



FULL PAPER

Physiology

Orally administrated D-arginine exhibits higher enrichment in the brain and milk than L-arginine in ICR mice

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ABSTRACT. D-Amino acids exert various physiological functions and are widely present in animals. However, they are absorbed to a lesser extent than L-amino acids. Little is known about D-arginine (D-Arg); however, its isomer L-Arg serves as a substrate for several metabolites and exhibits various functions including promotion of growth hormone secretion. Milk is the only nutrient source for infants; it plays an important role during their initial growth and brain development. No studies have evaluated the availability of D-Arg in the brain and milk in mammals. Here, we have studied the differential availability of orally administered D- and L-Arg in the brain and milk using ICR mice. Our results revealed that without D-Arg administration, D-Arg was undetectable in both plasma and brain samples. However, the plasma D-Arg was about twice the concentration of L-Arg post administration of the same. In the cerebral cortex and hypothalamus, L-Arg concentration remained almost constant for over period of 90 min after L-Arg treatment. Nevertheless, the L-Arg concentration decreased after D-Arg administration with time compared to the case post L-Arg administration. Contrastingly, D-Arg level sharply increased at both the brain regions with time after D-Arg treatment. Furthermore, L-Arg concentration in the milk hardly increased after L-Arg administration. Interestingly, oral administration of D-Arg showed efficient enrichment of D-Arg in milk, compared with L-Arg. Thus, our results imply that D-Arg may be available for brain development and infant nourishment through milk as an oral drug and/or nutrient supplement.

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Free amino acids (FAAs) exist in the D- and L-forms. However, most FAAs are present as L-forms in nature. D-Amino acids are found in living organisms [8, 23], but only a few of them have been studied for their functions and mechanisms of action. L-Arginine (L-Arg) is known to ameliorate lifestyle-related disease conditions [9], improve learning and memory [19], and promote secretion of growth hormones [3]. L-Arg is not an essential dietary amino acid owing to its endogenous synthesis in sufficient amount in healthy adults. However, L-Arg synthesis may not meet the requirement of infants and growing children as well as adults with dysfunctions of the small intestine or kidney. Thus, L-Arg is classified as a semi-essential or conditionally essential amino acid [12, 13].

D-Arg, on the other hand, is known to be present in the central nervous system [4]. More than 10 types of L- and D-amino acids are known to be absorbed, but the absorption of D-amino acids is inferior to that of L-amino acids [7]. However, there is no such information about L-Arg and D-Arg absorption in this study. D-Arg is believed to use a different transporter from that of L-Arg and recent evidence suggests that D-Arg may also produce nitric oxide (NO) and NO derivatives via a pathway different from that of nitric oxide synthase (NOS) [11]. On the other hand, the supply of FAAs to the whole body, including the central nervous system, mainly dependent on the plasma FAA levels rather than their absorption rates. It has been shown that the levels of several FAAs at different brain sites linearly correlate with their plasma levels [21]. Even with high absorption rates, some FAAs except branched chain amino acids are mainly catabolized in the liver [1]. Thus, evaluation of the plasma FAA levels rather than their absorption rates may directly explain their functions.

The growth of an offspring is affected by not only proteins but also FAAs available in the maternal milk. Milk is derived from the mother's blood constituents and is the only source of nutrition to infants during the suckling period. Recent reports have shown that nutrition during early infancy may affect the health of the infant during later stages [10, 22]. Thus, changes in the components

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of maternal milk may influence the health and growth of offspring.

However, no information is available on the availability of D-Arg in the brain and milk. In the present study, we have investigated the differences in the accumulation of orally administered L-Arg and D-Arg in the brain and evaluated the effects of orally administered L-Arg and D-Arg on their availability in mouse milk.

MATERIALS AND METHODS

Chemicals

L-Arg and D-Arg (purity over 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Seven-week-old male ICR mice used in Experiment 1 and 11-day pregnant female ICR mice used in Experiment 2 were purchased from SLC (Hamamatsu, Japan). In Experiment 1, six mice were housed per plastic cage (25 cm \times 40 cm \times 20 cm) without enrichment and had free access to standard feed for laboratory rodents (MF, Oriental Yeast, Tokyo, Japan) and water for 1 week for acclimatization. Mice were housed under the same conditions until the end of this experiment. In Experiment 2, one mouse was housed per plastic cage (12 cm \times 30 cm \times 13.5 cm) without enrichment until delivery and had free access to food and water. Mice were housed with their pups from delivery to the end of this experiment. Mice were maintained on a 12 hr light/ dark cycle (lights on at 08:00, lights off at 20:00) at a room temperature of $23 \pm 1^{\circ}$ C and 60% humidity. This study was performed according to the guidelines for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government.

Experimental procedures

Experiment 1: Mice were divided into three groups as follows: 1) no administration group (control group); 2) L-Arg (6 mmol/10 ml distilled water [DW]/kg body weight [BW]) administration group and; 3) D-Arg (6 mmol/10 ml DW/kg BW) administration group. Oral administration was performed using a plastic probe. Each Arg administration group was further divided into three groups (6 mice per group) based on the time from administration to sampling (30, 60, and 90 min). Mice were tested during the light period and were maintained in a closed room. All mice were euthanized under anesthesia with isoflurane (Escain[®], Mylan, Osaka, Japan), and blood samples were collected. The brains were immediately excised and dissected to collect the cerebral cortex and hypothalamus.

Experiment 2: Mice were divided into three groups as follows: 1) distilled water (10 ml/kg BW) administration group (control group); 2) L-Arg (6 mmol/10 ml DW/kg BW) administration group; and 3) D-Arg (6 mmol/10 ml DW/kg BW) administration group. Post 13 days after delivery, each solution was orally administered 1 hr before milking. After milking, all mice were euthanized under anesthesia with isoflurane and liver and blood samples were collected.

Milking

Before milking, the maternal mice were separated from their offspring for more than 4 hr to obtain sufficient amount of milk for analysis. Mice were then subcutaneously injected with 0.1 ml (1 oxytocin unit) oxytocin (ZENOAQ, Fukushima, Japan) to promote milk release. After 20 min post injection, the mice were anesthetized using isoflurane and milked using KN-591 milking equipment for mice and rats (Natsume Seisakusho Co., Ltd., Tokyo, Japan) for 10 min. Milk (1.0–1.3 ml) was obtained from each mouse.

Analysis of free L-Arg and D-Arg concentrations in the plasma, milk, liver, and brain

Plasma samples were prepared by centrifugation of blood samples at $3,000 \times g$ for 15 min at 4°C (KUBOTA 3740) and filtration through ultrafiltration tubes (Millipore, Bedford, MA, USA). Milk was prepared by centrifuging at $14,000 \times g$ for 15 min at 4°C and filtration through ultrafiltration tubes. Brain and liver samples were homogenized in ice-cold 0.2 M perchloric acid solution containing 0.01 mM EDTA·2Na and incubated for de-proteinization in ice. After 30 min, the samples were centrifuged at $20,000 \times g$ for 15 min at 4°C and filtered through a 0.20- μ m filter. The pH of the filtrate was adjusted to approximately 7.0 with 1 M sodium hydroxide.

To investigate the influence of Arg administration, free L-Arg and D-Arg concentrations in the plasma and brain were analyzed with ultra-performance liquid chromatography (UPLC; the Acquity UPLC system comprised of Waters Binary Solvent Manager, Waters Sample Manager, and Waters FLR Detector [Waters, Milford, MA, USA]) with the fluorescent detection method described in a previous study [15] with some modifications. Each sample (10 μ l) was transferred to a UPLC tube and mixed with 20 μ l of a derivatization solution (10 mg of N-acetyl-L-cysteine and 8 mg of o-phthalaldehyde in 1 ml of methanol) and 70 μ l of 0.4 M borate buffer (pH 10.4). The mixtures were incubated in a dark room for 2 min. An aliquot of 1 μ l of derivatized samples was loaded into the UPLC system. The excitation and emission wavelengths for fluorescent detection were 350 nm and 450 nm respectively. The system was operated at a flow rate of 0.25 ml/min at 30°C. The UPLC gradient system (A=50 mM sodium acetate [pH 5.9], B=100% methanol) comprised 10–20% B over 3.2 min, 20% B for 1 min, 20–40% B over 3.6 min, 40% B for 1.2 min, 40–60% B over 3.8 min, 60% B for 1 min, and 60–10% B over 0.01 min. L-Arg and D-Arg levels in the plasma were expressed as nmol/ml, and those in brains were expressed as pmol/mg wet tissue.

In Experiment 2, liquid chromatography mass spectrometry (LC-MS; Xevo TQD, Waters) was used for the analysis of free L-Arg and D-Arg in the plasma, milk, and liver. The system was operated at a flow rate of 0.20 ml/min at 30°C. The LC gradient

system (A=10 mM ammonium bicarbonate, B=100% acetonitrile) included 5% B for 2 min, 5–12.5% B over 6 min, 12.5–20% B over 3.5 min, 20–40% B over 3 min, 40–10% B over 0.01 min, and 10–5% over 5.49 min. L-Arg and D-Arg levels in the plasma and milk were expressed as nmol/ml, and those in the liver were expressed as pmol/mg wet tissue.

Statistical analysis

All data were expressed as means \pm standard error of the mean (SEM). In Experiment 1, changes in L-Arg and D-Arg concentrations with respect to the control group were analyzed by one-way analysis of variance (ANOVA) after the oral administration of L-Arg and D-Arg respectively. When a significant effect was detected, the Dunnett test was applied as post-hoc test. The effects of both L-Arg and D-Arg were analyzed by two-way ANOVA. Amino acids (L-Arg versus D-Arg) and time (30, 60, and 90 min) were main effects and amino acid × time served as an interaction. Upon detection of a significant interaction, the Tukey-Kramer test was applied to the same treatment groups. In Experiment 2, one-way ANOVA was used for plasma and milk L-Arg and D-Arg level analyses. The Tukey-Kramer test was applied to the same treatment groups when the effect was significant. Statistical criteria for significant difference were set at *P*<0.05. All analyses were performed with StatView (version 5, SAS Institute, Cary, NC, USA, 1998). Outlying data were eliminated by Thompson's test criterion for outlying observations (*P*<0.01).

RESULTS

Oral administration of D-Arg highly enriched its levels in the plasma and brain of ICR mice

In order to determine the changes in the availability of L-Arg and D-Arg after the oral administration of L-Arg and D-Arg, their respective levels in the plasma, cerebral cortex, and hypothalamus in ICR mice were measured using UPLC (Figs. 1–3). In each figure, the scale of Y-axis for L-Arg and D-Arg is constant to allow comparison with the control group.

Our results revealed that while endogenous D-Arg was undetectable in any sample, the concentration of L-Arg post oral administration of the same increased with approximately 2.4-fold with respect to the control group at 30 min. Thus, L-Arg administration significantly (*P*<0.0001) enhanced the plasma levels of L-Arg as compared with D-Arg administration. However, the plasma levels of L-Arg gradually decreased with time in L-Arg administration group while remaining constant in D-Arg administration group (Fig. 1A). In addition, the undetectable plasma levels of D-Arg strikingly increased to around 1,000 nmol/m*l* after 30 min post D-Arg administration and the levels were maintained until 90 min post administration (Fig. 1B).

In the cerebral cortex, significant changes in L-Arg levels from the control group were detectable only after 90 min of D-Arg administration. On the other hand, L-Arg treatment significantly (P<0.0001) enhanced the L-Arg levels as compared with D-Arg treatment, but the values significantly decreased (P<0.01) with time (Fig. 2A). D-Arg in the cerebral cortex was detectable only after D-Arg administration and its level gradually increased over 90 min with highest concentration of 94 pmol/mg wet tissue (Fig. 2B). In the hypothalamus, there was absence of any significant changes in L-Arg levels as compared with the control group, but L-Arg treatment significantly (P<0.01) enhanced the L-Arg concentration as compared with D-Arg treatment (Fig. 3A). The pattern of changes in D-Arg was similar to that detected in the cerebral cortex (Fig. 3B) with the level sharply increasing from 0 to 162 pmol/mg wet tissue.



Fig. 1. Changes in (A) L-arginine (L-Arg) and (B) D-arginine (D-Arg) concentration in the plasma after the oral administration of L-Arg and D-Arg. The values for each amino acid are expressed as means ± SEM in nmol/ml (n=5–6). *P<0.05 versus control. The results of two-way ANOVA. (A) Arg, P<0.0001; time, P<0.001; and Arg × time, P<0.01. (B) Arg, P<0.0001; time, P>0.05; and Arg × time, P>0.05. Groups with different letters (a, b, c) are significantly different (P<0.05).</p>



Fig. 2. Changes in (A) L-arginine (L-Arg) and (B) D-arginine (D-Arg) concentration in the cerebral cortex after the oral administration of L-Arg and D-Arg. The values for each amino acid are expressed as means ± SEM in pmol/mg wet tissue (n=5–6). *P<0.05 vs control. The results of two-way ANOVA. (A) Arg, P<0.0001; time, P<0.01; and Arg × time, P>0.05. (B) Arg, P<0.0001; time, P<0.0001; and Arg × time, P<0.001. Groups with different letters (a, b, c, d) are significantly different (P<0.05).</p>



Fig. 3. Changes in (A) L-arginine (L-Arg) and (B) D-arginine (D-Arg) concentration in the hypothalamus after the oral administration of L-Arg and D-Arg. The values for each amino acid are expressed as means ± SEM in pmol/mg wet tissue (n=5–6). *P<0.05 vs control. The results of two-way ANOVA. (A) Arg, P<0.01; time, P>0.05; and Arg × time, P>0.05. (B) Arg, P<0.001; time, P<0.05; and Arg × time, P<0.05. Groups with different letters (a, b, c) are significantly different (P<0.05).</p>

L-Arg and D-Arg showed differential availability in the milk and liver of ICR mice post respective oral administration To determine the levels of L-Arg and D-Arg in the plasma and milk samples of ICR mice, their levels were assessed at 60 min post oral administration of L-Arg or D-Arg (Fig. 4). Our results revealed that D-Arg was detectable only in the D-Arg administration group in all samples. Further, the D-Arg concentration in the plasma exceeded until 1,000 nmol/ml, but that it was mere 63 nmol/ml in the milk upon D-Arg administration. Moreover, while the concentration of D-Arg in the liver was about 719 pmol/mg wet tissue after D-Arg administration (Fig. 5B), L-Arg was undetectable in the same (Fig. 5A). In the L-Arg administration group, the L-Arg concentration in the milk showed a meagre increase of 5 nmol/ml, while it was significantly increased by about 150 nmol/ml in the plasma as compared to the control group.



Fig. 4. The concentration of arginine (Arg) in the plasma and maternal milk samples. (A) L-Arg in the plasma, (B) D-Arg in the plasma, (C) L-Arg in milk, and (D) D-Arg in milk. The values are expressed as means \pm SEM in nmol/ml (n=4–7). Groups with different letters (a, b) are significantly different (*P*<0.05). ND: not detected.



Fig. 5. The concentration of (A) L-arginine (L-Arg) and (B) D-arginine (D-Arg) in the liver. The values are expressed as means \pm SEM in pmol/mg wet tissue (n=5–7). ND: not detected.

DISCUSSION

In the plasma, the concentrations of L-Arg and D-Arg sharply increased at 30 min after oral administration of the respective amino acids. While L-Arg level gradually decreased from 30 min post administration, the D-Arg level was maintained until 90 min after administration. Gibson and Wiseman [7] have shown that while both L- and D-amino acids are known to be absorbed, the absorption of several D-amino acids is inferior to that of corresponding L-amino acids. Although these results did not describe about L-Arg and D-Arg absorption, we hypothesized that the absorption of D-Arg was lower than that of L-Arg. Our results imply that the metabolism of L-Arg and D-Arg may be different irrespective of their absorption rates. L-Arg is used for protein synthesis and metabolized into several metabolites such as ornithine and urea in the liver and intestine by arginase [18] and L-amino acid oxidase (LAAO) [5, 17]. Since the plasma samples were obtained from the trunk blood in the present study, the results suggest that L-Arg had already passed through the liver. As a result, the increased plasma level of L-Arg was lower than that of D-Arg and that L-Arg was rapidly metabolized with time. Undetectable levels of L-Arg in the liver in Experiment 2 (Fig. 5) further supports this hypothesis. On the other hand, D-amino acids, including D-Arg, are not used for protein synthesis. Our study further demonstrated

that the level of L-Arg was not significantly changed post L-Arg administration in the brain of ICR mice, while the concentration of D-Arg gradually raised from 30 to 90 min post D-Arg administration. The efficient transport and significant availability of D-Arg in the brain may be attributed to the changes in the plasma L-Arg and D-Arg levels with respect to the control group. The increased level of L-Arg was observed to be 375 nmol/m/ higher, while that of D-Arg was around 1,000 nmol/m/ higher than that in control plasma groups. The increase in L-Arg concentration in the brain may require administration of higher level of L-Arg, as the concentration of FAAs in the brain linearly correlates with the concentration of plasma FAAs [21]. However, administration of L-Arg may pose certain risks including increase in the expression and activity of arginase that may reduce the effectiveness of L-Arg therapy or decrease L-Arg concentration [18]. Hence, the dose and administration period must be carefully examined to achieve desired effectiveness. In addition, D-Arg is believed to be present in the central nervous system [4] but was undetectable in the brains of the control and L-Arg-administered mice in our study. This finding may be attributed to the difference in the mouse strain used, as the previous study used ddY mice [4] versus the ICR mice used in the present study. It is possible that the racemase for Arg is not present in ICR mice. However, further study is necessary to confirm this speculation.

After L-Arg administration, L-Arg concentration in the plasma raised by about 150 nmol/ml as compared to the control nursing ICR mice. However, the increase in milk samples was only about 4–5 nmol/ml, probably owing to the presence of LAAO in the mammary gland of mice [5, 20]. Post administration of D-Arg, the D-Arg levels in the plasma increased by more than 1,000 nmol/ ml as observed in male ICR mice and that in the milk increased by about 63 nmol/ml. Although the same amount of L-Arg and D-Arg was administered, the increase in the concentration of D-Arg in milk samples was higher than that of L-Arg. Thus, D-Arg may be metabolically less pliant than L-Arg. Besides, D-Arg concentration in milk was much lower than that in the plasma. Hence, it is possible that D-Arg may have been catabolized by D-amino acid oxidase (DAAO) upon its entry into the mammary gland. However, the rate of catabolism may have been lower than that of L-Arg, considering that DAAO activity against basic amino acids including Arg is low [6, 16]. Similar results have been confirmed about serine (Ser). In maternal milk, L-Ser levels increased by about 120 nmol/ml in mice fed the diet containing 2% L-Ser as compared to the control diet [14]. On the other hand, milk D-Ser levels in the mice fed with 0.5% D-Ser diet was over 2,000 nmol/ml higher than that in the mice fed with control diet (Aso *et al.*, unpublished data). Both D-Arg and D-Ser are efficiently enriched in the milk, but the efficacy may be different between the two amino acids. However, further experiments are needed to provide better clarification.

In conclusion, our study has shown that orally administered D-Arg is efficiently transported to and significantly enriched in the brain and milk in ICR mice as compared with the same dose of L-Arg. D-Arg is believed to exert neuroprotective effects against neurotoxicity induced by high levels of glucocorticoids in the central nervous system [2]. To the best of our knowledge, this report for the first time has shed some light on the function of D-Arg. However, if D-Arg competes with L-Arg, it is assumed to affect the function and metabolism of L-Arg. Thus, D-Arg may play a functional role in the brain development and infant nourishment through milk in the form of an oral drug and/or nutrient supplement.

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