Intestinal renal metabolism of L-citrulline and L-arginine following enteral or parenteral infusion of L-alanyl-L-[2,15N]glutamine or L-[2,15N]glutamine in mice

Petra G. Boelens,1 Paul A. M. van Leeuwen,1 Cornelis H. C. Dejong,2 and Nicolaas E. P. Deutz2

1Department of Surgery of the Vrije Universiteit University Medical Center, Amsterdam; and
2University of Maastricht and Nutrition and Toxicology Research Institute, Maastricht, The Netherlands

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A NEED FOR L-ARGININE (L-ARG) may develop under conditions of metabolic stress such as surgical trauma and sepsis (19). Renal L-ARG synthesis is completely dependent on the availability of circulating L-citrulline (CIT), and the small intestine is the major source of circulating L-CIT (12, 26). L-Glutamine (GLN) and/or L-glutamate (GLU) are the substrates for L-CIT production in the gut (25). This pathway, also described as the intestinal-renal axis, was also suggested from the findings of increased plasma L-ARG levels in trauma patients receiving enteral L-GLN (15). Because the L-ARG contents of the feedings in that study were similar in the study group and in the control group, we suggested that renal L-ARG synthesis accounted for the increase in L-ARG plasma values in patients receiving L-GLN-enriched nutrition (15). This pathway of exogenous delivered L-GLN serving as a substrate for intestinal L-CIT production and renal L-ARG synthesis has not yet been quantified by stable isotope technology across the intestinal-renal axis in mice in vivo.

It has been recognized that an adequate availability of L-ARG is important during metabolic stress because L-ARG is the physiological precursor for the synthesis of nitric oxide (NO), which is identified as the endothelium-dependent relaxing factor, a mediator of immune responses, a neurotransmitter, and a signaling molecule (9). L-ARG depletion compromises these important functions. Several studies provide evidence that supports the clinical relevance of supplementing L-ARG (19). Sufficient supply of L-ARG is thus of nutritional and physiological importance, and, for that reason, our hypothesis is that by increasing the availability of L-GLN, via increased intestinal L-CIT production, renal L-ARG synthesis might increase.

Although physiological concentrations of L-GLN are the highest of all circulating amino acids, metabolic stress such as surgery is responsible for a critical reduction of L-GLN’s bioavailability (7). Oudemans-van Straaten et al. (21) concluded in patients admitted to the intensive care unit that low-plasma L-GLN relates to higher age, shock as primary diagnosis, and higher hospital mortality. Moreover, they showed that low-plasma L-GLN represents a risk of poor outcome. Thus it is of crucial importance to prevent or improve low-plasma L-GLN, and many studies show that adding L-GLN to the nutrition of diverse patient populations can improve outcome (7).

Unfortunately, free L-GLN is relatively unstable, and the pharmacological complexity implies that it is therefore less suitable to be a compound of a shelf-stored, water-based nutritional formula (14). The L-CIT dipeptide L-alanyl-L-GLN, which is stable in watery solution, is rapidly hydrolyzed in mammals to free L-GLN and L-alanine (L-ALA) and seems to be a good alternative (2–4). However, it is not known whether the metabolic fate of enteral dipeptide-derived L-GLN is similar to the fate of enteral free L-GLN in relationship to L-ARG production.

Although we found that free L-GLN-enriched enteral nutrition increased plasma L-ARG concentrations (15), most of the studies investigating GLN-enriched parenteral nutrition did not show changes in plasma L-ARG. Because the gut has a key role in GLN’s conversion to L-CIT, we hypothesized that L-GLN would enhance L-CIT production when luminally offered to the intestines compared with parenterally delivered L-GLN, and,

Address for reprint requests and other correspondence: P. A. M. van Leeuwen, Dept. of Surgery, Vrije Universiteit Univ. Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands (e-mail pam.vleeuwen@vumc.nl).
Experimental Design

To better understand 1) the metabolic basis of the nutritional requirement of l-GLN, 2) the extent to which GLN serves as a substrate for l-CIT or l-ARG, and 3) whether feeding route (enteral or parenteral) alters this pathway, it is necessary to explore the quantitative aspects of CIT and ARG metabolism during GLN supply. This has, to our knowledge, not been studied before. Therefore, we used a combination of stable isotope tracers with arteriovenous substrate-metabolite balance measurements across the intestines and kidneys to gain more insight into the interorgan metabolism of these specific amino acids.

The purpose of this study was to elucidate whether different routes of delivery of GLN and biological availability coming from the free amino acid or the dipeptide influences the intestinal l-CIT production and, thereby, renal l-ARG production.

MATERIALS AND METHODS

Experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, and they were approved by the Ethical Committee of Animal Research of Maastricht University.

Animals

Male Swiss mice [n = 43, 20 ± 0.5 g body wt (mean ± SE)] were obtained from IFFA Credo Broekman (Someren, The Netherlands). The mice were fed standard lab chow (SMRA 2131, Hope Pharmas, Woerden, The Netherlands) before the day of the experiments and were subject to standard 12:12-h light-dark cycle periods (7:30 AM to 7:30 PM). Room temperature was maintained at 25°C. Radio music was on to acquaint the animals with noise in the environment to reduce their stress toward noise induced by the caretakers/researchers. Experiments were performed between 7:30 AM and 7:30 PM. Room temperature was maintained at 25°C. Radio music was on to acquaint the animals with noise in the environment to reduce their stress toward noise induced by the caretakers/researchers. All experiments were performed in the postabsorptive state.

Experimental Design

Mice received one of four infusions. Mice in the first series were assigned to group 1 or 2, and mice in the second series were assigned to group 3 or 4; group 1 received parenteral l-ALA-[2-15N]GLN infusion, n = 11; group 2 received enteral l-ALA-[2-15N]GLN infusion, n = 12; group 3 received parenteral l-[2-15N]GLN infusion, n = 10; and group 4 received enteral l-[2-15N]GLN infusion, n = 10.

A primed continuous infusion of l-[2-15N]GLN or l-ALA-[2-15N]GLN, depending on the group, was administered for 1 h simultaneously with intravenous infusion of l-[ureido-13C-2H2]CIT and l-[guanido-15N2-2H2]ARG (steady-state model). The plasma flow through the intestines and kidneys was measured by using an indicator dilution technique with a primed continuous infusion of [14C]para-aminohippuric acid ([14C]-PAH) (13).

The protocol of Hallemeesch et al. (13) was used. Briefly, after the mice were weighed, anaesthesia was induced by an intraperitoneal injection of a mixture of ketamine (62.5 mg/kg body wt; Nimatek, AUV, Cuyk, The Netherlands) and medetomidine (400 μg/kg body wt; Domitor, Farmos, Espoo, Finland). A subcutaneous injection of 1.5 ml saline was given in the neck. Anesthesia was maintained with a continuous subcutaneous infusion of a mixture of ketamine (17.5 mg·kg body wt·−1·h−1) and medetomidine (112 μg·kg body wt·−1·h−1). During the surgical procedures, the mice were kept at 37°C using a temperature controller (Technical Service, Maastricht University) and heat pads. The jugular vein, carotid artery, portal vein, or renal vein. Arterial amino acid concentrations and whole body results of the same series of mice were described separately (P. G. Boelens, unpublished observations).

Blood Sampling and Processing

For hematocrit determinations, a microhematocrit tube (Vitrex, Herlev, Denmark) was filled with blood and centrifuged for 3 min at 6.8 g at room temperature in an EBA 12 centrifuge (Hettich, Dépex, de Bilt, Netherlands). Hematocrit was determined by dividing the length (in mm) of the red blood cell section by the totally filled length of the tube. Blood was collected in heparinized cups (Sarstedt, Nümbrecht, Germany) on ice and centrifuged (4 min, 4°C, 8,500 g) to obtain plasma.

For [14C]-PAH determinations, 20 μl of whole blood were added to 280 μl of 8.6% trichloracetic acid and thoroughly mixed for deproteinization and centrifuging (4 min, 4°C, 8,500 g, Eppendorf 5413 centrifuge).

For determination of amino acid concentrations and tracer-tracer ratios (TTR), 65 μl of plasma were added to 4 mg solid 5′-sulfosalicylic acid, vortexed, frozen in liquid nitrogen, and stored at −80°C. Analysis of Samples

For [14C]-PAH determinations, 250 μl of supernatant were mixed with 6 ml scintillation fluid (Ultima Gold, Packard, Groningen, The Netherlands), and radioactivity was counted on a Winspectral 1414 counter (Wallac, EG&G, Breda, The Netherlands).

Table 1. Tracer prime and infusion rates

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Prime, nmol (in bolus of: 0.04 ml/mouse)</th>
<th>Infusion, nmol/min (in: 0.2 ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-[2-15N]GLN</td>
<td>3,000</td>
<td>250</td>
</tr>
<tr>
<td>l-ALA-[2-15N]GLN</td>
<td>3,000</td>
<td>250</td>
</tr>
<tr>
<td>l-[ureido-13C-2H2]CIT</td>
<td>36</td>
<td>3</td>
</tr>
<tr>
<td>l-[guanido-15N2-2H2]ARG</td>
<td>60</td>
<td>5</td>
</tr>
</tbody>
</table>

GLN, l-glutamine; ALA, l-alanine; CIT, l-citrulline; ARG, l-arginine.
Plasma amino acid concentration TTR were measured using a fully automated LC-MS system, using precolumn derivatization with o-phthalaldehyde. Detailed information on the accuracy and precision of this method were given before (23, 24).

Calculations

Net balances across organs. Plasma flows across the portally drained viscera (Fig. 1A) and the kidneys (Fig. 1B) were calculated using the indicator dilution technique with [13C]PAH as described recently (13). As shown in Fig. 1A, portally drained viscera (PDV) amino acid fluxes were calculated by multiplying the portal venous-arterial concentration differences with the mean PDV plasma flow. Amino acid fluxes are expressed in nanomoles per 10 g body wt per minute. As shown in Fig. 1B, renal substrate fluxes were calculated by multiplying the renal venous-arterial concentration difference with the mean renal plasma flow and are expressed in nanomoles per 10 g body wt per minute.

\[
NB = \text{plasma flow} \times ([\text{venous}] - [\text{arterial}])
\]

where NB is net balance. A positive flux indicates net release (efflux) and a negative flux reflects net uptake (influx) by the organ.

Organ Balance Tracer Methodology

TTR is an equivalent of specific activity. Therefore, formulas were derived from metabolic studies using radioactive tracers; when necessary, a correction for the contribution of lower isotopomers was performed as described by Wolfe (27). The amino acid stable isotope tracers were used to obtain information regarding the arterial substrate enrichment. With the use of these tracers, organ disposal and production rates can be calculated.

Intestinal-renal amino acid turnover was calculated in a two-compartment model (27). Substrate NB or fluxes were calculated by multiplying the plasma venous-arterial concentration difference by the plasma flow. The tracer NB across an organ (TNB), the disposal, and production rate (nmol·10 g body wt −1·min −1) across the portally drained viscera and kidneys were calculated as

\[
\text{TNB} = \text{plasma flow} \times ([\text{arterial}] \times \text{TTR}_A - ([\text{venous}] \times \text{TTR}_A))
\]

where TTR and TTR are the TTRs of the measured amino acid in the arterial plasma and venous plasma, respectively.

Disposal was calculated as

\[
\text{disposal} = \text{TNB}/\text{TTR}_A
\]

TTR was used as a surrogate precursor pool enrichment, because TTR, in contrast to TTR, more accurately represents the precursor pool TTR (27).

Production of an amino acid across the organ was calculated as

\[
\text{Production} = \text{disposal} + \text{NB}
\]

where NB is the plasma venous-arterial concentration difference multiplied by plasma flow. PDV are defined as the total of all portal-drained organs, which, in addition to the spleen and pancreas, largely represent the intestines. These are therefore referred to as the “gut” or “intestine” in the RESULTS and DISCUSSION.

Organ Amino Acid Kinetics

Calculations on the organ de novo synthesis rate of ARG are based on the same principle of isotopic conversion used for whole body calculations. The organ TNB of the isotopic metabolic product must be corrected for its loss by extraction across the organ. A good approximation of this fractional extraction can be obtained by the following partial dilution of the isotopomers.

Fractional extraction (FE) is the fraction of the total product that bypasses metabolism in the organ and so appears in the output and is used for correction of NB.

\[
\text{FE}_{\text{ARG}} = \frac{([\text{TTR}_A_{\text{15N}} \times [\text{A}] - \text{TTR}_{\text{R}_{\text{15N}}} \times [\text{R}])}{\text{TTR}_A_{\text{15N}} \times [\text{A}]}
\]

where R is the ARG concentration from the renal vein or the renal TTR (TTR) and A is the arterial ARG concentration or arterial TTR (TTR) of ARG or CIT. The term 1 – FE represents the fraction of total product that bypasses metabolism and so appears in the output and is used for correction of NB. \[
\text{NB}_{\text{15NARG}} = \text{PF} \times ([\text{R}] × \text{TTR}_{\text{R}_{\text{15NARG}}} - [\text{A}] × \text{TTR}_{\text{A}_{\text{15NARG}}} \times (1 - \text{FE}_{\text{ARG}}))
\]

With the use of this corrected NB, the organ de novo synthesis of the metabolic product can be calculated similar to disposal in which the arterial substrate enrichment TTR is used as the precursor pool because the upstream compartment is thought to reflect best the intracellular delivered enrichment.

Renal ARG synthesis from 15N-CIT = NB_{15NARG}/TTR_{A_{15NCT}}

According to the same methodology as renal ARG de novo synthesis, intestinal CIT synthesis was calculated.

Statistical Analysis

Results are presented as means ± SE as indicated in the text. For each series, normality was tested by means of the Shapiro-Wilk’s test, and, when data were normal distributed, a Student’s t-test was used to compare differences between the groups for feeding route (enterally vs. parenterally). If data were not normally distributed, a Mann-Whitney U-test for comparisons between the feeding routes was used (SPSS for Windows, release 11.0.1 2001; SPSS, Chicago, IL). Significance was defined as P < 0.05.

RESULTS

Plasma Flow

Intestinal and renal plasma flow was not affected by route of administering L-GLN (Table 2).

Net Amino Acid Organ Fluxes

L-GLN fluxes. Intravenous infusion of L-GLN resulted in intestinal GLN influx, whereas enteral administration of L-GLN led to L-GLN efflux (P < 0.05; Fig. 1). Similarly, the dipeptide infusion showed this difference (P < 0.05).

Table 2. Plasma flow and net balance over PDV and kidney

<table>
<thead>
<tr>
<th></th>
<th>Series 1</th>
<th></th>
<th>Series 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-ALA-L-GLN IV</td>
<td>L-ALA-L-GLN EN</td>
<td>L-GLN IV</td>
<td>L-GLN EN</td>
</tr>
<tr>
<td>Intestinal PF</td>
<td>1.1±0.4</td>
<td>0.9±0.2</td>
<td>1.5±0.6</td>
<td>1.5±0.4</td>
</tr>
<tr>
<td>Renal PF</td>
<td>1.3±0.4</td>
<td>1.0±0.3</td>
<td>0.7±0.2</td>
<td>1.5±0.4</td>
</tr>
<tr>
<td>GLU PDV*</td>
<td>47±19</td>
<td>9±7</td>
<td>23±6</td>
<td>43±13</td>
</tr>
<tr>
<td>ALA PDV*</td>
<td>186±89</td>
<td>369±127</td>
<td>330±45</td>
<td>328±125</td>
</tr>
<tr>
<td>EAA PDV</td>
<td>49±7.9</td>
<td>-10±37</td>
<td>-10±66</td>
<td>165±190</td>
</tr>
</tbody>
</table>

Values are means ± SE in ml·10 g body wt −1·min −1 for plasma flow (PF) and in nmol·10 g body wt −1·min −1 for net balance. Positive amino acid fluxes indicate release in portal vein or renal vein, and negative amino acid fluxes indicate uptake by the splanchic viscera or kidney. IV, intravenous infusion; EV, arterial infusion; PDV, portally drained viscera or intestine. List of all amino acid fluxes available on request. Student’s t-test was used for parametric data. *P < 0.05 is significant for the feeding route. Series 1 had significant differences among the groups. Mann-Whitney U-test was used when data were nonparametric.
L-CIT fluxes

All groups released L-CIT in the portal vein, and, in all groups, uptake of L-CIT by the kidney was observed, but there were no differences among the groups (Figs. 2 and 3).

L-ARG fluxes

L-GLN or the dipeptide, by either the enteral or parenteral route, resulted in intestinal ARG uptake and renal ARG release without differences between the groups (Figs. 2 and 3).

Effect of L-GLN on Renal L-CIT and L-ARG Metabolism

Renal L-CIT. The kidneys showed more disposal of L-CIT than production, representing the net uptake of L-CIT. Renal L-CIT production was higher in mice receiving the dipeptide intravenously compared with the enteral group (P < 0.05; Fig. 3).

Renal L-ARG. Renal production of L-ARG was higher than renal L-ARG disposal, reflecting the net release of L-ARG in all groups. Renal L-ARG disposal and production were higher in the intravenous group after dipeptide infusion but were lower in the intravenous group when free L-GLN was given (P < 0.05; Fig. 3).

De Novo Intestinal L-[2,15N]CIT From L-[2,15N]GLN

L-[2,15N]GLN and L-ALA-L-[2,15N]GLN, given either enterally or parenterally, were both converted into L-[2,15N]CIT by the PRV. De novo 14 ± 2 nmol·10 g body wt⁻¹·min⁻¹ L-[2,15N]CIT was produced when L-ALA-L-[2,15N]GLN was given intravenously and enterally (16 ± 3 nmol·10 g body wt⁻¹·min⁻¹).
Almost twice as much de novo L-[2,15N]CIT was measured in the free L-[2,15N]GLN groups [intravenous group: 34.5±5 nmol·10 g body wt⁻¹·min⁻¹; enteral group: 35.6±6 nmol·10 g body wt⁻¹·min⁻¹ (Fig. 4)].

De Novo Renal L-[2,15N]ARG From L-[2,15N]CIT

Approximately 25% of L-[2,15N]GLN and L-ALA-L-[2,15N]GLN, given either enterally or parenterally, were both converted via L-[2,15N]CIT into L-[2,15N]ARG by the kidney. The rate of ARG production by the kidney was not significantly different among the groups (Fig. 4).

DISCUSSION

Major Observations

Our study explored for the first time the interorgan pathway from exogenous L-GLN to endogenous L-ARG in mice using stable isotope technology. We quantified the effect of the feeding route, enterally or parenterally, on de novo intestinal L-CIT production and de novo renal L-ARG production following free L-GLN or dipeptide L-alanyl-L-GLN administration.

Validity of the Model

The use of a multicatheterized mice model under steady-state conditions enabled in vivo flux measurements across the organs, crucial for our hypothesis (8, 13). Moreover, the unique combination of different types of stable isotope tracers in mice allowed the simultaneous quantification of the important metabolic routes. The model was used to study the disposal, production, and net balances of L-ARG and L-CIT as well as de novo synthesis by the kidneys and gut.

Net Substrate Balance

There was a remarkable difference between the routes of administering L-GLN or the dipeptide with respect to the intestinal flux of L-GLN. It is well established that the gut is a major GLN-consuming organ, because it has high glutaminase activity (key enzyme able to hydrolyze GLN) and only low glutamine synthetase activity (5, 17, 22). For example, intravenous L-GLN infusion demonstrated the expected intestinal L-GLN uptake, but enteral free L-GLN infusion showed an opposite effect. Enteral free L-GLN caused an efflux of L-GLN from the PRV, which was larger than the enteral infusion rate of L-GLN, suggesting “production or release” of GLN contrary to “GLN consumption.” Therefore, the most likely explanation for the intestinal L-GLN efflux in the enteral free L-GLN group seems to be that L-GLN is just passing through and is not from either L-GLN release from protein breakdown or L-GLN de novo production. Regarding protein breakdown, no differences were found among the four groups in intestinal efflux of essential amino acids (a measure of net protein breakdown in the postabsorptive state). Furthermore, the products of GLN breakdown, e.g., L-GLU, L-ALA, and L-CIT, were not different with respect to the route of administration of L-GLN, pointing out that at the same time a similar L-GLN uptake or consumption occurs in all groups. This renders it also less likely that intestinal L-GLN de novo production from its own degradation products, L-GLU or L-ALA, might add to the GLN efflux. In rats, during acute liver insufficiency-induced hyperammonemia, a comparable net PRV GLN release was found at very
high ammonia levels, possibly related to glutaminase inhibition and glutamine synthetase stimulation. In our experiment, ammonia was not measured, and, although theoretically degradation of L-GLN in L-GLU and ammonia could have played a role in recycling of L-GLN in the enteral free L-GLN group, the importance of this route is yet unclear (11).

Consistent with previous studies (29, 30), the intestines released L-CIT, and the kidneys took up L-CIT and released L-ARG. The results of the fluxes are an extension of the findings of Houdijk et al. (16). They found an increase in arterial CIT and ARG concentrations concomitant with increased renal CIT uptake and ARG release in rats fed a GLN-enriched diet compared with rats fed a control diet.

Organ Balance

Intestinal CIT and ARG metabolism. Consistent with the intestinal substrate fluxes of L-CIT and L-ARG mentioned before, the data coming from the combined tracer methodology showed the same pattern, being a release of L-CIT by the intestines and an uptake of L-ARG. Feeding route of free L-GLN or the dipeptide did not result in significant differences with regard to the intestinal CIT metabolism. However, the two series of experiments might point at a higher L-CIT production when free L-GLN is given compared with the dipeptide. An explanation for higher L-CIT production could be that degradation of GLN coming from the dipeptide L-alanyl-L-GLN occurs via other pathways than when free L-GLN is directly given. In this light, it could be hypothesized that GLN dipeptides or their metabolites hinder the enzyme systems necessary for the uptake of GLN or the conversion to L-CIT. Dipeptide given intravenously will be cleared from the plasma mostly by the kidney, and most of the dipeptide will be subsequently cleaved rapidly by cytosolic peptidases because the kidney exhibits the highest intracellular hydrolase activity against short-chain peptides (1, 10, 18). Consequently, part of the administered dipeptides will not reach the gut because they are hydrolyzed in the kidney. Besides that, in the human intestine, the predominant mechanism for assimilation of GLN dipeptides is absorption as intact dipeptide rather than hydrolysis (20). For mice, to the best of our knowledge, this has not been studied before, but a certain amount of the dipeptide could be suggested to be transported to the circulation as such and might consecutively be degraded in plasma or the kidney. If this is true, free L-GLN might be metabolized more efficiently by the enterocytes, but this would have to be investigated more extensively.

Renal L-CIT and L-ARG metabolism. The kidneys showed L-CIT influx and L-ARG efflux. Renal L-ARG disposal and production were higher in the intravenous dipeptide group than when free L-GLN is given compared with the dipeptide. An explanation for higher L-ARG production by the kidneys, most of the dipeptide will be subsequently cleaved rapidly by cytosolic peptidases because the kidney exhibits the highest intracellular hydrolase activity against short-chain peptides (1, 10, 18). Consequently, part of the administered dipeptides will not reach the gut because they hydrolyzed in the kidney. Besides that, in the human intestine, the predominant mechanism for assimilation of GLN dipeptides is absorption as intact dipeptide rather than hydrolysis (20). For mice, to the best of our knowledge, this has not been studied before, but a certain amount of the dipeptide could be suggested to be transported to the circulation as such and might consecutively be degraded in plasma or the kidney. If this is true, free L-GLN might be metabolized more efficiently by the enterocytes, but this would have to be investigated more extensively.

In conclusion, stable isotope measurements showed that exogenous intravenous or enteral L-GLN or L-alanyl-L-GLN generate de novo L-CIT production by the intestines and de novo L-ARG production by the kidneys during surgery. The intestinal-renal axis is hereby proven in mice. Although further research is required, it would seem justified on the basis of the present data to state that the route of administration of exogenous L-GLN does influence the metabolism of L-CIT and L-ARG but that not all L-CIT and L-ARG is coming from GLN by the intestinal-renal axis in this model.

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