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Contribution of Carboxylesterase in Hamster to the Intestinal First-Pass Loss and Low Bioavailability of Ethyl Piperate, an Effective Lipid-Lowering Drug Candidate

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ABSTRACT:

Ethyl piperate is an effective lipid-lowering drug candidate synthesized from piperine. However, its pharmacokinetic characteristics and oral absorption process remain unclear. A liquid chromatography-tandem mass spectrometry method was applied to determine the oral bioavailability of ethyl piperate. Simulated gastrointestinal pH conditions and intestinal washings were prepared to investigate their contributions to the loss of ethyl piperate. Hydrolysis by carboxylesterase (CES) was evaluated in vitro using microsomes and S9 fractions. In situ intestinal single-pass perfusion experiments were performed to estimate the role of CES in ethyl piperate absorption. The bioavailability of ethyl piperate was extremely low (0.47%) in hamster independent of gastrointestinal environmental effects. Ethyl piperate was a typical substrate of CES with kinetic parameters K_m and V_{max} of 7.56 ± 1.491 μM and 0.16 ± 0.008 nmol · min^{-1} · mg protein^{-1}, respectively. CES was responsible for 85.8% of the intestinal hydrolysis of ethyl piperate. Specific inhibition of CES with bis-*p*-nitrophenyl phosphate (BNPP), decreased degradation clearance to 36% of control with no significant change in absorption clearance. This contrasted with the results of Caco-2 monolayer experiments, which showed a dramatic increase in the apparent permeability coefficient after BNPP treatment. mRNA levels for the CES isozyme, CES2A3, were similar among the three regions of hamster intestine and 60% less than those in liver; CES1B1 mRNA levels were even lower in the intestine and showed a proximal-to-distal decrease. In conclusion, CES markedly contributes to intestinal first-pass hydrolysis of ethyl piperate that is sufficient, but not necessary, to cause the observed extremely low bioavailability.

Introduction

Atherosclerosis is a complex process that leads to multiple pathologies, including heart attack and stroke (Lusis, 2000), with the highest prevalence of such diseases occurring in affluent industrialized countries. Piperine, a major active component of black and long peppers, has been reported to protect against myocardial damage (Hu et al., 2009), cure acute gouty arthritis (Sabina et al., 2010), and inhibit tumor progression (Manoharan et al., 2009) in experimental animal models. Reports from Vijayakumar and Nalini, 2006 showed that piperine has a lipid-lowering effect in drug-induced hypercholesterolemic rats, and Matsuda et al. (2008) found that it inhibits lipid droplet accumulation in macrophages. However, piperine has been reported to be cytotoxic to cultured embryonic rat brain neurons (Unchern et al., 1998) and cause extensive immunotoxicological effects in mice (Dogra et al., 2004). Ethyl piperate is a successful modification of piperine that not only eliminates the toxicity of the parent compound but also retains its lipid-lowering function (Borjihan and Wu, 2005). It efficiently reduces the levels of plasma total cholesterol and triglycerides in the hamster.

Pharmacokinetic analyses have become increasingly important in the early stages of drug research and development, in part because they may provide insight into pharmacodynamic mechanisms (Nix, 2003). To date, the pharmacokinetics of ethyl piperate has not been studied, and we have found that the bioavailability of ethyl piperate after oral administration is extremely low. The extent of drug absorption through oral administration may be affected by a number of physiological factors, including volume and composition of gastrointestinal fluids, the pH and buffer capacity of these fluids, digestive enzymes, and bacterial flora in the gut (Dressman and Lennernas, 2000). To better understand the ethyl piperate absorption process and the modification and disposition of ethyl piperate in vivo, we extended our preliminary observations to include an analysis of factors with

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ABBREVIATIONS: CES, carboxylesterase; BNPP, bis-*p*-nitrophenyl phosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; LC, liquid chromatography; MS/MS, tandem mass spectrometry; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

potential relevance to the pharmacokinetic profile of ethyl piperate. Our results showed that digestive enzymes and physiological pH conditions were not major contributors to the low bioavailability of ethyl piperate. Using in situ intestinal single-pass perfusion techniques in hamsters, we found that more than half of orally delivered ethyl piperate, including its hydrolysate piperinic acid, passed through the wall of intestine and entered the blood circulation. Of importance, carboxylesterase (CES) in enterocytes played an important role in hydrolyzing ethyl piperate and reducing its level in the blood. Inhibition of CES with the specific inhibitor BNPP reduced the level of the hydrolysate without producing the expected increase in the level of the parent drug, possibly indicating that other ethyl piperate metabolites were produced in the absence of CES activity. We also provide the first report of the intestinal expression of different CES isozymes in hamsters, which shows that their mRNA distribution is different from that in rats.

Materials and Methods

Materials. Ethyl piperate and its hydrolysis product piperinic acid (both ≥99.0% pure) were supplied by the Institute of Macromolecular Chemistry and Mongolian Medicine, Inner Mongolia University (Hohhot, Inner Mongolia, China). The chemical structures of these compounds are illustrated in Fig. 1. MES was obtained from Bio Basic Inc. (Amherst, NY). BNPP was purchased from Sigma-Aldrich (St. Louis, MO). HEPES was obtained from AMRESCO (Solon, OH). All other reagents used in this study, unless otherwise specified, were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Animals. Male hamsters (110–175 g), obtained from Shanghai Institute of Biological Products (Shanghai, China) were housed in a temperature-controlled room (23 ± 2°C) under a 12-h light/dark cycle and were handled according to the *Guidelines for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Absolute Bioavailability of Ethyl Piperate. Twelve male hamsters, fasted overnight before experiments, were randomly divided into two treatment groups ($n = 6$ per group): in one group, 30 mg/kg ethyl piperate was administered orally; in the other, 10 mg/kg ethyl piperate was delivered by sublingual vein injection. Blood, collected before treatment and at 2, 5, 10, 20, and 30 min and 1, 1.5, 2, 4, 6, 10, 15, and 24 h after administration was centrifuged to prepare plasma, which was maintained at −80°C until analysis. The entire sampling process was completed within 1 h.

Stability in Simulated Gastrointestinal pH Conditions. The method for simulated gastrointestinal pH conditions was described previously (Chaurasia et al., 2006) with removal of the effect from digestive enzyme. Simulated gastric pH fluid consisted of NaCl (34 mM) and HCl (71 mM), and pH was adjusted to 1.2 ± 0.5. Simulated intestinal pH fluid consisted of KH_2PO_4 (50 mM) and NaOH (200 mM), and pH was adjusted to 6.8 ± 0.1. Simulated gastric-intestinal pH fluid (pH 4.5) was prepared by mixing simulated gastric fluid and simulated intestinal fluid in a ratio of 39:61. The stability study was initiated by incubating 1 mM ethyl piperate in simulated pH fluids at 37°C for 3 h, followed by collection of 50-μl aliquots every hour for analysis.

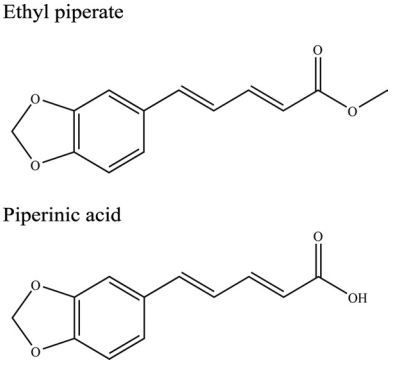

FIG. 1. The chemical structure of ethyl piperate and its hydrolysate piperinic acid.

Degradation Study in Hamster Intestinal Washings. Intestinal washings were prepared as described previously (Crauste-Manciet et al., 1998). In brief, six male hamsters were anesthetized, and the intestines were exposed and ligated at both ends. The ligated segment was filled with 0.5 ml of washing buffer (10 mM HEPES, pH 7.0, and 300 mM mannitol) and left standing for 10 min. The intestine was then evacuated, and the particulate material was removed by centrifuging at 3800*g* for 10 min. The washings (1 mg/ml protein, 180 μl) were preincubated at 37°C for 5 min before initiation of the assay by addition of 20 μl of 1 mM ethyl piperate solution (final ethyl piperate concentration, 100 μM). After incubation for 1 h, the reaction was stopped by addition of 200 μl of acetonitrile, and the supernatant was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Identification of Ethyl Piperate as a CES Substrate. Intestine, liver, and renal microsomes were prepared by the following method. Six hamsters were sacrificed to obtain whole intestines, livers, and kidneys. The intestinal mucosa was scraped and homogenized in buffer A (154 mM KCl and 50 mM Tris-HCl) on ice, followed by sequential centrifugation steps at 9000*g* for 30 min and 105,000*g* for 60 min. The final pellet containing microsomes was resuspended and stored in buffer B (20% glycerol and 100 mM K_2HPO_4). Microsomes from liver and kidney were also prepared using the same procedure.

BNPP was used as a specific inhibitor of CES to confirm that the ethyl piperate hydrolysis reaction was mediated by CES. The reaction was initiated by addition of the ethyl piperate substrate (final concentration, 50 μM) to preincubated microsome solutions with or without 400 μM BNPP (0.5 mg/ml protein dissolved in 100 mM K_2HPO_4 buffer solutions containing ≤0.5% residual organic solvent) and stopped 30 min later by addition of an equal volume of acetonitrile. After centrifugation, the supernatant was retained for analysis.

In Situ Intestinal Single-Pass Perfusion. The perfusion study was performed using the method of Masaki et al. (2007) with some modifications. The trial was performed as follows. Twenty-four hamsters were randomly divided into two groups, and each group was divided in half including control and BNPP-treated groups. In the first group, after hamsters were anesthetized, both ends of the intestine were cannulated to permit single-pass perfusion. Animals of the control and treated groups were perfused at 0.3 ml/min with MES buffer alone (pH 6.5, containing 0.03 mM phenol, which was added as a nonabsorbable marker and detected spectrophotometrically at 550 nm, outflow-methanol-100 mM NaOH, 1:1:8, v/v/v) and MES buffer plus BNPP (400 μM) for 40 min, respectively, before sacrifice to prepare the intestinal S9 fraction. After the same treatment as in the first group, animals in the second group were subsequently perfused with test compound (ethyl piperate, 400 μM) to collect samples from intestinal outflow at 10-min intervals for 1 h. In a modification of the original protocol, 0.3 ml of portal vein blood was drawn concurrently with a closed intravenous catheter system (24 gauge × 0.75 in., 0.7 × 19 mm; BD Medical Devices Co., Ltd., Franklin Lakes, NJ), and an equal volume of saline was injected as supplement. The portal vein flow rate during the sampling period was determined with another six hamsters using a T206 small animal blood flowmeter (Transonic Systems Inc., Taiwan, China) as described previously (Schmandra et al., 2001). During the whole process, animals were kept anesthetized, and the intestine remained viable throughout the experimental period. The outflow was immediately mixed with an equal volume of acetonitrile, and the blood was processed to obtain plasma for analysis. The data were analyzed as described previously (Kim et al., 1993; Masaki et al., 2006).

Hydrolysis of Ethyl Piperate in the Intestinal S9 Fraction. The intestinal mucosal S9 fraction was prepared from the first group of the perfused intestines as described previously (Masaki et al., 2006). After preincubation of control and BNPP-treated S9 solutions (5 mg/ml) at 37°C for 5 min, reactions were initiated by addition of ethyl piperate solutions (final concentration, 400 μM) and stopped after a 5-min incubation. The remaining parent drugs were detected to calculate the inhibition ratio of CES after BNPP perfusion. The kinetic profile for the hydrolysis of ethyl piperate (final concentrations, 2–200 μM, incubated in control S9 solutions) was determined. The hydrolytic activity of intestinal control S9 was inhibited in vitro by incubation with 1 to 1000 μM BNPP (final concentration of ethyl piperate, 400 μM). The hydrolysates were determined, and plots were fitted. All parameters were obtained by using GraphPad software (GraphPad Software Inc., San Diego, CA).

Transport across Caco-2 Monolayers. Caco-2 monolayers were prepared by the method of Hubatsch et al. (2007). The effect of CES on ethyl piperate permeability was determined in transport assays using 200 μM BNPP as an inhibitor. The bidirectional assay was initiated by addition of 10 μM ethyl piperate at the donor side. Samples were collected from the receiver compartment 1 h later, and both piperinic acid and ethyl piperate concentrations were determined by LC-MS/MS.

LC-MS/MS Analysis. Plasma samples collected after oral administration of ethyl piperate were prepared by mixing 50 μl of plasma with 10 μl of internal standard solution (1.5 μg/ml megestrol acetate) and extracting with 1 ml of *tert*-butylmethylether. The organic layer was air-dried before reconstitution with 50 μl of mobile phase. Samples from in vitro and in situ experiments to simultaneously determine the total fractions of piperinic acid and ethyl piperate were precipitated with 3 volumes of acetonitrile (containing 2.4 μg/ml silibinin and megestrol acetate as internal standard) and centrifuged to obtain the supernatant. The supernatant was diluted 2-fold with deionized water, and a 5-μl sample was injected into a reverse-phase column (Gemini 5-μm C18 110A, 50 $\times$ 2.00 mm; Phenomenex, Torrance, CA). LC-MS/MS, with a high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) coupled to a QTRAP 3200 mass spectrometer (Applied Biosystems, Foster City, CA), was used for sample analysis. The mobile phase was a mixture of acetonitrile and deionized water and contained a constant 0.02% formic acid. The sample was initiated with a gradient program (for the first 0.5 min, elution with 70% acetonitrile and 0.1 min later changed to 100% acetonitrile for 2 min and then dropped to 70% acetonitrile in 0.1 min) and equilibrated (with 70% acetonitrile for 2.8 min) at a constant flow of 0.25 ml/min. The mass spectrometer was operated in multiple reaction monitoring mode, with monitoring of the precursor-to-product ion transitions of *m/z* 247.0 $\rightarrow$ 201.0 for ethyl piperate, *m/z* 481.0 $\rightarrow$ 152.0 for silibinin in positive mode, and *m/z* 217.0 $\rightarrow$ 143.0 for piperinic acid, and *m/z* 385.1 $\rightarrow$ 325.1 for megestrol acetate in negative mode.

The calibration curve of ethyl piperate in plasma samples was linear over the concentration range of 1 to 800 ng/ml, with a correlation coefficient (r) $>$ 0.99. The lower limits of quantification of piperinic acid and ethyl piperate were 40 and 20 ng/ml, respectively, for in vitro and in situ samples. The intra- and interbatch variation, a measure of precision, was less than 11.12% and the corresponding accuracy was 92 to 98%, evaluated by assaying the quality-control samples. The extraction recovery of ethyl piperate was in the range of 62 to 68% (n = 6).

mRNA Expression of CES Isozymes in the Whole Intestine. Total RNA was extracted from the intestine using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized from 3 μg of RNA using M-MLV reverse transcriptase according to the manufacturer's instructions (Promega, Madison, WI). Segments of the CES isozymes CES2A3 (GenBank accession number D50577), CES2A11 (GenBank accession number D28566), and CES1B1 (GenBank accession number D50578) were amplified from cDNA by reverse transcription (RT)-polymerase chain reaction (PCR) using TaqDNA polymerase (TIANGEN, Beijing, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession number X52123.1) was used as an internal control. PCR conditions and the sequences of primers used are listed in Table 1. Amplified PCR products were separated on 0.8 to 1% Biowest agarose gels and stained with ethidium bromide.

Statistical Analysis. Data are expressed as the mean $\pm$ S.D. Two-tailed Student's t tests were performed to compare results between control and BNPP treatment groups; $p < 0.05$ was considered statistically significant. Pharmacokinetic parameters were calculated from plasma levels of piperinic acid and ethyl piperate by applying a noncompartmental statistic using Drug and Statistics software, developed by the Mathematical Pharmacology Professional Committe of China (version 2.0; Anhui, China).

Results

Oral Bioavailability of Ethyl Piperate in the Hamster. The plasma mean concentration-time profiles of oral and intravenous administration are shown in Fig. 2, and the pharmacokinetic parameters are listed in Table 2. Samples were undetectable after 1.5 h of oral administration or 4 h of intravenous injection because the ethyl piperate level was lower than the lower limit of quantification (1 ng/ml in plasma). The absolute bioavailability (F) was only 0.47% by comparison of area under the concentration-time curve up to the last measured time point (AUC_{0-t}) between oral (34.5 $\pm$ 51.6 μg/l $\cdot$ h) and intravenous (2454.6 $\pm$ 489.1 μg/l $\cdot$ h) administration. The elimination half-life ($t_{1/2}$) calculated from intravenous data was 0.63 $\pm$ 0.25 h, indicating that ethyl piperate rapidly disappeared in vivo. The extremely low bioavailability raised a pivotal question: is ethyl piperate absorbed after oral administration and does it directly decrease triglyceride and cholesterol?

Stability Research of Ethyl Piperate before Absorption. Because ethyl piperate is a carboxylic acid ester that might be susceptible to hydrolysis in the gut, we investigated the impact of a simulated gut environment on the stability of the ethyl piperate ester bond. There was no significant degradation during 3 h under simulated gastrointestinal pH conditions, and greater than 80% of the parent drug could still be detected after a 1-h incubation in intestinal washings (Fig. 3). Thus, the pH and digestive enzyme encounter in the gastrointestinal tract do not appear to represent obstructions to the absorption of ethyl piperate.

CES Participation in the Hydrolysis of Ethyl Piperate. CES, which primarily catalyzes the hydrolysis of aliphatic esters, is highly expressed in the liver, kidney, and small intestine and is predominantly located in the endoplasmic reticulum of the cell (Satoh and Hosokawa, 2006). The robust generation of piperinic acid in control microsomes and its sharp reduction by BNPP-treated microsomes indicated that ethyl piperate was hydrolyzed in vivo mostly by CES in the intestine, liver, and kidney. The rank order of in vitro hydrolytic activity in microsomes was liver $>$ kidney $>$ intestine (Fig. 4).

CES Activity and mRNA Expression in the Hamster Small Intestine. S9 fractions were prepared from both control and BNPP-perfused intestines. The production of hydrolysate in intestines perfused with 400 μM BNPP was decreased to 0.060 $\pm$ 0.007 nmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1} from the control level of 0.196 $\pm$ 0.032 nmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1} (Table 3), a significant difference that corresponded to 69.4% inhibition. Comparison of this result with the maximal inhibition of 93.5% achieved in vitro by adding 1 mM BNPP to S9 preparations (Fig. 5B) indicated that perfusion with 400 μM BNPP in vivo could inhibit 74.2% of CES activity.

TABLE 1

RT-PCR conditions and the sequences of forward and reverse primers

	Sequence of Primer	Position	Denaturation	Annealing	Extension	Cycles
CES2A3	TACCGTCTGGGTGTCCT	573–589	94°C, 30 s	55°C, 60 s	72°C, 60 s	35
	CTGTGGGTCCTCATTGTC	1247–1230				
CES2A11	CAACCATGCCACTCAAT	22–38	94°C, 30 s	55°C, 60 s	72°C, 60 s	35
	GATACCCAAGCGATACTG	566–583				
CES1B1	GCAGGAGGTTTCAGTGTC	668–685	94°C, 30 s	55°C, 60 s	72°C, 60 s	40
	CACAAGAGGGAGTTAGCC	1146–1129				
GAPDH	GTCGGCGTGAACGGATTT	83–100	94°C, 30 s	55°C, 60 s	72°C, 60 s	28
	CATTTGATGTTGGCGGGAT	327–309				

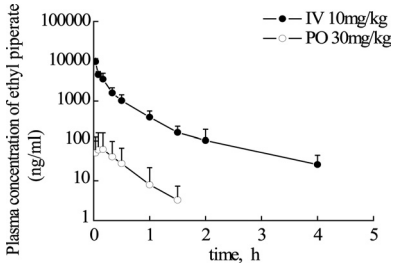

FIG. 2. Mean plasma concentration-time profile of ethyl piperate in the hamster after receiving a 30 mg/kg oral dose (○) or 10 mg/kg intravenous dose (●). Each point represents the mean + S.D. ($n = 6$).

The kinetic profile was estimated by comparison of R^2 among Michaelis-Menten, substrate inhibition, and allosteric sigmoidal equations. The kinetics in intestinal S9 fitted to the Michaelis-Menten equation ($R^2 = 0.97$) (Fig. 5A), and the kinetic parameters were 7.56 ± 1.491 μM (K_m) and 0.16 ± 0.008 nmol · min^{-1} · mg protein^{-1} (V_{max}).

The results of RT-PCR analyses of the three major CES isozymes in liver, duodenum, jejunum, and ileum are shown in Fig. 6A. The expression levels of CES isozymes CES1B1, CES2A3, and CES2A11 in the duodenum, jejunum, and ileum compared with those in liver are shown in Fig. 6, B, C, and D, respectively. The expression level of CES2A3 was similar in all three parts of the intestine and was approximately 60% of that in liver. CES1B1 expression in the duodenum and jejunum was approximately 10 to 20% of that in liver; no expression of CES1B1 was detected in ileum. The expression of CES2A11 varied greatly among animals.

Role of CES in the Absorption of Ethyl Piperate. Although the liver may be the main organ for the elimination of ethyl piperate according to the result of the microsome experiment, the total amount of parent drug that crosses the intestinal wall is the measure of bioavailability that most accurately predicts its utilization. An in situ single-pass perfusion assay was used to effectively simulate the absorption process in vivo. The blood flow rate in the portal vein during the sampling period was 3.29 ± 1.13 ml/min, and the absorption parameters are shown in Table 3. The appearance rates of ethyl piperate and its hydrolysate piperinic acid at steady state in the portal vein [$\nu_1 = Q_{blood} \times C_{blood}$, where Q_{blood} and C_{blood} are the flow rates of vascular perfusion and the concentration of ethyl piperate or piperinic acid in the portal vein, respectively] were 10.8 ± 3.27 and 52.1 ± 6.14 nmol/min, indicating that at least 52.4% of the total drug was absorbed. The ν_1 of piperinic acid was decreased to 21.7 ± 7.62 mol/min after treatment with BNPP, a value almost half that of the control group. The appearance rate of piperinic acid in the intestinal lumen [$\nu_3 = Q_{lumen} \times C_{metabolite, out}$, where Q_{lumen} is the flow rate of intestinal perfusion and $C_{metabolite, out}$ is the concentration of piperinic acid at the exit of the intestinal segment] after BNPP-treatment was the same as that in control group as was the appearance rate of ethyl piperate in the portal vein (ν_1). The disappearance rate of ethyl piperate from the perfusate [$\nu_2 = Q_{lumen} \times (C_{in} - C_{out})$, where C_{in} and C_{out} are the concentrations of ethyl piperate at the entrance and exit of the intestinal segment, respectively] decreased from 76.5 ± 7.70 to 62.7 ± 11.49 nmol/min, possibly due to the inhibition of hydrolysis. Because the hydrolysis could be inhibited largely by BNPP in vitro (almost 90% of the hydrolytic activity was inhibited by 100 μM BNPP in S9 solutions), differences between ν_2 and the sum of ν_1 and ν_3 in the absence of the drug may be due to the reduced formation of piperinic acid and the production of other metabolites in vivo. The absorption clearance [$CL_{app} = (AUC_{parent, blood}/AUC_{parent, lumen}) \times Q_{blood}$, where $AUC_{parent, blood}$ and $AUC_{parent, lumen}$ are the areas under the curve of the parent drug in the portal vein and intestinal lumen, respectively, at the steady state] in BNPP treatment groups (66.7 ± 24.71 μl/min) trended lower than that in the control group (55.0 ± 42.86 μl/min). The reduction of hydrolysis by BNPP treatment led to a decrease in degradation clearance [$CL_{deg} = (AUC_{metabolite, lumen}/AUC_{parent, lumen}) \times Q_{lumen} + (AUC_{metabolite, blood}/AUC_{parent, lumen}) \times Q_{blood}$, where $AUC_{metabolite, lumen}$ and $AUC_{metabolite, blood}$ are the areas under the curve of piperinic acid in the intestinal lumen and portal vein, respectively, at the steady state] from 339.9 ± 71.12 to 121.6 ± 48.90 μl/min, indicating that hydrolysis was inhibited by 63.7 ± 13.49% after BNPP treatment. This result suggests that CES accounts for 85.8% of the

TABLE 2

Pharmacokinetic parameters of ethyl piperate after oral and intravenous administration in the hamster

For $AUC_{(0-t)}$, the value of t is defined as the last quantifiable point (1.5 and 4 h after oral and intravenous administration of ethyl piperate, respectively).

Parameters	30 mg/kg p.o.	10 mg/kg i.v.
$AUC_{(0-t)}$ (μg/l · h)	34.5 ± 51.6	2454.6 ± 489.1
$MRT_{(0-t)}$ (h)	0.40 ± 0.08	0.43 ± 0.09
$t_{1/2}$ (h)	N.A.	0.63 ± 0.25
T_{max} (h)	0.15 ± 0.11	N.A.
CL_z/F (l · h^{-1} · kg^{-1})	N.A.	4.17 ± 0.77
V_z/F (l/kg)	N.A.	3.76 ± 1.57
C_{max} (μg/l)	70.4 ± 93.4	N.A.
F (%)	0.47	

MRT, mean retention time; T_{max}, time to reach maximum concentration; C_{max}, maximum concentration; CL_z/F, clearance; V_z/F, volume of distribution; N.A., not available.

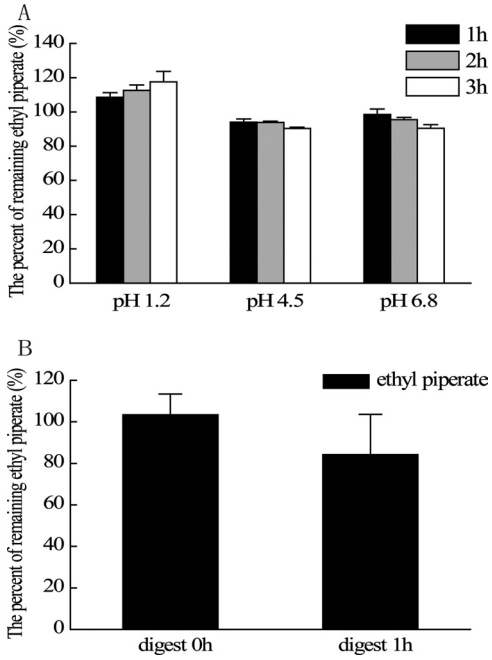

FIG. 3. The stability of ethyl piperate in simulated gastrointestinal pH buffers (A) and intestinal digestive fluid (B). Each point represents the mean ± S.D. ($n = 6$).

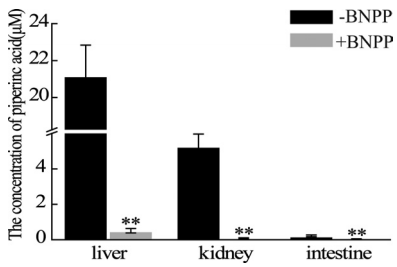

FIG. 4. Effects of BNPP on ethyl piperate hydrolysis in tissue microsomes. Each point represents the mean ± S.D. (**, $p < 0.01$; $n = 6$).

TABLE 3

Kinetic parameters for ethyl piperate absorption in the hamster measured in single-pass perfusion experiments with or without BNPP treatment

Parameter	Explanation	Control	400 μM BNPP
v_1 (nmol/min)	Appearance rate of piperinic acid in portal vein	52.1 ± 6.14	21.7 ± 7.62**
	Appearance rate of ethyl piperate in portal vein	10.8 ± 3.27	9.8 ± 5.44
v_2 (nmol/min)	Disappearance rate of ethyl piperate in intestine	76.5 ± 7.70	62.7 ± 11.49*
v_3 (nmol/min)	Appearance rate of piperinic acid in intestine	2.3 ± 1.22	2.0 ± 0.94
CL_{app} (μl/min)	Absorption clearance	66.7 ± 24.71	55.0 ± 42.86
CL_{deg} (μl/min)	Degradation clearance	339.9 ± 71.12	121.6 ± 48.90**
P_{app} (cm/min)	Apparent permeability coefficient of piperinic acid	$2.24 \times 10^{-2} \pm 5.10 \times 10^{-3}$	$7.64 \times 10^{-3} \pm 3.57 \times 10^{-3}$**
	Apparent permeability coefficient of ethyl piperate	$4.62 \times 10^{-3} \pm 1.65 \times 10^{-3}$	$3.59 \times 10^{-3} \pm 2.93 \times 10^{-3}$
S9 activity (nmol · min^{-1} · mg^{-1})	Production of piperinic acid	0.196 ± 0.032	0.060 ± 0.007**

* $p < 0.05$, control versus BNPP group.
** $p < 0.01$.

intestinal hydrolysis of ethyl piperate during absorption in vivo, given that pretreatment with BNPP inhibited 74.2% of CES activity. The apparent permeability coefficients of ethyl piperate [$P_{app} = X_{blood}/AUC_{parent,\,lumen}/2\pi RL$, where X_{blood} is the cumulative amount of ethyl piperate or piperinic acid in the portal vein, R, the radius of the segment, is 0.15 cm, and L, the length of the segment, is 22 cm] of ethyl piperate were the same between the control group ($4.62 \times 10^{-3} \pm 1.65 \times 10^{-3}$ cm/min) and BNPP treatment group ($3.59 \times 10^{-3} \pm 2.93 \times 10^{-3}$ cm/min), an outcome different from that observed in Caco-2 monolayers after addition of BNPP, whereas the P_{app} of piperinic acid dropped from $2.24 \times 10^{-2} \pm 5.10 \times 10^{-3}$ to $7.64 \times 10^{-3} \pm 3.57 \times 10^{-3}$ cm/min. In Caco-2 monolayers, the apparent permeability coefficient [$P_{mono} = (dQ/dt)/(A \times C_0)$, where dQ/dt is the rate of appearance of drugs in the receiver compartment, A is the surface area of cell monolayer (i.e., 1.11 cm^2), and C_0 is the initial drug concentration in the donor compartment] across Caco-2 cell monolayers of ethyl piperate was dramatically elevated in both directions by BNPP; in contrast, the P_{mono} of piperinic acid was decreased as expected (Fig. 7). The recovery of the drug including its hydrolysate in cell experiment was >90%.

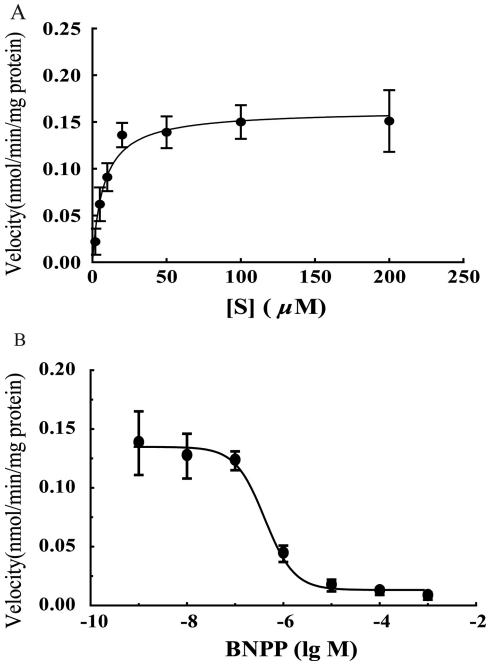

FIG. 5. Concentration dependence of hydrolysis (A) and inhibition (B) of ethyl piperate in the intestinal S9 fraction. A, K_m and V_{max} were calculated to be 7.56 ± 1.491 μM and 0.16 ± 0.008 nmol · min^{-1} · mg protein^{-1}, respectively. Ethyl piperate was used at concentrations from 2 to 200 μM. B, the IC_{50} of BNPP, determined by plotting the remaining activity against the logarithm of BNPP concentration, was 4.99×10^{-7} M. The substrate concentration was 400 μM, and BNPP was used at concentrations of 1 μM to 1 mM. Each point represents the mean ± S.D. ($n = 6$).

Discussion

Ethyl piperate is a drug candidate that efficiently down-regulates cholesterol and triglyceride, but its low systematic exposure, undetectable in plasma at times later than 1.5 h after oral administration, reduces its absolute bioavailability to less than 1%. The short $t_{1/2}$ after intravenous injection indicates that ethyl piperate is quickly eliminated in vivo, reflecting rapid metabolism and/or excretion (Yang et al., 2006).

Preliminary results with orally administered ethyl piperate have shown promising lipid-lowering efficacy, and efforts to understand the pharmacodynamic mechanism have focused on ethyl piperate absorption (Nix, 2003). The gut is a complex environment with a variable pH and a mixture of digestive enzymes that can sometimes undermine the stability of ester bonds (Crauste-Manciet et al., 1997). In our study, we found that ethyl piperate was stable in acidic, neutral, and alkaline conditions for 3 h and was resistant to intestinal washings, indicating that the gastrointestinal environment contributed little to ethyl piperate degradation. Using BNPP as a tool to identify substrates of CES, which typically hydrolyzes carboxylic esters in vivo (Testa and Waterbeemd, 2007), we showed that the enzymes in microsomes from hamster liver, kidney, and intestine had high hydrolytic activity toward ethyl piperate in vitro. We further demonstrated that CES extensively metabolized ethyl piperate in intestine, which may be the main reason for the low oral bioavailability and short $t_{1/2}$ of ethyl piperate.

Exploiting an experimental design described by Mainwaring et al. (2001) in which BNPP (intraperitoneal) was used to assess the role of CES in the resistance to methyl methacrylate toxicity in rat nasal epithelium, we introduced BNPP into the single-pass perfusion experiment to uncover the role of CES in the disposition of ethyl piperate in the intestinal tract. Compared with the appearance rates of ethyl piperate and piperinic acid in portal vein, the bioavailability of piperinic acid was greater than that of the original drug. BNPP inhibited the hydrolysis of ethyl piperate and diminished its degradation clearance. The reduced P_{app} and P_{mono} of piperinic acid indicated that hydrolysis by CES was inhibited by BNPP both in Caco-2 cells and intestinal tissues, whereas the P_{app} and appearance rate of ethyl piperate in portal vein were not affected. These results were inconsistent with those of Caco-2 monolayer experiments, in which there was an apparent increase in P_{mono} after inhibition of CES. This discrepancy may reflect inherent differences in the composition of Caco-2 cells and enterocytes. Cancer cell lines, such as Caco-2, are known to have low enzymatic activity, especially with respect to metabolic enzymes such as CYP3A4 (Sun et al., 2008). In contrast,

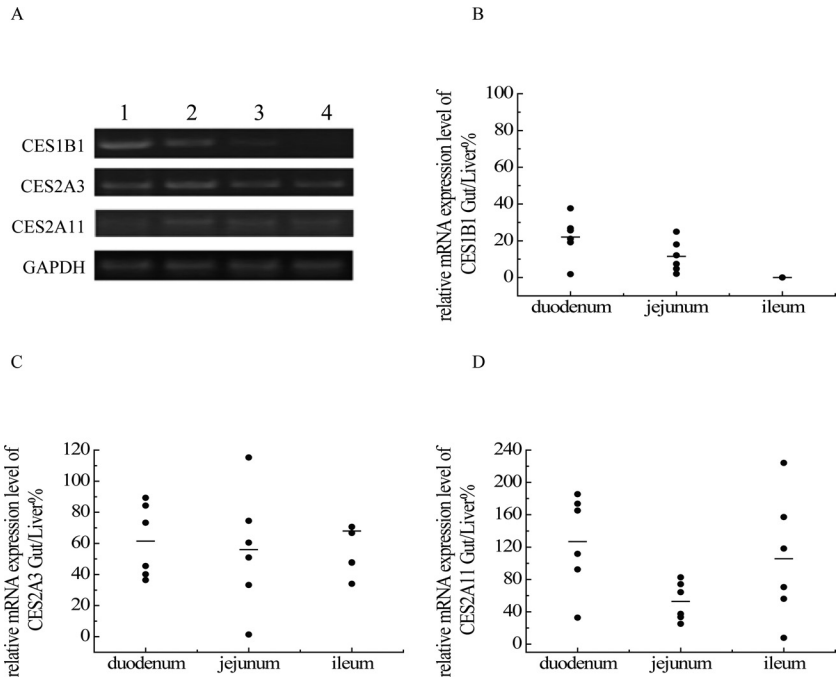

FIG. 6. Expression of CES isozyme transcripts in the hamster intestines. A, mRNA levels of the CES isozymes CES1B1, CES2A3, and CES2A11 in the duodenum, jejunum, and ileum were detected by RT-PCR; GAPDH mRNA was used as an internal control ($n = 6$). Lanes 1, liver; lanes 2, duodenum; lanes 3, jejunum; lanes 4, ileum. The expression levels of CES isozymes CES1B1 (B), CES2A3 (C), and CES2A11 (D) in intestinal regions relative to that in liver are shown.

previous reports have shown that the enterocyte is one of the main drug-metabolizing cells in the body and expresses multiple metabolic enzymes (Lin and Lu, 2001). Our latter study provided support for the idea that these differences in metabolic capacity might account for the contrasting results obtained in the two different models (Y. Lu and Y. Wang, unpublished data). In any case, the P_{mono} values of piperinic acid and ethyl piperate on Caco-2 monolayers revealed that both the parent drug and its hydrolysate should be absorbed well (Lennernäs et al., 1996). As is shown in Table 3, the loss of ethyl piperate from intestine was much more than the gain of both original drug and the hydrolysate in the portal vein when perfused with BNPP, almost half of the total disappearance of ethyl piperate. Although the predominant reason was the partial replacement of hydrolysis by alternative metabolic pathways in vivo, a few drugs may be left in the intestinal tissue, according to the difference between ν_2 and the sum of ν_1 and ν_3 in control group. More work should be performed to confirm whether the ethyl piperate or its hydrolysate piperinic acid was trapped in enterocytes by some influx or efflux transporters when they passed through the intestinal wall and whether the BNPP affected the process.

CES has seldom been studied in hamsters, and only one liver cDNA encoding CES2A11 has been reported previously (Sone et al., 1994). On the basis of high homology and similarity of characteristics, this isozyme was inferred to be the main CES expressed in the hamster intestinal tract (Satoh and Hosokawa, 2006). Our analysis of CES isozymes expressed in the hamster intestine, which to the best of our knowledge is the first analysis of its kind, showed that the distribution of CES isozymes in the hamster was different from that in the Wistar rat (Masaki et al., 2007). CES2A3 mRNA levels were similar among the three regions of the hamster intestine and were 60% less than those in liver. CES1B1 mRNA levels were even lower in the intestine (10–20% of liver levels), and there was a proximal-to-distal decrease in the expression of CES1B1, which reached undetectable levels in ileum. Although the expression of CES2A11 exhibited substantial interindividual variation, it was the only isozyme expressed at comparable levels in both liver and intestine.

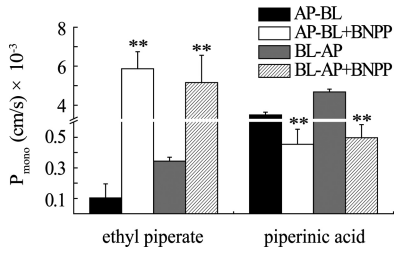

FIG. 7. The effect of CES inhibition on the permeability of ethyl piperate and its hydrolysate across Caco-2 monolayers in both apical-to-basolateral (AP-BL) and basolateral-to-apical (BL-AP) directions. The ethyl piperate concentration applied to the donor side was 5 μM; monolayers were treated with 200 μM BNPP or dimethyl sulfoxide (control) for 1 h at pH 7.4. Each point represents the mean ± S.D. (**, $p < 0.01$; $n = 3$).

A number of studies have attempted to interpret the action of therapeutics in the context of the complex molecular mechanisms underlying the pathogenesis of atherosclerosis (Tavridou and Manolopoulos, 2008). The ongoing efforts of cardiovascular specialists have yielded numerous drugs and drug candidates to slow disease progression or facilitate its turnover. The sites of actions of these drugs are extensively distributed throughout the body, but the main focus of previous studies has been on the liver, intestine, and blood circulation, especially the artery wall where plaques may occur. Although the curative effect of ethyl piperate in hypercholesterolemic hamsters is unambiguous, the molecular target of ethyl piperate has not been clearly defined. Our research into the status of ethyl piperate on the two sides of the absorption equation, intestine and systemic circulation, indicated that the parent drug may not be the principle antiatherosclerosis component if the lipid-lowering active sites are distributed in the liver or any other organs except the gut where drugs do not need to be absorbed (Kitayama et al., 2006). Additional studies to fully characterize the metabolites of ethyl piperate and define the pharmacokinetics and distributions will clearly be required to fully explain the behavior of ethyl piperate after oral administration. However, the results of the current study have provided the framework for our ongoing investigations into the pharmacodynamic mechanism of ethyl piperate and its metabolites.

Authorship Contributions

Participated in research design: Lu, Bao, and Wang.

Conducted experiments: Borjihan, Hu, Yu, and Wang.

Contributed new reagents or analytic tools: Bao, Borjihan, Ma, Hu, and Yang.

Performed data analysis: Lu, Ma, Jia, and Wang.

Wrote or contributed to the writing of the manuscript: Lu, Li, and Wang.

Other: Wang acquired funding for the research.

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