Characterization of L-Leucine Transport System in Brush Border Membranes From Human and Rabbit Small Intestine

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The branched-chain amino acids (BCAAs) leucine, isoleucine, and valine are beneficial to catabolic patients by improving hepatic protein synthesis and nitrogen economy, yet their transport from the intestinal lumen is not well-defined. The leucine transport system in human and rabbit small intestine was characterized using a brush border membrane vesicle (BBMV) model. Sodium and pH dependence and transport activity along the longitudinal axis of the small bowel were determined. Transport kinetics and inhibition profiles were defined. Although previous studies in other tissues show leucine transport to be mostly a Na^+-independent process, our studies show that leucine transport is a predominantly Na^+-dependent process occurring mainly via a single saturable pH-independent transporter resembling system B^0 in the intestine. This system B^0 transporter demonstrates stereoisomeric specificity. There is also a minor Na^+-independent transport component (<6% in rabbits). Leucine uptake in both rabbits and humans is significantly greater than the uptake of other clinically relevant nutrients such as glutamine. In the rabbit, ileal leucine transport is significantly greater than jejunal uptake. While the affinities of the human and rabbit transporters are similar, the rabbit transporter has greater carrier capacity (maximal transport velocity [V_{max}]). These findings suggest that the transport of leucine in the gut mucosa is significantly different from the transport in other tissues.

AMINO ACIDS have long been known to have a pivotal metabolic role in critically ill patients. The branched-chain amino acids (BCAAs) are essential amino acids consisting of leucine, isoleucine, and valine. They have been reported to have beneficial effects in states of sepsis, systemic inflammation, trauma, burns, and renal and hepatic failure.[1] The BCAAs are vital as metabolic fuels and can act to provide energy even in the case of hepatic dysfunction. Protein degradation rates are commonly elevated during states of physiologic stress. By providing an energy and nitrogen source, BCAAs slow the degradation of lean body mass, stimulate de novo synthesis of proteins, especially acute-phase reactants, and create a more anabolic milieu during the hypercatabolism of critical illness.[2]

To date, the properties of intestinal BCAA transport have been characterized piecemeal in animal models. Human BCAA transport remains poorly defined and difficult to compare with animal data. This study comprehensively defines and compares the physicochemical properties of BCAA transport in normal human and rabbit small intestine.

MATERIALS AND METHODS

Animals

Two-kilogram juvenile male New Zealand White rabbits (Hazelton Research Products, Denver, PA) were housed in individual cages on a light-dark cycle in a temperature-controlled room in accordance with institutional guidelines and *The Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services, National Institutes of Health). All experimental protocols were approved by the Institutional Animal Care and Use Committee.

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Submitted January 15, 1999; accepted May 25, 1999.

Supported by National Institutes of Health Grant No. R29-DK47989-02 (H.C.S.).

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0026-0495/99/4811-0017$10.00/0

The rabbits were anesthetized with xylazine (25 mg/kg intramuscularly [IM]) and ketamine (50 mg/kg IM). The small bowel was rapidly harvested through a midline celiotomy and rinsed in ice-cold normal saline solution. Mucosa was scraped with a glass slide and immediately frozen at −70°C. When appropriate, mucosa from the proximal, middle, and distal gut was separately stored for further processing.

Adult (average age, 42 years; range, 25 to 70) human proximal jejunum (5-cm segments) was obtained from patients on the surgical service at Strong Memorial Hospital, University of Rochester Medical Center, Rochester, NY. Informed consent was obtained from each patient after approval by the University of Rochester Medical Center Research Subject Review Board (no. 6230). Specimens were obtained during gastric partitioning with Roux-en-Y gastrojejunostomy for morbid obesity (n = 9), partial gastrectomy with gastrojejunostomy for peptic ulcer disease (n = 1), and common bile duct reconstruction with Roux-en-Y hepaticojejunostomy after common bile duct injury (n = 1). No patient had concomitant neoplasia or uncontrolled diabetes. The segment of small bowel was rinsed in ice-cold normal saline solution immediately upon resection. Mucosa was scraped with a glass slide and immediately frozen at −70°C.

Reagents and Chemicals

All reagents and chemicals were of analytic quality and were purchased from Sigma Chemical (St Louis, MO). Radiolabeled L-[4,5-^{3}H]-leucine, L-[G-^{3}H]-glutamine, L-[2,3-^{3}H]-alanine, L-[2,3,4,5-^{3}H]-arginine, and D-[6-^{3}H]-glucose were purchased from Amersham (Arlington Heights, IL). Mannitol, glutamine, L-alanine, D-alanine, histidine, methyl-amino-isobutyric acid (MeAIB), leucine, lysine, glycine, isoleucine, valine, and proline were purchased from Sigma Chemical.

Membrane Vesicle Preparation

Nutrient transport across human and rabbit small intestinal mucosa brush border membrane vesicles (BBMVs) was determined. BBMVs were prepared from pooled small intestinal mucosa by magnesium aggregation and serial differential centrifugation at 0° to 5°C as previously reported.[3] Briefly, each gram of thawed mucosal scrapings was homogenized in 8 mL buffer containing 300 mmol/L mannitol and 1 mmol/L HEPES/Tris (pH 7.4) with a Polytron homogenizer (setting no. 6) for 45 seconds (Brinkman Instruments, Westbury, CT). Homogenates from each group were then treated with 100 mmol/L magnesium dichloride/1 mmol/L HEPES/Tris to yield a final magnesium concentration of 10 mmol/L. After stirring for 20 minutes, the homogenate was centrifuged for 10 minutes at 2,200 × *g*. The supernatant containing

brush border material was collected and centrifuged for 5 minutes at 3,300 × *g*. The supernatant was then centrifuged at 45,000 × *g* for 35 minutes. The BBMV pellet was resuspended in 350 mmol/L mannitol and 50 mmol/L HEPES/Tris and centrifuged again at 45,000 × *g* for 35 minutes. The final pellet was resuspended in the same buffer to yield a final protein concentration of 10 to 15 mg/mL. The protein concentration was determined by the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA) against a γ-globulin protein standard according to the instructions of the manufacturer.

BBMV Purity

Vesicle purity was determined by assessing the specific activity enrichment of brush border membrane–specific enzyme, alkaline phosphatase (AP),[4] and basolateral membrane–specific enzyme, ouabain-sensitive Na^+/K^+ adenosine triphosphatase (ATPase).[4] Reagents at the following concentrations were used for AP determination: 5 mmol/L *p*-nitrophenolphosphate in Tris maleate buffer (5 mmol/L $MgCl_2$, 250 μmol/L $CaCl_2 \cdot H_2O$, and 200 μmol/L $ZnCl_2$) at 37°C. Reagents at the following concentrations were used for Na^+/K^+ ATPase assay: 6 mmol/L ATP with or without 2 mmol/L ouabain in incubation solution (100 mmol/L NaCl, 100 mmol/L Tris, 20 mmol/L KCl, and 5 mmol/L $MgCl_2$, pH 7.3) at room temperature. The specific activities of both enzymes were quantified by spectrophotometric analysis. AP was read at 410 nm and Na^+/K^+ ATPase at 340 nm.

Transport Measurements

The uptake of tritiated substrates (glutamine, glucose, leucine, alanine, and arginine) was measured by a rapid mixing/filtration technique in the presence and absence of a sodium gradient at 4°C. For each uptake measurement, 10 μL BBMVs and 40 μL radioactive uptake buffer were placed separately at the bottom of a 12 × 75-mm polystyrene tube (VWR Scientific, West Chester, PA). The uptake buffer components were adjusted so that the final concentration mixture contained initial gradients of 120 mmol/L NaCl or KCl and 100 μmol/L substrate for all transport measurements, with the exception of kinetics (described later). An electronically controlled device initiated the reaction by rapidly vibrating the tube. After a 10-second reaction period, 1 mL ice-cold stop buffer (150 mmol/L NaCl and 10 mmol/L HEPES/Tris) was added to quench the reaction. The quenched reaction mixture was vacuum-filtered with a premoistened and chilled 0.45-μm nitrocellulose filter (Gelman Sciences, Ann Arbor, MI) to separate intravesicular and extravesicular radiolabeled substrate. The filters were rapidly washed with 8 mL ice-cold stop buffer and dissolved in Cytoscint scintillation cocktail (ICN Biomedicals, Irvine, CA). The radioactivity trapped by the vesicles (representing transport) was measured by liquid scintillation spectrometry (LS 8000; Beckman Instruments, Fullerton, CA). Values for nonspecific retention of radioactivity by the filter and the vesicles were obtained from time 0 uptakes and subtracted from total filter radioactivity. The radioactivity was converted to units of uptake and expressed as picomoles of substrate per milligram of vesicle protein per unit time.

The Na^+-dependent component of substrate transport was calculated by subtracting uptake in the presence of potassium (Na^+-independent uptake, passive diffusion) from that determined in the presence of sodium (total uptake). Osmolarity differences as a result of varying concentrations of amino acids were corrected with mannitol. Uptake data were normalized to BBMV protein concentration.

Kinetic Measurements

Leucine transport kinetic parameters were determined by assaying the uptake of ^{3}H-leucine over a range of increasing unlabeled leucine concentrations (25 to 5,000 μmol/L; rabbit, n = 3; human, n = 4). Kinetic experiments were also performed at the 10-second time point to ensure measurement of the initial maximal uptake before dissipation of the sodium gradient.

Leucine Uptake Inhibition Profiles

BBMVs were coincubated with tritiated leucine and individual potential competitive inhibitors (10 mmol/L each) to determine leucine transporter specificity. Inhibition profiles were generated for various amino acids, ie, glutamine, L-alanine, D-alanine, histidine, MeAIB, lysine, glycine, isoleucine, valine, and proline. Mannitol and unlabeled leucine served as the positive and negative controls, respectively.

Transport Activity Along the Longitudinal Axis of the Small Bowel

The regional variation of leucine transport in the rabbit small intestine was assessed by measuring the uptake from mucosa harvested from the proximal, middle, and distal thirds of the small intestine (n = 4 per anatomic section).

pH Dependence

Leucine transport into BBMVs prepared from proximal, middle, and distal rabbit small intestinal mucosa was determined (n = 4 per anatomic section) over a range of uptake buffer pH (5 to 9).

Statistical Analysis

The data are reported as the mean ± SEM, with significance ($P < .05$) determined by ANOVA with the post hoc Student-Newman-Keuls pairwise multiple-comparison procedure where appropriate.

RESULTS

BBMV Purity

The specific activities for AP (brush border membrane marker) and Na^+/K^+ ATPase (basolateral membrane marker) in vesicles were compared with those of crude homogenates. The purity of the BBMV preparation was confirmed by enrichment of the activity of the brush border enzyme AP with no change in the activity of the basolateral enzyme Na^+/K^+ ATPase. The AP enrichment was 12-fold in human jejunum and sixfold in rabbit whole-gut mucosa ($P < .05$). In rabbits, no significant differences in AP enrichment were noted between proximal or distal bowel (6.2 ± 0.5 and 5.8 ± 0.85, respectively). There was a decrement in Na^+/K^+ ATPase activity in all groups (data not shown).

Transport Measurements

Sodium-dependent leucine transport in humans and rabbits (218 ± 17 and 611 ± 29 pmol/mg protein/10 s, respectively) was 3.9- and 16.5-fold higher than the Na^+-independent component of transport (56 ± 3 and 37 ± 3 pmol/mg protein/10 s, respectively). The mean uptake of radiolabeled leucine in the presence of sodium was higher ($P < .05$) in rabbits than in humans. Leucine uptake was compared with the uptake of glutamine, alanine, arginine, and glucose in normal rabbit (n = 4) and human (n = 11) small intestinal mucosa (Fig 1). Sodium-dependent leucine transport was markedly greater than the transport of other amino acids in both species.

Kinetic Measurements

To quantify the K_m and maximal transport velocity (V_{max}), sodium-dependent transport was determined in rabbit BBMVs

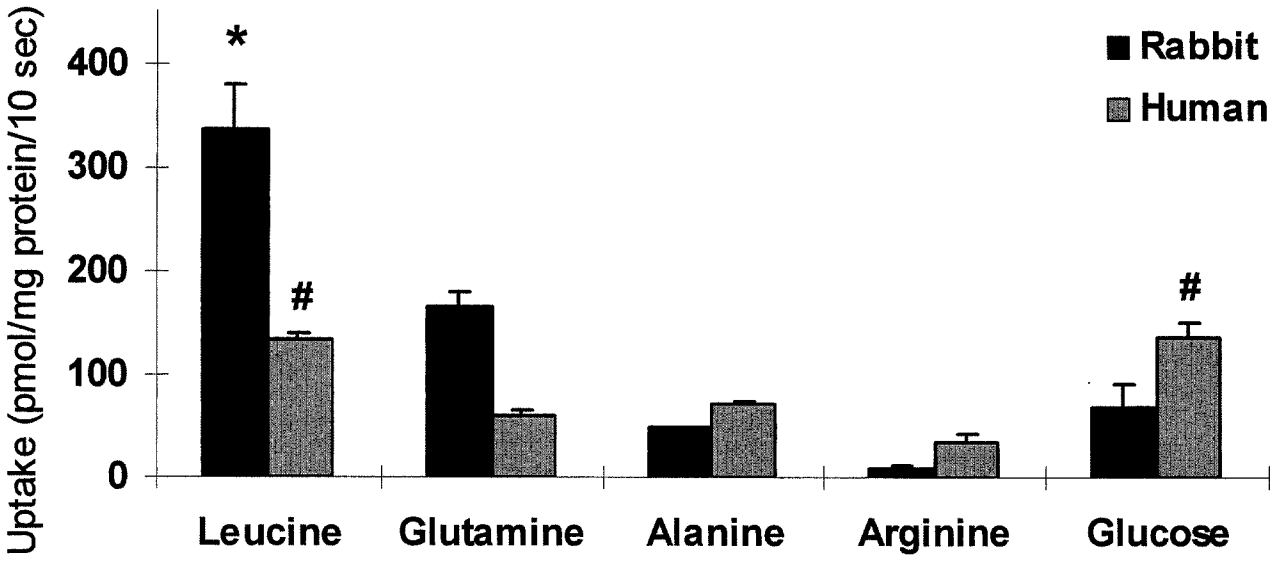

Fig 1. Sodium-dependent neutral amino acid transport (glutamine, glucose, alanine, leucine, and arginine) into BBMVs measured in pmol/mg protein/10 s. BBMVs were coincubated with nutrient uptake buffer containing 100 μmol/L substrate. The figure is a composite of quadruplicate BBMV preparations. *$P < .05$ *v* unmarked substrates (rabbit). #$P < .05$ *v* unmarked substrates (human).

(Fig 2) and human BBMVs (Fig 3) over a range of leucine concentrations (25 to 5,000 μmol/L). Eadie-Hofstee transformation of the kinetic data revealed a V_{max} of 1,965 ± 33 pmol/mg protein/10 s and a K_m of 203 ± 3 μmol/L in rabbit ($r = .99$) and a V_{max} of 697 ± 6 pmol/mg protein/10 s and a K_m of 364 ± 11 μmol/L in human ($r = .94$).

Inhibition Profiles

Sodium-dependent leucine uptake inhibition profiles were generated for various amino acids, ie, glutamine, L-alanine, D-alanine, histidine, MeAIB, leucine, lysine, glycine, isoleucine, valine, and proline (Fig 4). There was a marked inhibition ($P < .05$) of leucine transport in both species by L-alanine, asparagine, and glutamine, as well as the BCAAs leucine, valine, and isoleucine. Mild to moderate inhibition was noted by coincubation with glycine, lysine, histidine, and proline. While L-alanine produced a marked inhibition ($P < .05$) of leucine uptake, D-alanine, a stereoisomer, produced almost no inhibition. Likewise, the system A–specific substrate MeAIB produced very limited inhibition.

Regional Variation in Transport

Sodium-dependent uptake of leucine in the rabbit distal segment (ileum, 677 ± 23 pmol/mg protein/10 s) was significantly greater ($P < .05$) versus either the proximal (jejunum,

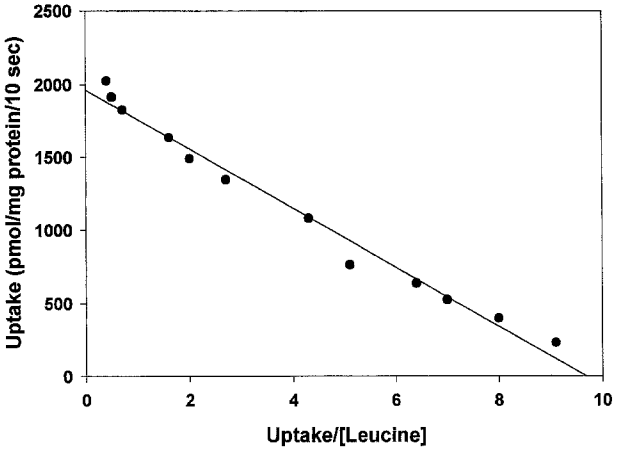

Fig 2. Eadie-Hofstee transformation of rabbit leucine transport kinetic data. BBMVs were incubated with varying concentrations of leucine in uptake buffer from 25 to 5,000 μmol/L for a period of 10 seconds. Sodium-dependent leucine uptake is plotted as a function of leucine uptake/leucine concentration. The y-axis intercept represents the V_{max}, and the negative slope of the line corresponds to transport affinity (K_m).

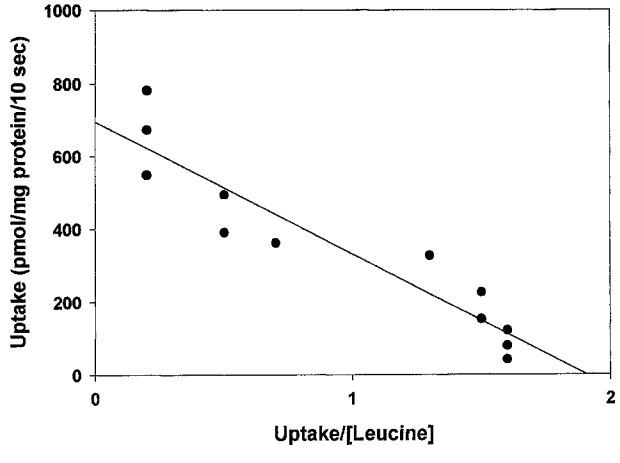

Fig 3. Eadie-Hofstee transformation of human leucine transport kinetic data. BBMVs were incubated with varying concentrations of leucine in uptake buffer from 25 to 5,000 μmol/L for a period of 10 seconds. Sodium-dependent leucine uptake is plotted as a function of leucine uptake/leucine concentration. The y-axis intercept represents the V_{max}, and the negative slope of the line corresponds to transport affinity (K_m).

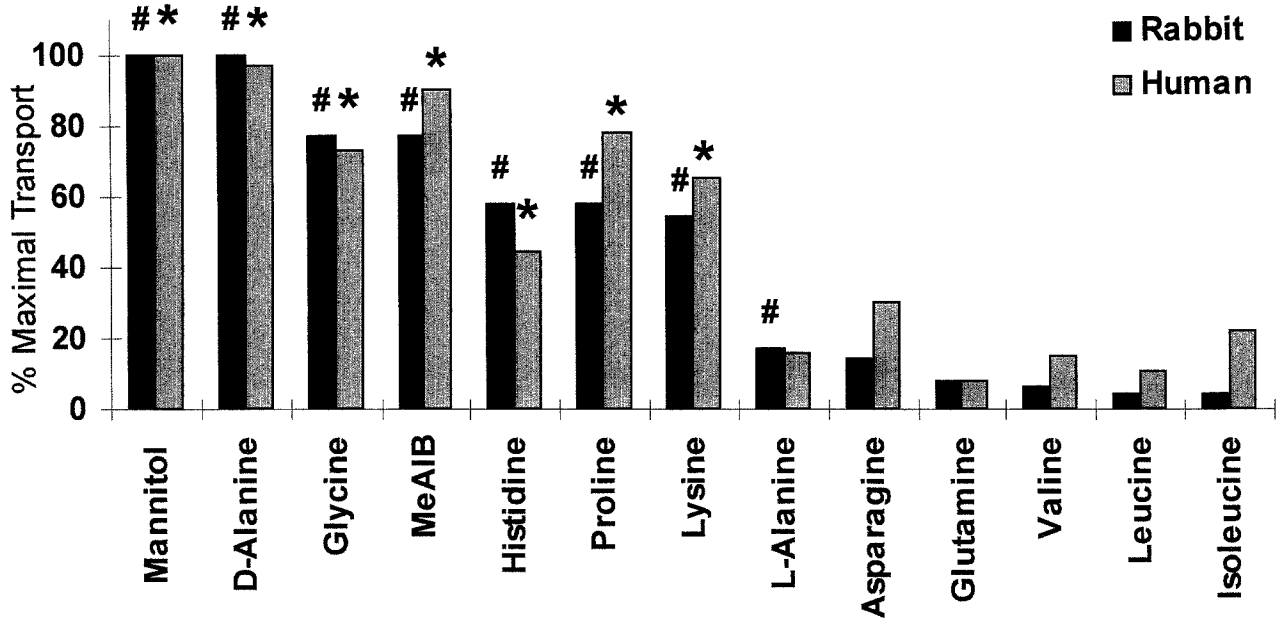

Fig 4. Amino acid inhibition profiles of leucine transport in human and rabbit brush border membranes. Leucine transport inhibition profiles were generated for single amino acids by coincubating 10 μL BBMVs with 40 μL radioactive uptake buffer consisting of 100 μmol/L unlabeled leucine and 10 mmol/L excess potential competitive inhibitor. The inhibition data are reported as % maximal leucine transport obtained in the presence of a positive control (mannitol). Excess unlabeled leucine served as a negative control. *$P < .05$ *v* rabbit leucine uptake. #$P < .05$ *v* human leucine uptake.

443 ± 22 pmol/mg protein/10 s) or middle (jejuno-ileal, 485 ± 36 pmol/mg protein/10 s) segments.

pH Dependence

There was no pH effect (pH range, 5 to 9) on leucine uptake along the intestinal longitudinal axis (data not shown).

DISCUSSION

Small intestinal amino acid transporters play a major role in whole-body nitrogen metabolism during absorptive and postabsorptive states.[5] The transport of amino acids into the cytoplasm occurs via functionally and biochemically distinct amino acid transport systems defined on the basis of their amino acid selectivities and biochemical kinetic properties.[6] Leucine, isoleucine, and valine are neutral amino acids characterized by side chains of branched hydrocarbons. As these amino acids are considered essential, their uptake by the small intestine is extremely important and highly regulated.[2] The substrate specificity, kinetic parameters (V_{max} and K_m), and inhibition analysis with a broad range of amino acids comprise the essentials of the functional characterization of a transport system.[5]

Leucine transport in many non-gut tissues has been previously described as a Na^+-independent process that occurs by facilitated diffusion. Leucine transport and aromatic amino acid transport was ascribed to system L.[6,7] Our data indicate that in rabbits and humans, leucine brush border transport is mostly Na^+-dependent. In this instance, we replicate the Na^+-dependent leucine absorption reported in the small intestine and other polarized epithelial cells.[8] While one might postulate that Na^+-independent leucine transport becomes significant when Na^+-dependent transport is saturated, this does not appear to be relevant. The BCAA transporter is not dependent on a proton gradient because it is insensitive to pH over a range of 5 to 9, further confirming that amino acids do not cotransport with protons.

Although glutamine metabolism has received a great deal of attention during the past decade, leucine uptake is 100% greater than glutamine uptake in both rabbit and human tissue. Sodium-dependent leucine uptake is 300% greater in rabbit intestine versus human intestine. The greater specific absorptive efficiency of the rabbit intestine supports the importance of BCAAs in this species, which perhaps confers a survival advantage during periods of starvation or limited dietary intake.

Our kinetic studies suggest that a single saturable system is responsible for Na^+-dependent BCAA transport in both rabbits and humans, although we cannot rule out the existence of a system with a similar K_m (Figs 2 and 3). The rabbit carrier exhibited a higher capacity (V_{max}) than the human carrier, yet the affinities (K_m) are not significantly different between these species. The findings may have been influenced by both interspecies and developmental-stage differences between rabbits and humans. However, increased leucine transport most likely does not represent the simple effect of the increased basal metabolic rate in rabbits compared with humans. In fact, alanine, arginine, and glucose transport were higher in humans (Fig 1). In relation to other species, the V_{max} of intestinal leucine transport in sheep and pigs has been shown to be of the same magnitude as in rabbits and significantly greater than in humans. While the porcine K_m is similar to that of rabbits and humans, the ovine K_m suggests a markedly reduced affinity for its substrate.[9]

BCAA transport was further characterized by determining the inhibition profiles of leucine transport with potential competitive inhibitors (Fig 4). The marked inhibition of leucine

transport by the BCAAs valine, leucine, and isoleucine implies that the BCAAs share a common transporter system. Furthermore, the inhibition of leucine uptake by coincubation with glutamine and L-alanine suggests that BCAA transport is mediated by system B^0.[8] BCAAs are transported also by the Na^+-dependent system $B^{0,+}$, a previously described neutral and cationic amino acid transporter. $B^{0,+}$ is characterized by the inhibition of neutral and basic amino acid transport via a wide spectrum of neutral and dibasic amino acids.[6,10] A system resembling system $B^{0,+}$ has been described also in rabbit ileum.[11-13] While L-alanine produced marked leucine transport inhibition, D-alanine, inhibitory to $B^{0,+}$,[11] had minimal effects on leucine transport. The partial inhibition of Na^+-dependent leucine transport by lysine in our study suggests that system $B^{0,+}$ may also function in human and rabbit intestine. However, since the inhibition is relatively minor, the contribution of $B^{0,+}$ may be minimal or nonspecific. Nonetheless, the data collectively indicate that system B^0 mediates the majority of leucine transport from the intestinal lumen.

As the small intestine differs anatomically and histologically along its length, it is not unexpected that regional variation in transport activity along the longitudinal axis exists. Leucine transport is significantly greater in the distal (ileum) than in the proximal (jejunum) or middle (jejuno-ileal) rabbit small intestine. The distal small intestine might demonstrate more efficient leucine transport as a result of carrier upregulation because of the decreased amount of nutrients reaching that portion of the gut.

In summary, we have characterized some of the physicochemical properties of leucine transport in human and rabbit small intestinal mucosa using the BBMV model. Leucine transport in both species is mainly a Na^+-dependent process that occurs by a stereospecific pH-independent transporter resembling system B^0. There is a minor Na^+-independent component, whereas in nonepithelial tissues, transport is mostly Na^+-independent. The magnitude of leucine uptake is significantly greater than that of the other individual nutrients, including glutamine, another system B^0 substrate. Regional variation in transport activity along the longitudinal axis of the rabbit small intestine also exists. The characterization of such processes in healthy subjects allows a basis of comparison for catabolic states or malabsorptive states, which is an active area of study.

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