Arginine Can Be Synthesized from Enteral Proline in Healthy Adult Humans\textsuperscript{1–3}

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\section*{Abstract}
There is considerable controversy recently in identifying dietary precursors for arginine synthesis. We have previously shown in human neonates and piglets that proline is the sole dietary precursor for arginine synthesis. It is unclear in adult humans whether proline is a dietary precursor for arginine. We performed a multi-tracer stable isotope study in adults using \textsuperscript{15}N\textsubscript{2}-ureido arginine and \textsuperscript{15}N proline to elucidate synthesis of citrulline and arginine and determine whether proline is a precursor for arginine. Primed, intermittent infusions of the labeled amino acids were given enterally to 5 healthy men consuming a standardized milkshake diet. Blood was sampled during plateau enrichment between 1.5 and 3 h. Plasma enrichment occurred for both tracers, giving enteral turnover estimates of 93 \mu mol·kg\textsuperscript{-1}·h\textsuperscript{-1} for arginine and 154 \mu mol·kg\textsuperscript{-1}·h\textsuperscript{-1} for proline. Appearance of the label from proline in arginine and the intermediaries, ornithine and citrulline, was measured in all participants. The rate of synthesis of arginine from proline was 3.7 \mu mol·kg\textsuperscript{-1}·h\textsuperscript{-1}, which is estimated to be \textasciitilde40\% of newly synthesized arginine. In this first study in adult humans using an enteral proline tracer, we have demonstrated synthesis of arginine from this dietary amino acid. Therefore, as in newborns, proline must now be considered a dietary precursor for arginine in healthy adults. J. Nutr. 141: 1432–1436, 2011.

\section*{Introduction}
There has been considerable interest in arginine synthesis recently, recognizing its conditionally indispensable status and its multiple metabolic roles, specifically in the critically ill. We and others have investigated arginine synthesis from glutamine in adults in different states of health (1,2). However, to our knowledge, there have been no studies investigating arginine synthesis from proline in healthy or critically ill adults.

Proline, too, is classified as a conditionally indispensable amino acid (3). In adult humans, Jaksic et al. (4) showed no ill effects from a diet devoid of proline for 1 wk and, more recently, Tharakaran et al. (5) demonstrated the safety of 4 wk of a diet lacking in arginine, proline, glutamate, and aspartate. However, in several species, notably chicks (6) and piglets (7), proline is necessary for maximal growth.

Proline’s main function appears to be as a structural component of protein and it had previously been thought to have few metabolic properties. However, it is now clear that proline can function as a neurotransmitter and play a role in the development of schizophrenia (8); also, proline oxidase has been implicated as a regulator of apoptosis in carcinogenesis (9).

There have been few studies using isotopically labeled proline in humans and none in large mammals. The most in-depth investigations were those of Jaksic et al. (4,10,11) who performed a series of studies in both healthy men and patients after burn injury. However, these studies primarily assessed proline synthesis and whole body flux rates and did not determine synthesis of other amino acids from proline. Furthermore, all these studies used i.v. tracers; there have been no studies using labeled proline provided enterally to assess first pass metabolism by the splanchnic organs, which are important in arginine synthesis.

Given the importance of arginine, especially in the critically ill, our laboratory has focused on arginine synthesis from dietary precursors and has shown that at least in neonates, arginine was only synthesized from proline and not from glutamine/glutamate (12–14). Others have reported that arginine is synthesized from glutamate/glutamine in adult humans but have done so using a nitrogen tracer, which could potentially reflect transamination rather than use of the carbon chain from glutamate into arginine (1,15). Very recent studies in rodents have also challenged glutamate/glutamine as a dietary precursor for arginine synthesis (16). Hence, we thought it important to investigate whether proline is a dietary precursor for arginine synthesis in healthy participants, potentially preparing the way for studies in disease. Therefore, as a preliminary study, we assessed arginine synthesis from dietary proline performed using stable isotope-labeled proline and arginine given enterally to healthy men.

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\textsuperscript{2} Author disclosures: C. Tomlinson, M. Rafii, R. O. Ball, and P. B. Pencharz, no conflicts of interest.
\textsuperscript{3} Supplemental Figures 1–3 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
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Methods

Participants. Five healthy men were recruited for the study. Their characteristics are summarized in Table 1. At the time of the study, they were in good health, as determined by medical history and a simple physical exam and were taking no medications. To ensure uniformity and to habituate to a standardized intake, the participants were asked to eat only milkshakes (Sanskesh, Xanxan pharmaceutical) for 48 h prior to the study. This provided 10 MJ/d energy and 52 g·kg⁻¹·d⁻¹ protein. No other food was allowed, but the participants could drink water ad libitum. Participants provided informed consent and were given remuneration for taking part in the study, which was approved by the Research Ethics Board at the Hospital for Sick Children.

Study protocol. On the day of the study, the participants were admitted to the Clinical Investigation Unit at the Hospital for Sick Children. The study lasted 8 h. To simulate normal physiology, this study was carried out in the fed state. For the duration of the study, the participants drank hourly amounts of milkshake providing them with an energy intake of 630 kJ/h and a protein intake of 3.25 g/h protein, which is equivalent to 1 g·kg⁻¹·d⁻¹ protein. In addition, the participants could drink water ad libitum but no other food or drink for the duration of the study. For convenience of blood sampling, an i.v. line was inserted in the antecubital fossa of each participant.

At 5 h into the study, the isotope infusions were commenced. The isotoxams were consumed orally by the participants and the medicine containers were rinsed with water afterwards to ensure the entire dose was given. A constant infusion was simulated by giving the participants a prime dose of the labeled amino acids followed by half-hourly amounts for 2.5 h. Three blood samples were collected into EDTA-containing vacutainers at baseline and 4 h blood samples were collected during plateau enrichment at 90, 120, 150, and 180 min after starting the isotope infusion (Supplemental Fig. 1).

Blood samples were kept on ice until they were centrifuged to obtain plasma, which was then stored at −20°C until analysis.

Amino acid labeled tracers. The labeled amino acid tracers were obtained from Cambridge Isotope Laboratories. The 2 tracers used in this study were: guanidino-¹⁵N₂ arginine and 2-¹⁵N proline. The proline tracer had an isotope enrichment of 99.4% of ¹⁵N and a D-isomer content of 1.2%. The D-amino acid tracer had an isotope enrichment of 98.7% of ¹⁵N and a D-isomer content of <0.5%. The guanidino-¹⁵N₂ arginine tracer had an enrichment of 99.4% and a D-isomer content of 1.2%. The D-amino acid content was high enough to affect the enrichment and necessitated the use of chiral chromatography (17).

The ¹⁵N proline tracer was given with a priming dose of 4 μmol/kg followed by q30-min bolus doses to simulate an infusion rate of 7 μmol·kg⁻¹·h⁻¹. There is no human data on which to base these; hence, the doses were double those from the i.v. studies of Jaksic et al. (10). The equivalent doses for the guanidino-¹⁵N₂ arginine were 5.9 μmol/kg and 5.9 μmol·kg⁻¹·h⁻¹. These doses were the same as those used by Castillo et al. (18). Both tracers were given simultaneously to each participant.

The solutions of the tracer were prepared in sterile water, stored at 4°C until use, and dispensed by the pharmacy at The Hospital for Sick Children. The concentration of the amino acid solutions were 30 g/L arginine and 25 g/L proline. Before use, solutions were confirmed to be sterile and pyrogen free; the concentrations were confirmed by HPLC.

Table 1 Characteristics of the men

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>20.8 ± 1.9 (19–24)</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.76 ± 0.07 (1.66–1.85)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>73.2 ± 9.7 (62.5–88.2)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.7 ± 2.7 (20.5–27.2)</td>
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</table>

1 Values are mean ± SD (range), n = 5.

Analysis of enrichment. Plasma samples were thawed and 25 μL was deproteinized by the addition of 500 μL of methanol, mixed on a vortex for 15 s, and then centrifugation at 13,000 × g for 15 min. For the urine samples, 250 μL of urine was used. The supernatant was dried under N₂ and reconstituted in 0.1% formic acid.

The isotope analysis was performed as previously described (17). Briefly, enrichment was measured using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems/ MDS Sciex) operated in positive electrospray ionization mode. This was coupled to an Agilent 1100 HPLC system (Agilent Technologies). All aspects of the system operation were controlled using The Analyst NT v 1.4.1 software (Applied Biosystems/ MDS Sciex). Maximum sensitivity for L- and D-arginine, citrulline, ornithine, and proline was achieved by measuring product ions Multiple Reaction Monitoring from the fragmentation of the protonated [M+H⁺] molecules of each amino acid. Only the enrichments of the L-isofoms of the amino acids were analyzed for this study.

The [M+H⁺] precursor (parent) ion for each of the amino acids was (m/z) 175.1 for arginine, 176.1 for citrulline, 133.1 for ornithine, and 116.1 for proline. The signal for the most abundant product (daughter) ions (m/z) 70.1 for arginine, 70.1 for proline, 70.1 for citrulline, and 84.1 for ornithine were also optimized. Chiral separations were performed with a Chirobiotic T (teicoplanin) HPLC column, 25 cm × 4.6 mm, 5 μm (Astec) using an isocratic gradient with 95:5 (10 mmol/L ammonium acetate, pH 4.1):[2-methylamino1.0% formic acid in acetonitrile) buffer at 700 μL/min. The injection volume was 20 μL with an overall analysis time (injection to injection) of 35 min.

The labeled [¹⁵N₂-guanidino] arginine has a parent ion with a m/z of 177.1 and a daughter ion m/z of 70.1. Labeled [¹⁵N] proline has a precursor ion with a m/z of 117.1 and a product ion of 71.1.

The conversion of the [2-¹⁵N] proline tracer in [2-¹⁵N] arginine was determined by measuring the precursor/product m/z ion pair of 176/71. Similarly, the conversion of the proline tracer into [2-¹⁵N] citrulline was determined using the precursor/product m/z ion pair of 177/71 and into [2-¹⁵N] ornithine from using the m/z ion pair of 134/71. We did not expect any other isopertomers of arginine, citrulline, or ornithine to be synthesized from the [2-¹⁵N] proline tracer; however, we also assessed the synthesis of the [5-¹⁵N] isopertomers of arginine, citrulline, and ornithine using the m/z ion pairs of 176/70, 177/70, and 134/70, respectively.

Data analysis. Isotope enrichment is expressed as molecule percent excess (MPE) calculated as enrichment at plateau minus the background measurement at baseline.

Turnover of the infused amino acids was calculated using the equation:

\[ Q = \frac{I(E_i/E_c) - 1}{t} \]

where Q is the flux of the amino acid through the free amino acid pool, t is the rate of infusion of the tracer, Eᵢ is the enrichment of the tracer in the infusion, and Eₓ is the MPE of the tracer in plasma at plateau.

The conversion rates of proline to arginine was calculated by the formula

\[ Q_{pro → arg} = E_{arg}/E_{pro} \times Q_{arg} \]

The fractional conversion of proline to ornithine and citrulline was calculated by the formula

\[ Q_{pro → product} = E_{product}/E_{pro} \]

The conversion rate of citrulline to arginine was calculated by the formula

\[ Q_{cit → arg} = E_{arg}/E_{cit} \times Q_{arg} \]

with Eₓᵢ being the enrichment of the citrulline from the proline tracer.

Statistical analysis. Data are expressed as mean with SD. Enrichment of amino acids above baseline was analyzed using paired Student’s t test. Differences between amino acids in enrichment also were compared using paired Student’s t test. Significance was assumed if \( P < 0.05 \). Statistical analysis was performed using SAS version 9.1 (SAS Institute).
Results

All participants tolerated the study, including the 48-h milkshake-only diet and isotope administration. There were no violations of the study protocol.

Enrichment in plasma of the 2 tracers was detectable with significant enrichment above baseline in all participants. Plateau enrichment was reached for both amino acids within 2.5 h as demonstrated by a visual inspection of the data and the absence of a significant slope (Supplemental Figs. 2 and 3). The mean enrichment of $^{15}$N proline was $4.3 \pm 0.5$ MPE, from which we calculated the turnover of enteral proline to be $154 \mu$mol·kg$^{-1}$·h$^{-1}$. The mean enrichment of $^{15}$N$_2$ arginine was $5.9 \pm 1.1$ MPE; the turnover of enteral arginine was $93 \mu$mol·kg$^{-1}$·h$^{-1}$.

There was measurable enrichment of the M+1 isomers of $[2,^{15}$N$_2]$ arginine, $[2,^{15}$N]$^m$ ornithine, and $[2,^{15}$N]$^m$ citrulline, indicating synthesis from proline; the enrichment of each was different from zero after correction for background (natural) enrichment ($P < 0.05$). These data are summarized in Table 2. There were no $[5,^{15}$N]$^m$ isotopomers of arginine, ornithine, or citrulline detected (data not shown). Hence, there was no evidence of transamination of the amino group from proline.

The fractional synthesis rate of ornithine and citrulline was 22 and 38%, respectively. The enrichment of ornithine was greater than that of ornithine ($P < 0.05$). No citrulline tracer was given in this study, so an absolute synthesis rate of citrulline cannot be directly calculated. However, using literature values for citrulline flux (19), which are consistent over a range of conditions for this nondietary amino acid, the interconversion rates of proline to citrulline and arginine can be estimated (Fig. 1). The measured citrulline to arginine conversion rate of $10.3 \mu$mol·kg$^{-1}$·h$^{-1}$ is nearly identical to the flux of citrulline as one would expect, because the only metabolic pathway for citrulline is conversion to arginine. Also, the estimated conversion rate of proline to citrulline of $3.95 \mu$mol·kg$^{-1}$·h$^{-1}$ is very similar to the measured rate of proline to arginine of $3.70 \mu$mol·kg$^{-1}$·h$^{-1}$, indicating a rapid turnover of citrulline to arginine.

The calculated intake of arginine (~7% of animal protein) from the milkshake diet used in this study was $17 \mu$mol·kg$^{-1}$·h$^{-1}$, indicating that de novo synthesis of arginine from proline contributes an important amount to arginine appearance.

Discussion

It is well recognized that the gut plays an important role in amino acid metabolism (20). However, there is little evidence to date in adults, that the splanchnic organs metabolize proline to a large extent. Indeed, there has been little work using stable isotope labeled proline in humans. This is likely reflective of the fact that proline has provided important information and was necessary for our study given the preference of the enterocyte for luminal amino acids (1), it does make interpretation of the turnover rates difficult to interpret, because they include first pass metabolism. The enteral turnover estimate in the current study of $154 \mu$mol·kg$^{-1}$·h$^{-1}$ is higher than the whole body flux measurements of Jaksic et al. (4) and Hiramatsu et al. (21). The protein intake in the current study was similar to that of Hiramatsu et al. (21) at 1 g·kg$^{-1}$·d$^{-1}$ compared with 0.6 g·kg$^{-1}$·d$^{-1}$ in the Jaksic et al. (4,11) studies.

From the Young group (4,10,11,21) studies, the mean flux of proline using the i.v. tracer in healthy adults receiving a complete enteral diet was variable, being ~70–75 μmol·kg$^{-1}$·h$^{-1}$ in the studies by Jaksic et al. (4,11) but considerably higher (99–125 μmol·kg$^{-1}$·h$^{-1}$) in the study by Hiramatsu et al. (21). This difference may have been due to the increased protein intake in the second study (21) 1 g·kg$^{-1}$·d$^{-1}$ compared with 0.6 g·kg$^{-1}$·d$^{-1}$ in the Jaksic et al. (4,11) studies.

Our study is the first to our knowledge to use an enteral tracer in adult humans. Although this provides important information and was necessary for our study given the preference of the enterocyte for luminal amino acids (1), it does make interpretation of the turnover rates difficult to interpret, because they include first pass metabolism. The enteral turnover estimate in the current study of $154 \mu$mol·kg$^{-1}$·h$^{-1}$ is higher than the whole body flux measurements of Jaksic et al. (4) and Hiramatsu et al. (21). The protein intake in the current study was similar to that of Hiramatsu et al. (21) at 1 g·kg$^{-1}$·d$^{-1}$, so the turnover rates measured should be comparable. However, the Hiramatsu et al. (21) study indicates that there was significant variation in proline flux measurements. The control group of 7 adults in this study was studied twice under identical conditions and, until recently, was thought to have few roles beyond that of a structural component of proteins. Our study is therefore the first to our knowledge to demonstrate that proline is significantly metabolized as a precursor for arginine in adult humans.

The studies of the Young group (4,10,11,21) are the most comprehensive assessment of proline metabolism and synthesis in humans to date. In all of these studies, an i.v. proline isotope tracer was used; consequently, the proline flux rates of these studies do not include first pass metabolism by the splanchnic organs. Three of these studies (4,10,21) examined the effects of a proline-deficient diet given by different routes and lengths of time on proline synthesis. In summary, during the proline-deficient diets, the plasma proline concentration fell along with flux and synthesis rates. These studies did not assess whether amino acids were synthesized from proline.

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Enrichment of M+1 isomers of proline, ornithine, citrulline, and arginine in men after administration of guanidino- $^{15}$N$_2$ arginine and 2- $^{15}$N proline$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPE</td>
<td></td>
</tr>
<tr>
<td>$[2,^{15}$N$]$ Proline</td>
<td>$4.3 \pm 0.5^*$</td>
</tr>
<tr>
<td>$[2,^{15}$N]$^m$ Ornithine</td>
<td>$0.95 \pm 0.2^d$</td>
</tr>
<tr>
<td>$[2,^{15}$N$]$ Citrulline</td>
<td>$1.63 \pm 0.2^*$</td>
</tr>
<tr>
<td>$[2,^{15}$N$]$ Arginine</td>
<td>$0.18 \pm 0.1^*$</td>
</tr>
</tbody>
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$^1$Values are mean ± SD, n = 5. *Greater than baseline, $P < 0.05$; $^d$different from ornithine, $P < 0.05$.
and there was a significant increase in proline flux from 99 \pm 10 to 125 \pm 22 \text{ mmol-kg}^{-1}\text{-h}^{-1} over the duration of the protocol; the authors could not explain this difference. The comparison of our enteral turnover rate with the i.v. flux rates from Hiramatsu et al. (21) suggests substantial first pass metabolism.

There have been only limited studies investigating the splanchnic metabolism of proline and none in humans. What evidence there is comes from piglets and indicates a highly variable metabolism, with first pass splanchnic metabolism between 9 and 57% depending on route and diet (12,22,23). It is unclear therefore whether the turnover value of 154 \text{ mmol-kg}^{-1}\text{-h}^{-1} measured in the current study is in fact higher than the published i.v. flux rates, indicating a high degree of first pass metabolism, or represents simply inter-individual variation. This needs to be a focus of further studies.

Arginine has been more extensively studied in adults and estimates of the enteral flux of arginine tracers have been published. From Castillo et al. (18), the flux of enteral arginine was 114 \text{ mmol-kg}^{-1}\text{-h}^{-1}, slightly greater than in our study at 93 \text{ mmol-kg}^{-1}\text{-h}^{-1}, but was, however, within the error expected by these methods.

This is the first study to our knowledge to look at synthetic products of proline in the adult human. It is clear from the results that there is conversion of proline to arginine in the intermediaries’ citrulline and ornithine. Because only proline and arginine tracers were given in this study, it was only possible to accurately quantify arginine synthesis from proline with a rate of synthesis of 3.7 \text{ mmol-kg}^{-1}\text{-h}^{-1}. This is ~4% of arginine turnover. The approximate intake of arginine with the milkshakes was 17 \text{ mmol-kg}^{-1}\text{-h}^{-1}; therefore, synthesis of arginine from proline occurs at a rate of ~20% of arginine intake. Furthermore, by comparing the proline to arginine synthesis rate of 3.7 \text{ mmol-kg}^{-1}\text{-h}^{-1} and the citrulline to arginine synthesis rate of 10.3 \text{ mmol-kg}^{-1}\text{-h}^{-1}, we demonstrate that dietary proline contributes ~40% of newly synthesized arginine.

There has been considerable debate recently about the validity of the route of the citrulline tracer in this study was i.v., the citrulline content of dietary protein is negligible. It is reasonable to assume, therefore, that this published flux rate will approximate to the participants in the current study. Using this value for citrulline flux, 10.4 \text{ mmol-kg}^{-1}\text{-h}^{-1}, the rate of synthesis of citrulline from proline is 3.95 \text{ mmol-kg}^{-1}\text{-h}^{-1}. Because the enrichment of citrulline from proline in each participant at plateau enrichment was stable, this value can be used to estimate the conversion rate of citrulline to arginine at 10.3 \text{ mmol-kg}^{-1}\text{-h}^{-1} (Fig. 1).

These conversion rates are very instructive and indicate a rapid turnover rate of citrulline to arginine, because the proline to citrulline (3.95 \text{ mmol-kg}^{-1}\text{-h}^{-1}) and proline to arginine (3.7 \text{ mmol-kg}^{-1}\text{-h}^{-1}) rates are so similar. This is in keeping with the finding of others that citrulline to arginine conversion, primarily in the kidneys, is efficient and makes up the majority if not all of citrulline flux (14,27,28).

The aim of this study was to determine whether enteral proline is utilized for arginine synthesis. We have shown that dietary proline does act as a precursor for arginine and contributes to ~40% of newly synthesized arginine. Additionally, our results suggest a high degree of splanchnic metabolism of proline with at least a portion of this splanchnic metabolism as a precursor for arginine synthesis.

Acknowledgments
C.T., M.R., R.O.B., and P.B.P. designed the study. C.T. performed the research and data analysis. C.T., M.R., R.O.B., and P.B.P. contributed to writing the paper. All authors have read and approved the final manuscript.

Literature Cited

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Conversely, the enrichment of citrulline is likely representative of the complete conversion of proline to citrulline. The studies of Windmueller and Spaeth (26) indicate that the majority of circulating citrulline comes from the gut. Furthermore, the role of the enterocyte in arginine synthesis is to release citrulline and the adult enterocyte does not express arginino-succinate synthase, the only enzyme known to metabolize citrulline.

The fractional conversion rate of proline to citrulline was 38%. Because there was no citrulline tracer used in this study, absolute synthesis rates for citrulline could not be calculated directly. However, Castillo et al. (19) measured citrulline plasma flux rates over a range of arginine intakes and whether the individuals were fasted or fed. Citrulline flux in the fed state with an arginine sufficient diet was 10.4 \text{ mmol-kg}^{-1}\text{-h}^{-1}. Although the route of the citrulline tracer in this study was i.v., the citrulline content of dietary protein is negligible. It is reasonable to assume, therefore, that this published flux rate will approximate to the participants in the current study. Using this value for citrulline flux, 10.4 \text{ mmol-kg}^{-1}\text{-h}^{-1}, the rate of synthesis of citrulline from proline is 3.95 \text{ mmol-kg}^{-1}\text{-h}^{-1}. Because the enrichment of citrulline from proline in each participant at plateau enrichment was stable, this value can be used to estimate the conversion rate of citrulline to arginine at 10.3 \text{ mmol-kg}^{-1}\text{-h}^{-1} (Fig. 1).

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Acknowledgments
C.T., M.R., R.O.B., and P.B.P. designed the study. C.T. performed the research and data analysis. C.T., M.R., R.O.B., and P.B.P. contributed to writing the paper. All authors have read and approved the final manuscript.

Literature Cited