cm/min at pH 5.4 and 6.4, respectively. On the other hand, the intramucosal concentration of temocapril was increased to 20.6 and 15.4 nmol/g tissue at pH 5.4 and 6.4, respectively, and the rate of appearance of temocapril in the mesenteric vein (v_2) was increased from 0.353 nmol/min to 0.862 nmol/min at pH 5.4, and from 0.147 nmol/min to 0.696 nmol/min at pH 6.4. $CL_{\text{abs, temocapril}}$ also increased with increasing v_2 .

Meanwhile, CL_{deg} was decreased by CES inhibition from 34.4 to 24.9 μ l/min at pH 5.4, and from 35.4 to 19.5 μ l/min at pH 6.4; the intracellular concentration of temocaprilat was therefore decreased. However, the intracellular concentration of temocaprilat under CES-inhibited conditions remained at 50–81% of the values in control intestine (Tables 1 and 3). The rates of appearance of temocaprilat into the jejunal lumen (v_3) and mesenteric vein (v_4) were relatively constant, and the overall rate of appearance of temocaprilat ($v_3 + v_4$) was more than 50% of control values. These data suggest that enzymes other than CES, contribute to the hydrolysis of temocapril in rat intestine.

Hydrolysis of temocapril in homogenate and S9 fraction of rat jejunum

pre-perfused with BNPP

In order to elucidate the non-CES enzyme activity in the rat jejunum, a hydrolysis experiment was carried out using the homogenate and S9 fraction prepared from three kinds of jejunum: intact jejunum (without any perfusion), jejunum perfused by buffer (without BNPP), and jejunum pre-perfused with 400 μ M BNPP for 40 min. The results are shown in Table 4. The hydrolysis of temocapril was nearly identical in the intact jejunum and the jejunum perfused by buffer (4.17 and 3.96 nmol/min/mg protein in the homogenate, and 4.46 and 4.28 nmol/min/mg protein in the S9 fraction, respectively). This confirmed that the single-pass perfusion procedure itself had no effect on hydrolytic activity. Interestingly, the activity in the homogenate was nearly the same as that in the S9 sample. The homogenate of whole jejunal mucosa contains cell membrane fragments and nuclei that are absent from the S9 fraction. Since CES is present in the endoplasmic reticulum, CES-mediated hydrolase activity (per mg protein) should be higher in the S9 fraction than in the homogenate. Our results, which show similar activity in the homogenate and S9 fraction, indicate the presence of proteins in the homogenate which possess comparable hydrolytic activity to CES. Jejunum pre-perfused with BNPP exhibited 15% and 52% of the hydrolysis of temocapril in the S9 fraction and homogenate, respectively, compared with the jejunum perfused by buffer. These values are in good agreement with the percentages of activity remaining following the addition of 1 mM BNPP (10.9% in S9 fraction and 47% in homogenate; Table 4). These data show that enzymes other than CES are partly responsible for the hydrolysis of temocapril in rat intestine.

To determine the properties of the enzymes which are involved in the hydrolysis of temocapril, an inhibition experiment was performed. As shown in Fig. 4, hydrolase

activity in homogenates was not inhibited by the further addition of BNPP; this is presumably due to the fact that CES activity had been almost completely inhibited by pre-perfusion with BNPP. The remaining hydrolase activity was nearly completely inhibited by the potent serine protease inhibitors DFP and paraoxon, at only 10 μ M. These data suggest that temocapril is hydrolyzed not only by CES but also by other serine esterases in rat intestine.

Discussion

The prodrug approach has been used successfully to improve the absorption of the ACE-inhibitor temocaprilat. The partition coefficients ($\log P$) between *n*-octanol and phosphate buffer (pH 7.4) are -2.5 and -0.1 for temocaprilat and its prodrug, temocapril, respectively (Shionoiri *et al.*, 2001). The greater membrane permeability of temocapril is due to its higher hydrophobicity. Although the majority of ACE-inhibitors are substrates for peptide transporters (Boll *et al.*, 1994), our preliminary findings suggest that temocapril is not a substrate for PEPT1, but rather an inhibitor thereof (data not shown). We have confirmed that temocapril is partially transported by organic anion transporting polypeptides (OATPs) in Caco-2 cell monolayers (data not shown). The fact that a decrease in the pH of the intestinal lumen led to an increase in the membrane permeability of temocapril indicates its absorption by passive diffusion, due to an increase in the nonionic fraction at lower pH (Vistoli *et al.*, 2009). From the relation between the *in vivo* fraction absorbed in man and the P_{eff} obtained from the rat *in situ* experiment, complete absorption might be predicted in human intestine (Fagerholm *et al.*, 1996).

Temocaprilat, formed from temocapril in epithelial cells, is transported both into blood and into the intestinal lumen. It has been reported that temocaprilat is carried by multidrug-resistance-associated protein 2 (MRP2; Ishizuka *et al.*, 1997) and OATPs (Ishizuka *et al.*, 1998). It is certainly transported across the double-transfected Madin-Darby canine kidney II monolayers that express rat *Oatp4* and *Mrp2* on the basal and apical membranes (Sasaki *et al.*, 2004). Indeed, low concentrations of temocaprilat ($1 \mu\text{M}$) are transported by the carriers mentioned in these reports, but higher concentrations saturate these transporters. When $100 \mu\text{M}$ temocaprilat was applied to

Caco-2 cell monolayers, it permeated equally well in apical to basolateral and basolateral to apical directions (data not shown), indicating that, at high concentrations, temocaprilat will cross the membrane by passive diffusion. As shown in Table 1, the intracellular concentration of temocaprilat in rat intestinal epithelial cells is 17.6–34.2 nmol/g tissue. Temocaprilat may passively diffuse out of epithelial cells, its dicarboxylic acid groups allowing diffusion from an acidic condition to one at a higher pH, according to pH partition theory.

When only the pH in the jejunum was varied (Fig. 3, Table 1), the transport of temocaprilat from epithelial cells into the intestinal lumen was shown to be affected by the pH of the luminal fluid. The fastest rate of transport of temocaprilat from epithelial cells into blood vessels was observed at a luminal pH of 5.4. The ratio of transport of temocaprilat into blood vessel (absorption), to transport into the lumen (secretion) was about 1.67 at a luminal perfusate pH of 5.4, 0.54 at pH 6.4, and 0.33 at pH 7.4. The greatest net absorption of both temocapril and temocaprilat was therefore observed at a luminal pH of 5.4.

When the pH of both the vascular and luminal perfusates was 7.4, temocaprilat formed in the mucosa was transported into the lumen at a three-fold faster rate than into the mesenteric vein. Since there is no pH gradient, the efflux of temocaprilat from the mucosal cell must be dependent on membrane characteristics. At high intracellular concentrations, temocaprilat is transported by passive diffusion, so the extent of the available surface area affects the amount transported. The brush border membrane has a far greater surface area than the plasma membrane due to the presence of microvilli. With propranolol, a passively diffused compound formed from isovaleryl propranolol in the intestinal mucosa, appearance in the lumen is four-fold greater than in the mesenteric vein

(Ohura and Imai, 2010). Hydrolysis in the intestinal mucosa has a negative effect on the absorption of prodrug, because of the 3–4-fold greater efflux of hydrolysate into the lumen.

The highest value (0.972) of the hydrolytic ratio of temocapril, calculated from $CL_{deg} / (CL_{deg} + CL_{abs,temocapril})$, was observed at a luminal fluid pH of 7.4 and the lowest value (0.900) at pH 5.4. As this value approaches 1.0, intracellular hydrolysis is limited almost exclusively by the uptake of prodrug; lower values indicate that intracellular hydrolysis is limited by the intrinsic mucosal hydrolysis. We previously reported that the hydrolytic ratio of isovaleryl propranolol was 0.983 (Masaki *et al.*, 2006). Isovaleryl propranolol is rapidly hydrolyzed in mucosal S9, with 2110 $\mu\text{l}/\text{min}/\text{mg}$ protein of intrinsic clearance; this is 20-fold greater than that of temocapril (108 $\mu\text{l}/\text{min}/\text{mg}$ protein; Table 2). The hydrolysis of temocapril to temocaprilat is limited by the uptake and the intrinsic mucosal hydrolysis of temocapril at luminal fluid pHs of 7.4 and 5.4, respectively. The present data indicate that prodrugs with a greater intrinsic hydrolytic clearance than temocapril (around 110 $\mu\text{l}/\text{min}/\text{mg}$ protein) may be completely hydrolyzed during intestinal absorption.

Both rat and human intestine express only CES2 isozymes (Taketani *et al.*, 2007); in the rat intestine, three major isozymes are present, AB010635, AY034877 and D50580, which have, respectively, 70%, 67% and 70% homology with the amino acid sequence of hCE2 (Sanghani *et al.*, 2002). Although temocapril is hydrolyzed in rat jejunum mucosal S9 at an intrinsic clearance of 108 $\mu\text{l}/\text{min}/\text{mg}$ protein (Table 2), its intrinsic hydrolytic clearance is only 0.324 $\mu\text{l}/\text{min}/\text{mg}$ protein in human intestinal S9 (Imai *et al.*, 2005); this value is 330-fold smaller than that in rat jejunum S9. Therefore, rat intestinal absorption of temocapril does not accurately reflect human intestinal absorption.

In order to predict human intestinal absorption in the rat model, we used the previously reported methodology for inhibition of CES-mediated hydrolysis by pre-perfusion with BNPP (Masaki *et al.*, 2007). The absence of CES-mediated hydrolysis led to an increase in the intramucosal concentration of temocapril, followed by an increase in CL_{abs} and decrease in P_{eff} (Table 3). Interestingly, under CES-inhibited conditions, the CL_{deg} is maintained at 55–72% of control, while the intracellular concentration of temocaprilat is kept at 14.6–17.0 nmol/g tissue. In contrast, the CL_{deg} of isovaleryl propranolol, which is mainly hydrolyzed by CES, was shown to be inhibited by 77% following treatment with BNPP (Masaki *et al.*, 2007). This supports our contention that enzymes other than CES are involved in the hydrolysis of temocapril in rat intestine. The enzyme involved in the residual hydrolysis of temocapril after treatment with BNPP was mainly present in the cell components in the homogenate but not the S9 fraction, and this activity was inhibited by organophosphates. These data suggest that temocapril is not only hydrolysed by CES, but also by other hydrolases, containing serine residues in their catalytic centres and present in cell membranes.

It is also possible that temocapril is hydrolyzed in the cell membrane before entering the mucosal cell. The CL_{deg} in a luminal perfusate at pH 5.4 was only inhibited by 30% after treatment with BNPP, suggesting that the membrane enzyme involved is more active at pH 5.4 than pH 6.4. However, in the homogenates of jejunum pre-perfused with BNPP, temocapril was hydrolyzed at rates of 0.535 and 1.19 nmol/min/mg protein at pH 5.4 and 6.4, respectively. These results suggest that the membrane-bound enzymes may also catalyze the hydrolysis of temocapril inside the cell, depending on its intracellular concentration. Consequently, CES must be responsible for about 50% of the hydrolysis of temocapril in rat intestine. This is the first time that esterases other than CES have been

shown to be involved in the intestinal hydrolysis of ACE inhibitors. It is expected that membrane enzymes are also present in human intestinal cells, and their contribution may be larger than CES-mediated hydrolysis, as might be expected from the extremely low levels of hydrolysis of temocapril in human intestinal S9.

The present data suggest that temocapril is absorbed in the proximal intestine. Its absorption will be increased by administration after meals, due to the decrease of pH caused by secretion of bile acids, the prolongation of gastric emptying times, and the interaction of serine proteases on the cell membrane with several compounds contained in food. Under these conditions, temocapril may even be absorbed in intact form, without hydrolysis, in man. In the present study, we were able to mimic the conditions of the human intestine in a rat perfusion model by inhibiting CES activity. In addition, we demonstrated the involvement of serine-containing hydrolases on the cell membrane in the hydrolysis of prodrugs. We will need to characterize these enzymes further, in order to optimize the use of this model in predicting the intestinal absorption properties of prodrugs in man.

Authorship Contributions

Participated in research design: TI

Conducted experiments: TN

Contributed new reagents or analytic tools: TI, TN

Performed data analysis: TN

Wrote or contributed to the writing of the manuscript: TI, TN

Other: none

References

- Boll M, Markovich D, Weber WM, Korte H, Daniel H, Murer H (1994) Expression cloning of a cDNA from rabbit small intestine related to proton-coupled transport of peptides, beta-lactam antibiotics and ACE-inhibitors. *Pflugers Arch* **429**: 146–149.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.
- Ettmayer P, Amidon GL, Clement B, Testa B (2004) Lessons learned from marketed and investigational prodrugs. *J Med Chem* **47**: 2393–2404.
- Fagerholm U, Johansson M, Lennernäs H (1996) Comparison between permeability coefficients in rat and human jejunum. *Pharm Res* **13**: 1336–1342.
- Furihata T, Hosokawa M, Fujii A, Derbel M, Satoh T, Chiba K (2005) Dexamethasone-induced methylprednisolone hemisuccinate hydrolase: its identification as a member of the rat carboxylesterase 2 family and its unique existence in plasma. *Biochem Pharmacol* **69**: 1287–1297.
- Imai T, Imoto M, Sakamoto H, Hashimoto M (2005) Identification of esterases expressed in Caco-2 cells and effects of their hydrolyzing activity in predicting human intestinal absorption. *Drug Metab Dispos* **33**: 1185–1190.
- Imai T (2006) Human carboxylesterase isozymes: catalytic properties and rational drug design. *Drug Metab Pharmacokinet* **21**: 173–185.
- Imai T, Taketani M, Shii M, Hosokawa M, Chiba K (2006) Substrate specificity of carboxylesterase isozymes and their contribution to hydrolase activity in human liver and small intestine. *Drug Metab Dispos* **34**: 1734–1741.
- Imai T, Hosokawa M (2010) Prodrug approach using carboxylesterases activity: catalytic

- properties and gene regulation of carboxylesterase in mammalian tissue *J Pestic Sci* **35**: 229–239.
- Ishizuka H, Konno K, Naganuma H, Sasahara K, Kawahara Y, Niinuma K, Suzuki H, Sugiyama Y (1997) Temocaprilat, a novel angiotensin-converting enzyme inhibitor, is excreted in bile via an ATP-dependent active transporter (cMOAT) that is deficient in Eisai hyperbilirubinemic mutant rats (EHBR). *J Pharmacol Exp Ther* **280**: 1304–1311.
- Ishizuka H, Konno K, Naganuma H, Nishimura K, Kouzuki H, Suzuki H, Stieger B, Meier PJ, Sugiyama Y (1998) Transport of temocaprilat into rat hepatocytes: role of organic anion transporting polypeptide. *J Pharmacol Exp Ther* **287**: 37–42.
- Koike H, Yanagisawa H, Ikeda T, Manabe S, Ishikawa S, Kawahara Y (1992) Temocapril (CS-622), a novel angiotensin-converting enzyme inhibitor. *Annu Rep Sunkyo Res Lab* **44**: 1–81.
- Liederer BM, Borchardt RT (2006) Enzymes involved in the bioconversion of ester-based prodrugs. *J Pharm Sci* **95**: 1177–1195.
- Masaki K, Taketani M, Imai T (2006) First-pass hydrolysis of a propranolol ester derivative in rat small intestine. *Drug Metab Dispos* **34**: 398–404.
- Masaki K, Hashimoto M, Imai T (2007) Intestinal first-pass metabolism via carboxylesterase in rat jejunum and ileum. *Drug Metab Dispos* **35**: 1089–1095.
- Ohura K, Sakamoto H, Ninomiya S, Imai T (2010) Development of a novel system for estimating human intestinal absorption using Caco-2 cells in the absence of esterase activity. *Drug Metab Dispos* **38**: 323–331.
- Ohura K, Imai T (2010) The role of intestinal carboxylesterase in the oral absorption of prodrugs. *Curr Drug Metab* **11**: 793–805.

- Püchler K, Sierakowski B, Roots I (1998) Single dose and steady state pharmacokinetics of temocapril and temocaprilat in young and elderly hypertensive patients. *J Clin Pharmacol* **46**: 363–367.
- Sasaki M, Suzuki H, Aoki J, Ito K, Meier PJ, Sugiyama Y (2004) Prediction of in vivo biliary clearance from the in vitro transcellular transport of organic anions across a double-transfected Madin-Darby canine kidney II monolayer expressing both rat organic anion transporting polypeptide 4 and multidrug resistance associated protein 2. *Mol Pharmacol* **66**: 450–459.
- Sanghani SP, Davis WI, Dumaul NG, Mahrenholz A, Bosron WF (2002) Identification of microsomal rat liver carboxylesterases and their activity with retinyl palmitate. *Eur J Biochem* **269**: 4387–4398.
- Shionoiri H, Takasaki I, Minamisawa K, Ishizuka H, Konno K, Naganuma H, Sasahara K, Kawahara Y (2001) Trandolaprilat, an angiotensin-converting enzyme inhibitor, is not excreted in bile via an ATP-dependent active transporter (cMOAT). *Hypertens Res* **24**: 235–240.
- Takai S, Matsuda A, Usami Y, Adachi T, Sugiyama T, Katagiri Y, Tatematsu M, Hirano K (1997) Hydrolytic profile for ester- or amide-linkage by carboxylesterases pI 5.3 and 4.5 from human liver. *Biol Pharm Bull* **20**: 869–873.
- Taketani M, Shii M, Ohura K, Ninomiya S, Imai T (2007) Carboxylesterase in the liver and small intestine of experimental animals and human. *Life Sci* **81**: 924–932.
- Testa B (2004) Prodrug research: futile or fertile? *Biochem Pharmacol* **68**: 2097–2106.
- Vistoli G, Pedretti A, Mazzolari A, Bolchi C, Testa B (2009) Influence of ionization state on the activation of temocapril by hCES1: a molecular-dynamics study. *Chem Biodivers* **6**: 2092–2100.

Yamaoka K, Tanigawara Y, Nakagawa T, Uno T (1981) A pharmacokinetic analysis program (multi) for microcomputer. *J Pharmacobiodyn* **4**: 879–885.

Yamashita S, Tanaka Y, Endoh Y, Taki Y, Sakane T, Nadai T, Sezaki H (1997) Analysis of drug permeation across Caco-2 monolayer: implication for predicting in vivo drug absorption. *Pharm Res* **14**: 486–491.

Legends for Figures

Fig. 1 Intestinal behaviour of temocapril in *in situ* single-pass perfusion experiment.

Q_l and Q_b are flow rates of intestinal and vascular perfusion, respectively. C_{in} and C_{out} are the concentrations of temocapril at the entrance and exit of the jejunal segment. C_l and C_b are drug concentrations in luminal and vascular fluid, respectively.

Fig. 2 Absorption of temocapril in rat jejunal single-pass perfusion experiment.

a) Rate of disappearance (closed squares) and appearance (closed circles) of temocaprilat in the jejunal lumen; b) rate of appearance of temocapril (open squares) and temocaprilat (open circles) in the mesenteric vein. Luminal perfusate (pH 6.4) contained 100 μ M temocapril. The flow rates were 0.2 ml/min and 2.5 ml/min for luminal and vascular perfusate (pH 7.4), respectively. Each point represents the mean \pm S.D. ($n = 4$).

Fig. 3 Absorption of temocapril under several different pH conditions of the luminal perfusate.

Luminal perfusate was adjusted to pH 5.4 (closed column), pH 6.4 (open column) or pH 7.4 (hatched lined column); 100 μ M of temocapril was perfused at 0.2 ml/min. The flow rate of the vascular perfusate (pH 7.4) was 2.5 ml/min. Results are expressed as the mean \pm S.D. ($n = 3-6$). Statistically significant differences are indicated by: * $p < 0.05$, ** $p < 0.01$ at pH 5.4 vs pH 6.4; † $p < 0.05$, †† $p < 0.01$ at pH 5.4 vs pH 7.4.

Fig. 4 Effect of serine protease inhibitors on hydrolysis of temocapril in homogenate prepared from rat jejunum perfused with BNPP.

An homogenate of rat jejunal mucosa was prepared following perfusion of rat jejunum with BNPP (400 μ M) for 40 min. The hydrolysis of 500 μ M temocapril was measured in the presence of more BNPP, and the serine protease inhibitors, diisopropyl fluorophosphonate (DFP) and paraoxon. Closed, open and hatched columns show the activities remaining in the presence of 10, 100, and 1000 μ M inhibitor, respectively. Results are expressed as the mean \pm S.D. ($n = 4$).

Table 1 Kinetic parameters for absorption of temocapril and temocaprilat and their intracellular content in rat jejunal single-pass perfusion experiments

The effective permeability coefficient (P_{eff}), apparent hydrolytic clearance (CL_{deg}) and apparent absorption clearance ($CL_{\text{abs, temocaprilat}}$, $CL_{\text{abs, temocapril}}$) were calculated from the equations given in the Materials and Methods section. Values are expressed as the mean \pm S.D. ($n = 3-6$). Statistically significant differences are indicated by: * $p < 0.05$, ** $p < 0.01$ at pH 5.4 vs. pH 6.4; †† $p < 0.01$ at pH 5.4 vs. pH 7.4.

Parameter	Temocaprilat		Temocapril	
	pH 6.4	pH 5.4	pH 6.4	pH 7.4
$P_{\text{eff}} (\times 10^{-3} \text{ cm/min})$		4.56 ± 0.67	$3.43 \pm 0.27^{**}$	$3.25 \pm 0.30^{\dagger\dagger}$
$CL_{\text{deg}} (\mu\text{l/min})$		34.4 ± 9.32	35.4 ± 3.97	43.2 ± 6.08
$CL_{\text{abs, temocaprilat}} (\mu\text{l/min})$	1.87 ± 0.88			
$CL_{\text{abs, temocapril}} (\mu\text{l/min})$		3.59 ± 0.93	$1.59 \pm 0.86^*$	$1.23 \pm 0.27^{\dagger\dagger}$
$CL_{\text{deg}} / (CL_{\text{deg}} + CL_{\text{abs, temocapril}})$		0.900 ± 0.032	0.957 ± 0.020	0.972 ± 0.008
Intracellular content (nmol/g tissue)				
Temocapril		5.46 ± 0.35	1.83 ± 0.90	1.02 ± 0.93
Temocaprilat	2.99 ± 0.60	34.2 ± 2.62	18.1 ± 4.57	17.6 ± 3.16

Table 2 Kinetic parameters for hydrolysis of temocapril in rat duodenal and jejunal S9 fractions

K_m and V_{max} values were calculated by the Michaelis–Menten equation by fitting nonlinear least-squares analysis. Hydrolysis of temocapril was measured in the range 10–500 μM . Values are mean \pm S.D. ($n = 3$).

	K_m (μM)	V_{max} (nmol/min/mg protein)	V_{max}/K_m ($\mu\text{l}/\text{min}/\text{mg}$ protein)
Duodenal S9	47.4 \pm 3.14	5.20 \pm 0.784	109 \pm 10.4
Jejunal S9	47.3 \pm 1.16	5.11 \pm 0.406	108 \pm 7.70

Table 3 Kinetic parameters for the absorption of temocapril and its intracellular concentration in a rat jejunal preparation following pre-perfusion with BNPP

The jejunal lumen was pre-perfused with BNPP (400 μ M) for 40 min. Following washout, temocapril (100 μ M) was perfused through the lumen. The rates of disappearance of temocapril (v_1) and appearance of temocaprilat (v_3) in the lumen, the rates of appearance of temocapril (v_2) and temocaprilat (v_4) in the blood vessel, the effective permeability coefficient (P_{eff}), apparent hydrolysis clearance (CL_{deg}) and apparent absorption clearance ($CL_{\text{abs, temocapril}}$) were determined. The luminal perfusate containing 100 μ M temocapril, was adjusted to pH 5.4 or pH 6.4 and perfused at 0.2 ml/min. The vascular perfusate (pH 7.4) flowed at 2.5 ml/min. Results are expressed as the mean \pm S.D. ($n = 3$).

Parameter	pH 5.4	pH 6.4
v_1 (nmol/min)	2.98 \pm 0.24	2.44 \pm 0.18
v_2 (nmol/min)	0.862 \pm 0.046	0.696 \pm 0.072
v_3 (nmol/min)	1.01 \pm 0.11	1.17 \pm 0.17
v_4 (nmol/min)	1.01 \pm 0.06	0.604 \pm 0.139
P_{eff} ($\times 10^{-3}$ cm/min)	2.99 \pm 0.15	2.32 \pm 0.11
CL_{deg} (μ l/min)	24.9 \pm 3.43	19.5 \pm 1.71
$CL_{\text{abs, temocapril}}$ (μ l/min)	10.6 \pm 1.11	7.64 \pm 0.59
$CL_{\text{deg}}/(CL_{\text{deg}}+CL_{\text{abs, temocapril}})$	0.701 \pm 0.012	0.718 \pm 0.030
Intracellular concentration (nmol/g tissue)		
Temocapril	20.6 \pm 2.37	15.4 \pm 1.08
Temocaprilat	17.0 \pm 3.26	14.6 \pm 1.23

Table 4 Hydrolysis of temocapril in rat jejunal homogenate and S9 fraction prepared after perfusion with and without BNPP

After perfusion, with or without BNPP (400 μ M) for 40 min, homogenates and S9 fractions were prepared from the jejunal mucosa and kept on ice. Intact jejunum was used as control. The hydrolysis of 500 μ M temocapril was measured in samples of homogenate and S9 fraction containing 400 μ g/ml protein. Results are expressed as the quantity of temocapril hydrolysed (nmol/min/mg protein) and given as mean \pm S.D. ($n=4$). The percentage in parentheses indicates the remaining activity to each control.

	Intact mucosa		Perfused mucosa	
	Homogenate	S9	Homogenate	S9
	nmol/min/mg protein			
Control	4.17 \pm 0.428	4.46 \pm 0.137		
+100 μ M BNPP	2.23 \pm 0.131	1.13 \pm 0.443		
	(53.4%)	(25.2%)		
+1000 μ M BNPP	1.97 \pm 0.194	0.488 \pm 0.105		
	(47.4%)	(10.9%)		
Control			3.96 \pm 0.056	4.28 \pm 0.029
+400 μ M BNPP pre perfusion			2.05 \pm 0.255	0.625 \pm 0.111
			(51.8%)	(15.2%)

Figure 1

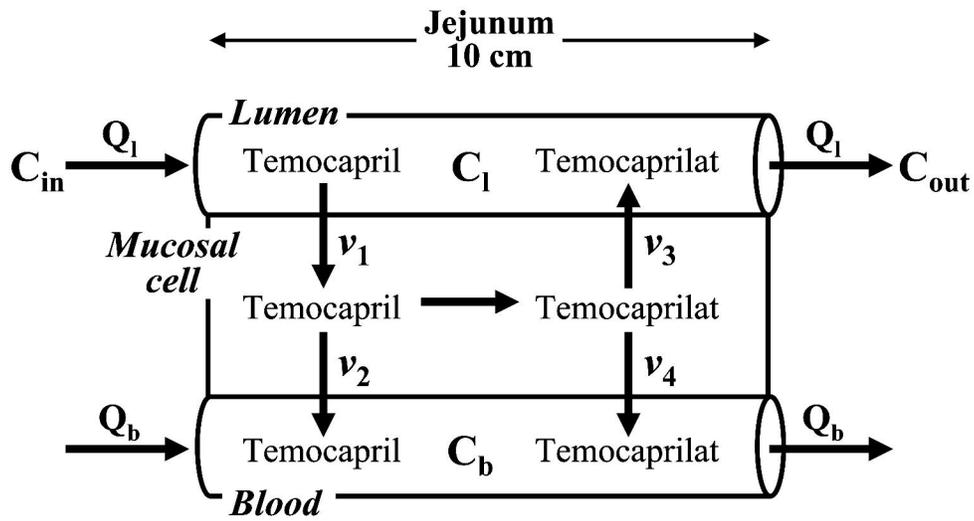
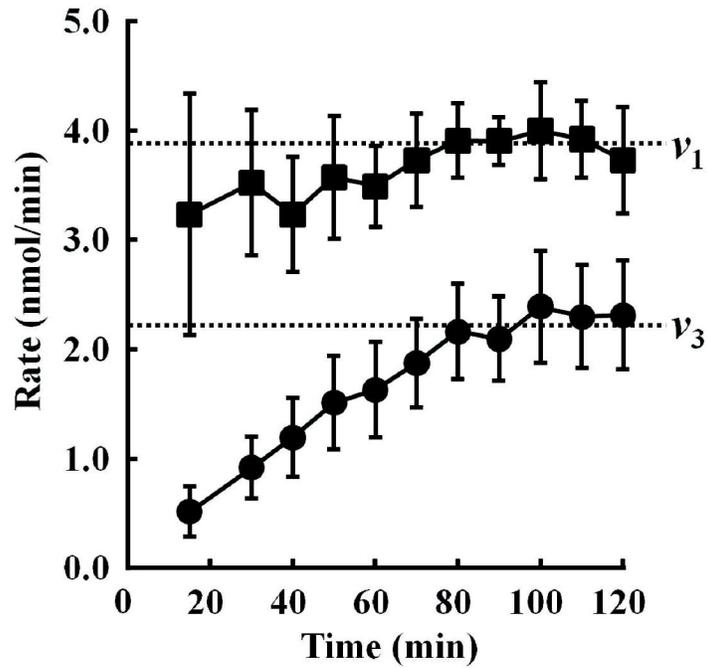


Figure 2

a) Jejunal lumen



b) Mesenteric vein

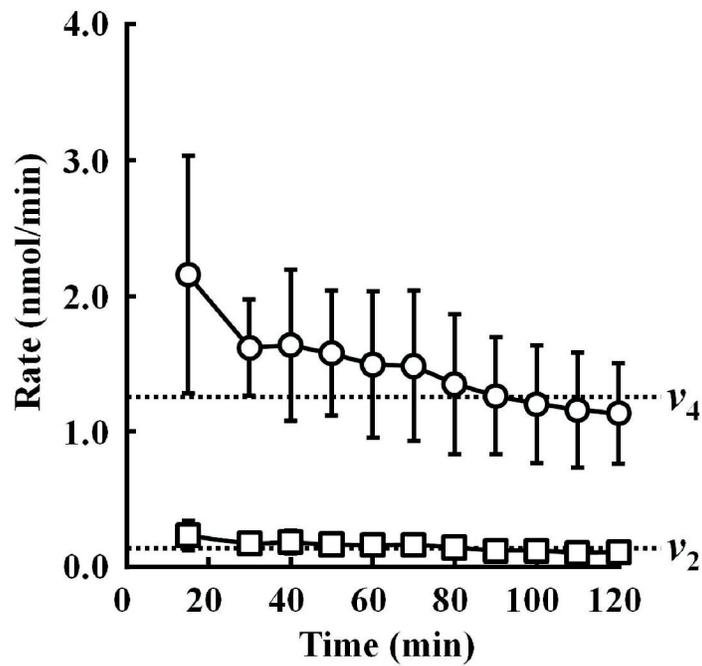


Figure 3

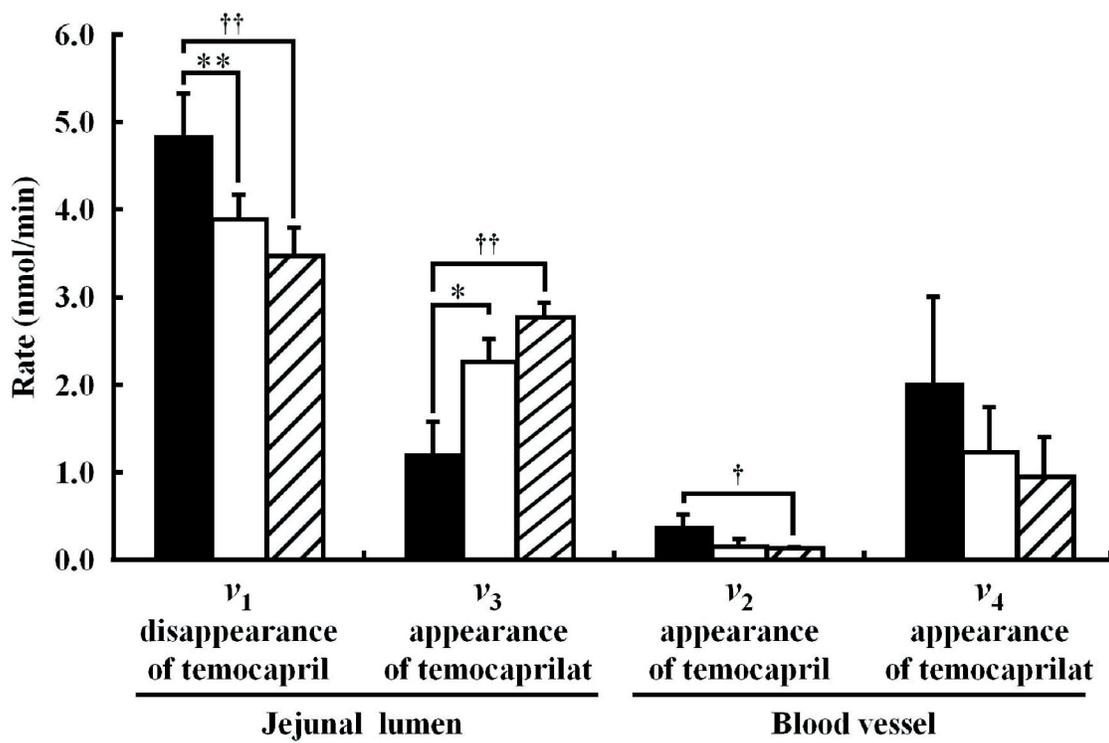


Figure 4

