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Transport of N^G-Nitro-L-Arginine Across Intestinal Brush Border Membranes by Na^+-Dependent and Na^+-Independent Amino Acid Transporters

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Purpose. To clarify the transport mechanism of N^G-nitro-L-arginine (L-NNA), a potent NO-synthase inhibitor, across intestinal brush border membranes (BBM).

Methods. Dog intestinal BBM vesicles were used.

Results. The time course of L-NNA uptake showed a Na^+-dependent overshoot phenomenon. Concentration-dependence curves of L-NNA initial uptake were saturable in the presence and absence of Na^+, indicating participation of Na^+-dependent and Na^+-independent carrier-mediated transport systems. The calculated kinetic parameters of L-NNA initial uptake indicate that the former is a low-affinity high-capacity system and the latter is a high-affinity low-capacity one, similar to those in neutral amino acid transport. Neutral and basic amino acids showed cis-inhibitory and trans-stimulatory effects on L-NNA uptake in the presence or absence of Na^+. N^G-Nitro-L-arginine methyl ester, another potent NO-synthase inhibitor, also had both effects, which were smaller than with amino acids.

Conclusions. The present study clearly indicates that transport of L-NNA across the intestinal BBM occurs in the same manner as neutral amino acid transport. However, it is affected by both neutral and basic amino acids in the presence or absence of Na^+ differently from that across plasma membranes of nonepithelial cells, because $B^{0,+}$ and $b^{0,+}$ amino acid transporters function partly in L-NNA transport across intestinal BBM.

KEY WORDS: N^G-nitro-L-arginine; neutral amino acid transport; basic amino acid transport; intestinal brush border membrane vesicles.

INTRODUCTION

Nitric oxide (NO) functions as a signaling molecule in various biological systems (1). NO is synthesized during the oxidation of the terminal guanidino nitrogen atoms of L-arginine by NO-synthase (NOS) (2). N^G-nitro-L-arginine (L-NNA, Fig. 1), an L-arginine analogue, has been shown to be a potent inhibitor of NOS, and is currently being tested as a therapeutic agent for various indications, such as prevention of morphine withdrawal, attenuation of ammonia toxicity, and reduction of and protection against hypoxic-ischemic damage (3). However, little information about the intestinal absorption of L-NNA after oral administration is available, except a report by Piotrovskij *et al.* (4), who have shown that the absolute bioavailability (BA) after oral administration of L-NNA was approximately 90% in rats.

Generally, low lipophilic substances such as L-NNA are considered to have low BA after oral administration, due to their low permeability across the brush border membranes (BBM) of intestinal epithelial cells. However, some solutes such as amino acids and hexoses are known to be transported through the intestinal BBM by the "carrier-mediated transport" process (5–7). L-NNA is an L-arginine analogue, and we can therefore expect L-NNA to be transported through the intestinal BBM by the "amino acid transporters". In nonepithelial cells, such as macrophages (8,9), cerebellar synaptosomes (3), neuroblastoma × rat glioma hybrid cells (10) and endothelial cells (11), uptake of L-NNA is mediated by the neutral amino acid transport system, rather than the basic amino acid transport system, because L-NNA behaves as a neutral amino acid in environments of physiological pH (3,9). In those reports, L-NNA uptake into those cells was not affected by L-arginine (3,8–11). However, in the intestines, neutral amino acids interact with basic ones via their common transporters across the BBM (7,12–15). In their review, Ganapathy *et al.* (7) classified the transport systems in the BBM of the small intestine for neutral amino acids (dipolar α-amino acids) and basic amino acids into four systems as follows: system B, a Na^+-dependent system for dipolar α-amino acids; system $B^{0,+}$, a Na^+-dependent system for neutral and basic amino acids; system $b^{0,+}$, a Na^+-independent system for neutral and basic amino acids; and system y^+, a Na^+-independent system for basic amino acids. That is, intestinal epithelial cells have different characteristics of amino acid transport from nonpolarized cells, because the BBM in the former cells possess various amino acid transport systems which have not been described in the latter cells (7,16).

In the present study, we used dog intestinal brush border membrane vesicles (BBMVs) to clarify the transport mechanism of L-NNA across the intestinal BBM. As a result, we showed that transport of L-NNA across intestinal BBM occurs in the same manner as that of neutral amino acids, but is affected by both neutral and basic amino acids differently from that across the plasma membranes of nonepithelial cells.

MATERIALS AND METHODS

Chemicals

L-[^{3}H]-NNA (1.89 TBq/mmol) was purchased from Amersham (Little Chalfont, Bucks., UK). All other chemicals were of at least analytical grade, and were obtained from Sigma (St. Louis, MO, USA) or Wako (Osaka, Japan).

Preparation of BBMVs

The experiments were approved by the Animal Welfare Ethics Committee of Chugai Pharmaceutical Co., Ltd. Three healthy beagle dogs (CSK Research Park, Suwa, Japan, weight 10.3–11.8 kg) were killed by exsanguination under anesthesia with sodium pentobarbital. BBMVs were isolated from the small intestine of the dogs, according to the calcium precipitation method, as described previously (12). Finally, BBMVs were suspended in suspension buffer (100 mM mannitol and 10 mM Hepes, pH 7.5 adjusted with KOH) to a concentration of approximately 15 mg protein/ml. Aliquots of the final suspension were stored at −80°C until use.

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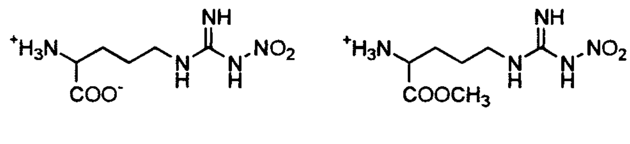

Fig. 1 Structures of N^G-nitro-L-arginine (L-NNA) and N^G-nitro-L-arginine methyl ester (L-NAME).

Transport Studies

Transport studies were performed using a rapid filtration technique, as described previously (12). The reaction was initiated by adding 100 μl of the incubation buffer (100 mM mannitol, 10 mM Hepes and 100 mM NaCl or KCl, pH 7.5 adjusted with KOH) containing labeled (1 μCi/100 μl) and unlabeled L-NNA, at various concentrations, to 10 μl of BBMV suspension, after pre-incubation for 2 min at 37°C. At the stated times, the reaction was stopped by adding 1 ml of the ice-cold stop solution (150 mM NaCl and 20 mM Hepes/Tris pH 7.5). The BBMVs were collected on a membrane filter (Millipore, HAWP), and kept under suction while being washed with 5 ml of the ice-cold stop solution. The cis-inhibition effects on L-NNA uptake were examined by the addition of various compounds to the incubation buffer at concentrations of 2.5 mM in the case of the amino acids, and 2.5 and 10 mM in the case of N^G-nitro-L-arginine methyl ester, another potent NOS inhibitor (L-NAME, Fig. 1).

The trans-stimulation effects on L-NNA uptake of various amino acids and L-NAME were also examined as described previously (12). 5 μl of BBMV suspension was added to 5 μl of the suspension buffer containing 10 mM of each stimulator. The mixture was incubated at room temperature for 1 hr to preload the stimulator at 5 mM into BBMVs, and used for transport studies: at 37°C, with 90 μl of the incubation buffer containing 0.5 mM of L-[^{3}H]-NNA (1 μCi/100 μl) as a substrate, for 15 s. To eliminate the inhibitory effect of the stimulator remaining outside of the BBMVs, we set up a control group using L-[^{3}H]-NNA at the final concentration of 0.95 mM without a stimulator, and calculated the uptake regarding L-NNA concentration as 0.5 mM, as described previously (12).

Radioactivity was counted using a liquid scintillation counter (Beckman, model LS5801, CA, USA), to determine the amount of L-[^{3}H]-NNA remaining on the membrane filter. Every experiment was carried out at least in duplicate, and was repeated three times with a different membrane preparation. Transport data are taken from three experiments, and are expressed as the mean ± S.E. in nmol/mg protein per unit time. Protein concentration was measured by the method of Bradford, using a commercial kit (Pierce, Rockford, IL, USA), with bovine serum albumin as a standard.

Statistical Analysis

The significance of difference was determined using a paired Students t test. A P value of <0.05 was considered statistically significant.

Calculation of Kinetic Parameters of Uptake

In our previous study, the total uptake of neutral and basic amino acids by intestinal BBMVs could be expressed as the sum of two saturable terms exhibiting Michaelis-Menten kinetics, and one non-saturable term (12). On this basis, the initial uptake rates (at 15 s) of L-NNA uptake as functions of substrate concentration ranging from 0.333 to 10 mM with the Na$^+$ and K$^+$ gradient were fitted simultaneously to the following equations, equation 1 and 2, respectively, using a nonlinear least squares regression analysis program, MULTI (17):

$$V_1 = \frac{V_{max} \cdot [S]}{K_m + [S]} + \frac{V'_{max} \cdot [S]}{K'_m + [S]} + k_d \cdot [S] \quad (1)$$

$$V_2 = \frac{V'_{max} \cdot [S]}{K'_m + [S]} + k_d \cdot [S] \quad (2)$$

where V_1 is the total velocity with the Na$^+$ gradient, V_2 is the total velocity with the K$^+$ gradient, [S] is the concentration of L-NNA, V_{max} and K_m are the maximal velocity and the Michaelis constant of the Na$^+$-dependent carrier-mediated transport, respectively, V'_{max} and K'_m are the maximal velocity and the Michaelis constant of Na$^+$-independent carrier-mediated transport, respectively, and k_d is the coefficient of non-saturable transport.

Calculation of Percentage Inhibition of Na$^+$-Dependent or Na$^+$-Independent Carrier-Mediated Transport

We resolved the inhibitory effect of various amino acids on total L-NNA uptake into effects on Na$^+$-dependent and Na$^+$-independent carrier-mediated uptake, as follows:

% inhibition (Na$^+$ dependent)

$$= 100 - \left[\frac{V_i(\mathrm{Na}^+) - V_i(\mathrm{K}^+)}{V(\mathrm{Na}^+) - V(\mathrm{K}^+)}\right] \times 100 \quad (3)$$

% inhibition (Na$^+$ independent)

$$= 100 - \left[\frac{V_i(\mathrm{K}^+) - V_d}{V(\mathrm{K}^+) - V_d}\right] \times 100 \quad (4)$$

where $V(\mathrm{Na}^+)$ and $V(\mathrm{K}^+)$ is the L-NNA initial uptake of the control group (uninhibited) in the cis-inhibition test, with Na$^+$ and K$^+$ gradients, respectively, $V_i(\mathrm{Na}^+)$ and $V_i(\mathrm{K}^+)$ is the corresponding L-NNA initial uptake in the presence of a inhibitor, and V_d is the L-NNA uptake by non-saturable transport ($k_d \times$ 0.5 mM).

RESULTS

Time Course of L-NNA Uptake with the Na$^+$ or K$^+$ Gradient

The time course of L-NNA uptake (1 mM) was determined with the inwardly directed Na$^+$ or K$^+$ gradient (Fig. 2). With the Na$^+$ gradient, L-NNA uptake showed a significant difference from that with the K$^+$ gradient at 30 s and 1 min, and a distinct overshoot phenomenon which was not present with the K$^+$ gradient. The maximum uptake of L-NNA with the Na$^+$ gradient was observed at 30 s, and the equilibrium was reached at 5 min.

Concentration Dependence of L-NNA Initial Uptake with the Na$^+$ or K$^+$ Gradient

The L-NNA initial uptake rates (at 15 s) by BBMVs were measured at various concentrations, ranging from 0.333 to 10

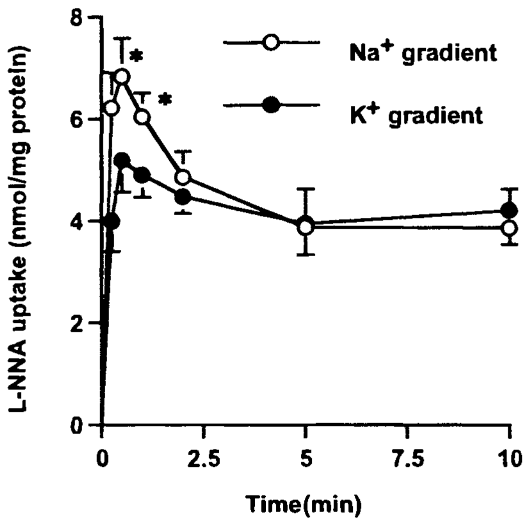

Fig. 2 Time course of N^G-nitro-L-arginine (1 mM) uptake by dog intestinal BBMVs in the presence (○) or absence (●) of the Na^+ gradient. Each value in the presence of the Na^+ gradient with $P < 0.05$ (*) is significantly different from each one in the absence of the Na^+ gradient at the same incubation time. Each point represents the mean ± S.E. of three experiments.

mM, with the inwardly directed Na^+ or K^+ gradient (Fig. 3). They were significantly different at all concentrations between with the Na^+ and K^+ gradients. Two different saturated curves were observed, indicating that L-NNA is transported across the intestinal BBM in both Na^+-dependent and Na^+-independent carrier-mediated pathways, similarly to amino acids (12,13). Therefore, we determined the kinetic parameters for L-NNA transport, resolving the total uptake into three components as we did for amino acid transport in our previous study (12). Briefly, the V_{max}, K_m, V'_{max}, K'_m and k_d values of the L-NNA initial uptake are 13.46 ± 1.33 nmol/mg protein/15 s, 3.63 ± 0.61 mM, 3.38 ± 0.31 nmol/mg protein/15 s, 0.318 ± 0.045 mM and 0.230 ± 0.036 μl/mg protein/15 s, respectively.

Cis-Inhibition Effects of Various Amino Acids and L-NAME on L-NNA Uptake

To ascertain whether transport systems for L-NNA through intestinal BBM are identical with those for amino acids, the cis-inhibition effects of various amino acids on L-NNA uptake by intestinal BBMVs were studied (Fig. 4). Basic amino acids (L-arginine and L-lysine), a small neutral amino acid (L-alanine) and bulky neutral amino acids (L-phenylalanine and L-leucine) were used as the inhibitors. All the amino acids (2.5 mM) used in this study significantly inhibited L-NNA uptake (0.5 mM) by BBMVs in the presence or absence of the Na^+ gradient, except L-NNA itself with the Na^+ gradient (P = 0.06) despite the equal value to those for other inhibitors. The inhibition percentages of Na^+-dependent and Na^+-independent carrier-mediated uptake by various amino acids were 15-56% and 75-89%, respectively, for all amino acids, including L-NNA itself (Table I).

L-NAME (Fig. 1, 2.5 and 10 mM) also had a significant cis-inhibition effect on L-NNA uptake in a concentration-dependent manner (Fig. 4, Table I). However, the effect at 2.5 mM was smaller than those for all amino acids used in this study.

Trans-Stimulation Effects of Various Amino Acids and L-NAME on L-NNA Uptake

To confirm directly that L-NNA is transported by the amino acid transporters, the trans-stimulation effects of various amino acids on L-NNA uptake by intestinal BBMVs were

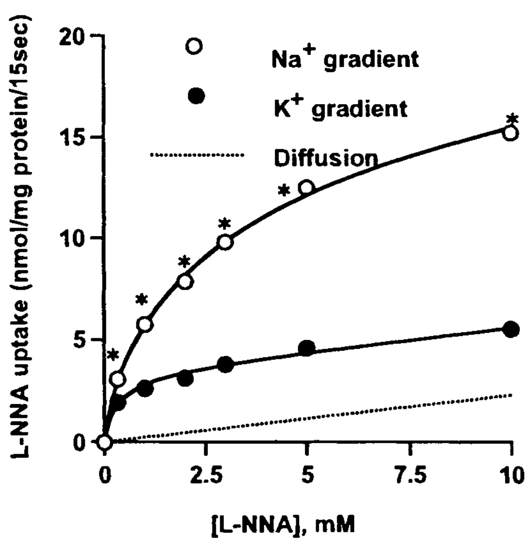

Fig. 3 Concentration dependence of the initial uptake rate (15 sec) of N^G-nitro-L-arginine by dog intestinal BBMVs in the presence (○) or absence (●) of the Na^+ gradient. The concentration-initial uptake rate curves and the lines expressing simple diffusion were obtained by nonlinear regression as explained in the text. Each value in the presence of the Na^+ gradient with $P < 0.05$ (*) is significantly different from each one in the absence of the Na^+ gradient at the same concentration. Each point represents the mean ± S.E. of three experiments.

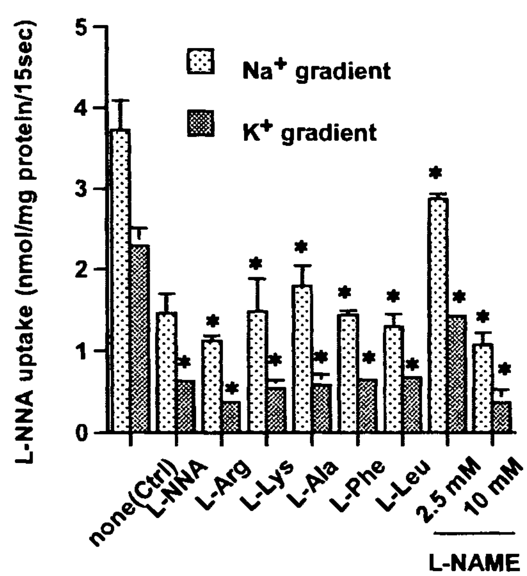

Fig. 4 Cis-inhibition effects of various amino acids, and L-NAME on L-NNA uptake by dog intestinal BBMVs. There are significant differences from each control value with the Na^+ or K^+ gradient at $P < 0.05$ (*). Each point represents the mean ± S.E. of three experiments.

Table 1. Inhibition of Na^+-Dependent and Na^+-Independent Carrier-Mediated L-NNA Transport (0.5 mM) by Various Amino Acids (2.5 mM) and L-NAME (2.5 and 10 mM)

								L-NAME	
	Ctrl	L-NNA	L-Arg	L-Lys	L-Ala	L-Phe	L-Leu	2.5 mM	10 mM
Na^+ dependent	0	42	47	34	15	44	56	0	50
Na^+ independent	0	76	89	81	79	76	75	40	89

(Percent inhibition)

examined (Fig. 5). The same amino acids as used in the cis-inhibition test were used in the trans-stimulation test as stimulators. All the amino acids, including both basic and neutral amino acids, accelerated L-NNA uptake by BBMVs in the presence or absence of the Na^+ gradient. However, the effects of L-arginine, L-alanine, L-phenylalanine and L-leucine with the Na^+ gradient were not statistically significant, despite the equal values to those for other stimulators ($P = 0.05$ to 0.09).

Preloaded L-NAME also stimulated L-NNA uptake significantly in the presence or absence of the Na^+ gradient.

DISCUSSION

The present study clearly indicates that transport of L-NNA across intestinal BBM occurs in the same manner as that of neutral amino acids. However, it is affected by both neutral and basic amino acids in the presence or absence of Na^+ differently from that across the plasma membranes of non-epithelial cells, because $B^{0,+}$ and $b^{0,+}$ amino acid transporters function partly in L-NNA transport across the intestinal BBM.

In this study, we used dog intestinal BBMVs, despite the difficulty of collecting a sufficient individual numbers, in order to investigate the interaction between L-NNA and basic (and neutral) amino acids transport across intestinal BBM clearly dividing into Na^+-dependent and Na^+-independent one. This was because dog intestinal BBMVs appeared to have larger capacity for $B^{0,+}$ than those of other animals (12). The time course of L-NNA uptake by dog intestinal BBMVs showed the Na^+-dependent overshoot phenomenon (Fig. 2). The kinetic parameters calculated from concentration-dependent curves of both Na^+-dependent and Na^+-independent uptake suggest that L-NNA passes through the intestinal BBM by at least three routes: low-affinity high-capacity Na^+-dependent carrier-mediated transport, high-affinity low-capacity Na^+-independent carrier-mediated transport, and non-saturable transport (Fig. 3). These transport characteristics for L-NNA are very similar to those observed for L-alanine, a neutral amino acid, and different from those for L-arginine, a basic amino acid, in our previous study (12), although L-NNA is an L-arginine analogue.

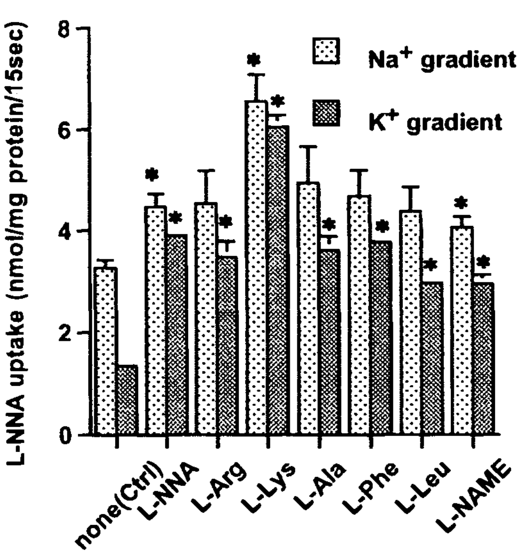

Fig. 5 Trans-stimulation effects of various amino acids and L-NAME on L-NNA uptake by dog intestinal BBMVs. There are significant differences from each control value with the Na^+ or K^+ gradient at $P < 0.05$ (*). Each point represents the mean ± S.E. of three experiments.

In the cis-inhibition and trans-stimulation tests, all the amino acids, including the basic amino acids L-arginine and L-lysine, inhibited and stimulated the L-NNA uptake, respectively, in the presence and absence of a Na^+ gradient (Figs. 4, 5 and Table 1). These results indicate directly that L-NNA is transported through intestinal BBM partly by neutral (B) and neutral/basic ($B^{0,+}$ and $b^{0,+}$) amino acid transporters.

In nonepithelial cells described above (3,8–11), L-NNA uptake was not affected by L-arginine. Christensen (16) reviewed that on the plasma membranes, transport interaction by neutral and basic amino acids is unlikely to be observed differently from intestinal BBM. The different interactions of L-NNA and basic amino acids across the intestinal BBM and across the plasma membranes of nonepithelial cells are well in accord with the differences in their amino acid transport characteristics.

Recently, Edwards *et al.* (18) reported that L-NNA inhibited L-arginine transport across the BBM of renal proximal tubules to a much lesser extent than basic amino acid-type NOS inhibitors, such as N^G-monomethyl-L-arginine and N-iminoethyl-L-ornithine, although these renal BBMVs are also considered to have $B^{0,+}$ and $b^{0,+}$ transporters. Moreover, L-NAME and citrullin (a neutral amino acid) had no effect on L-arginine transport across renal BBMVs. The reason for these results may be the differences in the tissue-specific proportion of $B^{0,+}$ and $b^{0,+}$ transporters between intestinal and renal BBM.

We resolved the inhibitory effects of various amino acids on total L-NNA uptake into effects on Na^+-dependent and Na^+-independent uptake by B and/or $B^{0,+}$, and $b^{0,+}$ transporters, respectively (Table 1). The contributions of the inhibitory effects of Na^+-independent carrier-mediated L-NNA transport were greater than those of the Na^+-dependent form in all the amino acids. These results are reasonable because the Na^+-independent neutral and basic amino acid transport system has a higher affinity to its substrates than the Na^+ dependent system (12,13). Similar results were obtained in the trans-stimulation test. The amounts of uptake trans-stimulated by various amino acids with the Na^+ gradient were similar to those with the K^+ gradient (Fig. 5). This indicates that the trans-stimulation effects of various amino acids observed in this study are largely attributable to the effects on the Na^+-independent transport system common to neutral and basic amino acids, system $b^{0,+}$. We previously observed a similar phenomenon in the trans-stimulation test when studying the effects of preloaded L-alanine and L-lysine on L-alanine uptake by the BBMVs (12). The most potent stimulator of L-NNA uptake was L-lysine, in accord with previous reports by us (12), although the reason for this is not well understood.

L-NAME is another potent NOS inhibitor which has been observed to inhibit L-NNA uptake into cerebellar synaptosome (3), macrophages (8) and endothelial cells (11). Therefore, it is also considered to be transported across these cell membranes by neutral amino acid transporters. In this study, L-NAME showed cis-inhibition and trans-stimulation effects on L-NNA uptake by intestinal BBMVs, in the presence or absence of the Na^+ gradient (Figs. 4, 5). This result indicates clearly that L-NAME is transported partly by amino acid transporters across intestinal BBM, although it does not have a free α-carboxyl group of amino acids (Fig. 1). It also shows a broad substrate specificity of both Na^+-dependent and Na^+-independent amino acid transporters on the intestinal BBM to the α-carboxyl group. However, the inhibitory effects of L-NAME on L-NNA uptake were less distinct than those of various amino acids and L-NNA itself at the same concentration of inhibitors. This indicates the lower affinity of L-NAME to the amino acid transport systems on intestinal BBM after changing a free carboxyl group of L-NNA to its methyl ester. Similar results have been reported with respect to the difference between the K_i values of L-NNA (0.34 mM) and L-NAME (0.53 mM) for L-citrullin transport by a neutral amino acid carrier in macrophages (8). Also in endothelial cells, L-NAME showed much lower K_i values for L-NNA and L-leucine transport than for L-NNA (11). The blockade of free carboxyl group in amino acids may decrease the affinity to the most types of amino acid transporters, but does not completely eliminate it.

From both quantitative and qualitative comparisons of the kinetic parameters, and cis-inhibition and trans-stimulation effects, between those for L-NNA and for neutral amino acids in our present and previous studies (12), we can conclude that L-NNA behaves as a neutral amino acid itself in transport across the intestinal BBM. Therefore, it is reasonable that efficient absorption of L-NNA by amino acid transporters brings about higher oral bioavailability (approx. 90%) when administered 48 hours after fasting, as reported by Piotrovskij *et al.*, in rats (4). However, the present study may also have an important practical implication as follows: when L-NNA is administered orally after a meal, its transport across the small intestine is interfered with by neutral and basic amino acids in the diet after the degradation of dietary protein, because amino acid transporters contribute to the greater part of L-NNA absorption (Fig. 3). The timing of administration may be very important for the consistent efficacy of L-NNA.

In summary, L-NNA is transported across the intestinal BBM in the same manner as neutral amino acids. However, L-NNA transport across the intestinal BBM is affected not only by neutral amino acids but also by basic amino acids differently from that across the plasma membranes of nonepithelial cells, because $B^{0,+}$ and $b^{0,+}$ amino acid transporters function partly in L-NNA transport across the intestinal BBM.

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