Enteral arginase II provides ornithine for citrulline synthesis

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Marini JC, Keller B, Didelija IC, Castillo L, Lee B. Enteral arginase II provides ornithine for citrulline synthesis. *Am J Physiol Endocrinol Metab* 300: E188–E194, 2011. First published October 26, 2010; doi:10.1152/ajpendo.00413.2010.—The synthesis of citrulline from arginine in the small intestine depends on the provision of ornithine. To test the hypothesis that arginase II plays a central role in the supply of ornithine for citrulline synthesis, the contribution of dietary arginine, glutamine, and proline was determined by utilizing multitracer stable isotope protocols in arginase II knockout (AII−/−) and wild-type (WT) mice. The lack of arginase II resulted in a lower citrulline rate of appearance (121 vs. 137 μmol·kg−1·h−1) due to a reduced availability of ornithine; ornithine supplementation was able to restore the rate of citrulline production in AII−/− to levels comparable with WT mice. There were significant differences in the utilization of dietary citrulline precursors. The contribution of dietary arginine to the synthesis of citrulline was reduced from 45 to 10 μmol·kg−1·h−1 due to the lack of arginase II. No enteral utilization of arginine was observed in AII−/− mice (WT = 25 μmol·kg−1·h−1), and the contribution of dietary arginine through plasma ornithine was reduced in the transgenic mice (20 vs. 13 μmol·kg−1·h−1). Dietary glutamine and proline utilization were greater in AII−/− than in WT mice (20 vs. 13 and 1.4 vs. 3.7 μmol·kg−1·h−1, respectively). Most of the contribution of glutamine and proline was enteral rather than through plasma ornithine. The arginase isoform present in the small intestinal mucosa has the role of providing ornithine for citrulline synthesis. The lack of arginase II results in a greater contribution of plasma ornithine and dietary glutamine and proline to the synthesis of citrulline.

arginase; glutamine; modeling; proline; urea cycle

ARGINASE IS THE ENZYME that catalyzes the hydrolysis of arginine into urea and ornithine. Two different arginase isoforms with different tissue distribution, subcellular localization, and physiological function have been characterized (5). Arginase I is present in high abundance in the liver, where it serves to detoxify ammonia and to regenerate ornithine within the urea cycle (14). New roles have been proposed for this isoform after it was discovered that it is also present in extrahepatic tissue (22).

Arginase II is widely expressed throughout the body and can be found in higher abundance in the intestine and kidney (5). The role of arginase II in macrophages and endothelial cells is critical for the regulation of nitric oxide production and vascular function (7). For this reason, arginase II has been proposed as a target for pharmacological intervention in essential hypertension (1), in the reversal of endothelial dysfunction during aging (8), in the treatment of atherosclerosis (17), and as cardioprotective during ischemia-reperfusion (9). The role of arginase II in the kidney seems to be different in males than in females, and it has been proposed that its activity is geared toward the production of polyamines and glutamate (10). In the small intestine, arginase II is colocalized with ornithine amino transferase (OAT), ornithine decarboxylase (ODC), and ornithine transcarbamylase (OTC), and thus the ornithine produced has the potential to be utilized for the synthesis of proline, polyamines, and citrulline, respectively (15).

Recently, we have shown that dietary arginine is the preferred precursor for citrulline synthesis in mice (9) and that, during arginine-free feeding, plasma arginine and plasma ornithine are the main precursors for citrulline synthesis (10). Although the utilization of both dietary and plasma arginine, together with the utilization of plasma ornithine, demonstrated that there is a preferential utilization of “preformed” ornithine for the synthesis of citrulline (9, 10), it also suggested that the role of arginase II in the small intestine is crucial for the synthesis of citrulline.

To test the hypothesis that arginase II plays a central role in the provision of ornithine for citrulline synthesis, the production of citrulline and the respective contributions of dietary arginine, glutamine, and proline to citrulline synthesis were determined utilizing multitracer stable isotope protocols in wild-type (WT) and arginase II knockout (AII−/−) mice.

MATERIALS AND METHODS

Animals and Treatments

Young adult male C57BL/6J AII−/− (18) and C57BL/6J mice were used in the experiments described below. The AII−/− mouse model utilized in these studies has no apparent phenotype (except higher plasma arginine concentration) and produces viable offspring (18). Mice were housed in a specific pathogen-free facility and had access to an irradiated 18% crude protein pelleted feed (Rodent Diet 2920X; Harlan Teklad). Dietary proximate analysis was as follows: protein (185 g/kg), gross energy (14.1 MJ/kg), fat (60 g/kg), fiber (28 g/kg), and ash (46 g/kg). Acetaldehyde reverse osmosis water was available at all times. Mice were under a 12-h light cycle (600–1800) in a temperature- (22 ± 2°C) and humidity-controlled (55 ± 5%) environment. All animal procedures were authorized by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Gastric catheterization. The procedure and postoperative care have been described in detail elsewhere (11). Mice recovered their presurgery body weight within 5 days, and infusions were conducted ≥7 days after surgery.

Infusions and sampling. On the day of the infusion, feed was removed at 7 AM, and mice were weighed at 9:30 AM. After a 3-h feed deprivation, mice were restrained and a tail vein catheter was inserted, as described previously (13). The tail vein catheter and the gastric catheter were then connected to syringe infusion pumps (PHD2000; Harvard Apparatus, Holliston, MA). In addition to the different tracers described below (Cambridge Isotope Laboratories, Andover, MA), a liquid amino acid and glucose solution similar to the one used in our previous report (11) was infused intragastrically at a rate of 20 ml·kg−1·h−1 to maintain a fed steady state.
Determination of the Precursors Utilized for the Synthesis of Citrulline

For this part of the study, 17 AII-/- and 19 WT mice were utilized. Each mouse was infused twice following two of the infusion protocols described below (which were randomly assigned to each mouse). A 7-day recovery period was allowed between infusions. The infusion protocols lasted 4 h and were designed to determine the contribution of dietary arginine, glutamine, and proline and plasma ornithine to the synthesis of citrulline.

Infusion 1 was an intragastric infusion of [U-13C6]arginine (50 µmol·kg⁻¹·h⁻¹, prime 50 µmol/kg; WT = 13 and AII-/- = 11 observations).

Infusion 2 was an intragastric infusion [U-13C₅]glutamine (200 µmol·kg⁻¹·h⁻¹, prime 200 µmol·kg⁻¹; WT = 12 and AII-/- = 12 observations).

Infusion 3 was an intragastric infusion of [U-13C₅]proline (240 µmol·kg⁻¹·h⁻¹, prime 240 µmol/kg; WT = 13 and AII-/- = 11 observations).

In addition, infusion protocols 1, 2, and 3 included the intravenous infusion of [5,5]D₂-ornithine (22 µmol·kg⁻¹·h⁻¹, prime 22 µmol/kg), [¹⁵N](ureido) citrulline (7 µmol·kg⁻¹·h⁻¹, prime 7 µmol/kg), and [ring-D₃]phenylalanine (10 µmol·kg⁻¹·h⁻¹, prime 10 µmol/kg).

Determination of First-Pass Utilization of Arginine

The first-pass utilization of arginine was studied in six mice of each genotype by infusing two different arginine tracers by the intragastric and intravenous routes.

Infusion 4 was a simultaneous, continuous infusion of [U-13C₆]arginine (enteral, 95 µmol·kg⁻¹·h⁻¹; prime, 95 µmol/kg) and 1,2-[¹⁵C₂]arginine (intravenous, 30 µmol·kg⁻¹·h⁻¹; prime, 30 µmol/kg; n = 6 WT and 6 AII-/- mice).

Tissue (liver, kidney, and small intestinal mucosa) was harvested from these mice to determine the presence of arginase I and II protein. Tissue samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Determination of the Effect of Dietary Ornithine Supplementation on Citrulline Entry Rate and Precursor Utilization

For this part of the study, nine AII-/- and nine WT mice were utilized. Each mouse was infused three times and the control (no supplementation), ORN1 (46 µmol·kg⁻¹·h⁻¹), or ORN2 (92 µmol·kg⁻¹·h⁻¹) ornithine supplementation treatment in a random sequence. Note that the ornithine supplemented was isotopically labeled. A 7-day recovery period was allowed between infusions. The infusion protocols lasted 4 h and were designed to determine the contribution of dietary proline, the contribution of supplemental ornithine to citrulline synthesis, and the rate of appearance of citrulline.

Infusion 5 (control) was an intragastric infusion of [U-13C₅]proline (240 µmol·kg⁻¹·h⁻¹, prime 240 µmol/kg; WT = 9 and AII-/- = 9 observations).

Infusion 6 (ORN1) was an intragastric infusion of [U-13C₅]proline (240 µmol·kg⁻¹·h⁻¹, prime 240 µmol/kg) and [5,5]D₂-ornithine (46 µmol·kg⁻¹·h⁻¹, prime 46 µmol/kg; WT = 9 and AII-/- = 9 observations).

Infusion 7 (ORN2) was an intragastric infusion of [U-13C₅]proline (240 µmol·kg⁻¹·h⁻¹, prime 240 µmol/kg) and [5,5]D₂-ornithine (92 µmol·kg⁻¹·h⁻¹, prime 92 µmol/kg; WT = 9 and AII-/- = 9 observations).

In addition, infusion protocols 4, 5, and 6 included the intravenous infusion of [¹⁵N](ureido) citrulline (7 µmol·kg⁻¹·h⁻¹, prime 7 µmol/kg), and [ring-D₃]phenylalanine (10 µmol·kg⁻¹·h⁻¹, prime 10 µmol/kg).

After a 4-h infusion, blood (~160 µl) was drawn from the submandibular bundle and centrifuged at 1,500 g for 15 min at 4°C, and plasma was kept frozen at −80°C until analysis. Blood samples from similar mice under identical housing conditions and infused with the amino acid and glucose mixture for 4 h were used to determine isotopic enrichment background.

Sample Analysis

Plasma amino acid concentrations were measured by reverse-phase HPLC of their phenyl isothiocyanate derivatives (PicoTag; Waters, Woburn, MA). Plasma citrulline, arginine, ornithine, and phenylalanine were determined as their dansyl derivatives by liquid chromatography-mass spectrometry utilizing a TSQ Quantum Ultra System (Thermo Finnigan, San Jose, CA). Citrulline isotopic enrichment was determined by monitoring the transitions m/z 409 to 392, 410 to 392 (ureido nitrogen), and 414 to 397. Arginine, ornithine, and proline isotopic enrichments were determined by a single reaction monitoring the transitions m/z 408 to 170, 413 to 170, and 414 to 170 for arginine, 599 to 170 and 604 to 170 for ornithine, and 399 to 170 and 404 to 170 for phenylalanine.

Protein from murine tissues was isolated using Celllytic MT Mammalian Tissue Lysis/Extraction Reagent (Sigma-Aldrich, St. Louis, MO) and quantified using Micro BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) using the suggested protocol. A standard Western blot was performed, loading 0.04–20 µg protein/lane on a 4–15% gel (Bio-Rad, Hercules, CA) and blotted on a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The blot was incubated overnight in a 1:200 dilution of arginase I and arginase II antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 1% dry milk/PBS or with a 1:5,000 dilution of mouse anti-β-actin antibody (Sigma-Aldrich) in 5% milk/PBS at 4°C. After washing, the blot was stained with a 1:5,000 dilution of peroxidase-conjugated anti-rabbit (GE Healthcare, Piscataway, NJ), anti-mouse (Bio-Rad), or anti-goat (Roche Applied Sciences, Indianapolis, IN) IgG antiserum. Bands were visualized by an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Calculations

Calculations have been described in detail elsewhere (12). Briefly, rate of appearance (Rₐ) of the different amino acids was calculated from the isotopic dilution of the intravenously infused tracer and first-pass extraction from the calculated disappearance of the intragastric tracer with respect of the peripherally infused tracer. The recovery of the intragastric tracer in circulating citrulline was calculated by multiplying the plasma citrulline enrichment that was due to the intragastric tracer by the Rₐ of citrulline. We used the precursor-intermediate-product approach (12) to determine the site of production of the ornithine used for citrulline synthesis. This model does not rely on any assumption regarding the enrichment of the precursor pool but rather on the rate of infusion of the intragastric tracers and their recoveries as plasma citrulline. Although plasma enrichments have been utilized in the past (3, 19), they do not reflect the enrichment of the precursors at the site of their utilization and thus result in an overestimation of the contribution of the different precursors. Furthermore, because it is likely that the different precursors undergo different first-pass extraction and dilution rates, the utilization of peripheral enrichments does not even allow for the determination of the relative rate of utilization of the different precursors.

Data Analysis

Data were analyzed statistically as a complete randomized design utilizing the Proc Mixed procedure of SAS (version 9.2, SAS Institute, Cary, NC), with the citrulline precursor (arginine, glutamine or proline) and genotype as fixed effects and mouse as random effect of the model. Means were tested for significance at the 5% level.
RESULTS

Arginase II and Plasma Amino Acid Fluxes

Arginase II was not present in the liver of mice and was absent in the kidney and intestinal mucosa of AII\textsuperscript{−/−} mice due to the targeted deletion of this enzyme (Fig. 1). Of the tissues analyzed, arginase I was present only in the liver of both AII\textsuperscript{−/−} and WT mice.

The R\textsubscript{e} of citrulline was greater in WT than in AII\textsuperscript{−/−} mice (P < 0.001) as well as the entry rate of phenylalanine (P = 0.012); however, there were no differences (P = 0.326) in the ornithine rate of appearance (Table 1). A trend for a lower first-pass extraction of arginine in AII\textsuperscript{−/−} mice (65 ± 3 vs. 75 ± 4%, P = 0.071) was detected, which was accompanied by a faster arginine Ra (P = 0.002) and higher plasma arginine concentration in AII\textsuperscript{−/−} vs. WT mice (P < 0.001; Table 2). No difference (P > 0.47) in citrulline and ornithine plasma concentrations was detected between the two genotypes.

Contribution of Plasma Ornithine to the Synthesis of Citrulline

There was a trend (P = 0.061) for a greater contribution of plasma ornithine to the synthesis of citrulline in AII\textsuperscript{−/−} mice than in WT mice (Table 1) that reached significance (P = 0.029) when expressed as percentage of the rate of ornithine entry (16.4 ± 0.4 vs. 14.9 ± 0.5%, respectively). A larger percentage (P = 0.003) of the entry rate of citrulline was derived from plasma ornithine in AII\textsuperscript{−/−} mice than in their WT counterparts (39.6 ± 3.2 vs. 28.8 ± 1.3%, respectively).

Contribution of Dietary Arginine to the Synthesis of Citrulline

There was a lower (P < 0.003) recovery of the arginine tracer infused intragastrically in plasma citrulline and ornithine in AII\textsuperscript{−/−} mice (Table 3). These two amino acids accounted for ∼30 ± 3 and 55 ± 3% of the infusion rate of the arginine tracer in AII\textsuperscript{−/−} and WT mice, respectively. The contribution of dietary arginine to the synthesis of citrulline was lower in AII\textsuperscript{−/−} mice compared with WT mice in absolute terms (P < 0.001; Table 3) or as percentage of circulating citrulline (8 ± 0.5 and 33 ± 1% R\textsubscript{a} citrulline, respectively, P < 0.001). Likewise, the contribution of dietary arginine to the synthesis of ornithine was lower in AII\textsuperscript{−/−} mice compared with WT mice in absolute terms (P < 0.001; Table 3) or as percentage of circulating ornithine (35 ± 0.5 and 54 ± 3% R\textsubscript{a} ornithine, respectively, P < 0.001).

There was less arginine tracer incorporated into citrulline through plasma ornithine in AII\textsuperscript{−/−} than in WT controls (P = 0.008; Table 3); this resulted in a lower calculated citrulline enrichment (3.1 vs. 4.2 molar percent excess, P < 0.014) due to this metabolic route. Although the measured citrulline enrichment for the AII\textsuperscript{−/−} mice was higher than the calculated enrichment, which results in unrealistic values (i.e., negative values), the rate at which arginine was utilized locally in the

Table 1. Rate of appearance of different amino acids, first pass-utilization of arginine, and contribution of plasma ornithine to the synthesis of citrulline in AII\textsuperscript{−/−} and WT mice

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Genotype</th>
<th>Rate of appearance, µmol·kg\textsuperscript{-1}·h\textsuperscript{-1}</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrulline</td>
<td>AII\textsuperscript{−/−}</td>
<td>121 ± 2</td>
<td>137 ± 3</td>
</tr>
<tr>
<td>Arginine†</td>
<td>WT</td>
<td>292 ± 23</td>
<td>267 ± 13</td>
</tr>
<tr>
<td>Phenylalanine†</td>
<td>WT</td>
<td>394 ± 8</td>
<td>433 ± 12</td>
</tr>
<tr>
<td>First-pass arginine extraction, %‡</td>
<td>WT</td>
<td>438 ± 12</td>
<td>381 ± 28</td>
</tr>
<tr>
<td>Rate of conversion of ornithine to citrulline, µmol·kg\textsuperscript{-1}·h\textsuperscript{-1}</td>
<td>WT</td>
<td>48.2 ± 4.1</td>
<td>39.5 ± 2.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. AII\textsuperscript{−/−}, arginase II knockout; WT, wild type. †AII\textsuperscript{−/−} 34 and WT 38 observations. ‡AII\textsuperscript{−/−} 6 and WT 6 observations.

Table 2. Plasma amino acid concentration in AII\textsuperscript{−/−} and WT mice

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Genotype</th>
<th>Plasma amino acid concentration, µmol/l</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrulline</td>
<td>AII\textsuperscript{−/−}</td>
<td>54 ± 3</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>Arginine†</td>
<td>WT</td>
<td>121 ± 9</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>Ornithine</td>
<td>WT</td>
<td>83 ± 5</td>
<td>79 ± 4</td>
</tr>
<tr>
<td>Proline</td>
<td>WT</td>
<td>107 ± 6</td>
<td>120 ± 9</td>
</tr>
<tr>
<td>Glutamine</td>
<td>WT</td>
<td>544 ± 22</td>
<td>567 ± 27</td>
</tr>
<tr>
<td>Glutamate</td>
<td>WT</td>
<td>52 ± 3</td>
<td>47 ± 5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>WT</td>
<td>84 ± 3</td>
<td>91 ± 7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>WT</td>
<td>46 ± 3</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>Leucine‡</td>
<td>WT</td>
<td>214 ± 7</td>
<td>228 ± 9</td>
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<tr>
<td>Isoleucine</td>
<td>WT</td>
<td>141 ± 6</td>
<td>140 ± 5</td>
</tr>
<tr>
<td>Valine</td>
<td>WT</td>
<td>365 ± 17</td>
<td>354 ± 16</td>
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<tr>
<td>Methionine</td>
<td>WT</td>
<td>71 ± 5</td>
<td>77 ± 5</td>
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<tr>
<td>Cystine</td>
<td>WT</td>
<td>181 ± 19</td>
<td>165 ± 18</td>
</tr>
<tr>
<td>Cysteine</td>
<td>WT</td>
<td>79 ± 18</td>
<td>72 ± 15</td>
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<tr>
<td>Aspartate</td>
<td>WT</td>
<td>12 ± 1</td>
<td>13 ± 2</td>
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<tr>
<td>Asparagine</td>
<td>WT</td>
<td>25 ± 1</td>
<td>29 ± 1</td>
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<tr>
<td>Tryptophan</td>
<td>WT</td>
<td>58 ± 2</td>
<td>63 ± 2</td>
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<tr>
<td>Threonine</td>
<td>WT</td>
<td>210 ± 13</td>
<td>222 ± 13</td>
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<tr>
<td>Lysine</td>
<td>WT</td>
<td>413 ± 14</td>
<td>417 ± 16</td>
</tr>
<tr>
<td>Alanine</td>
<td>WT</td>
<td>647 ± 32</td>
<td>629 ± 32</td>
</tr>
<tr>
<td>Glycine</td>
<td>WT</td>
<td>134 ± 6</td>
<td>156 ± 8</td>
</tr>
<tr>
<td>Serine</td>
<td>WT</td>
<td>131 ± 6</td>
<td>129 ± 8</td>
</tr>
</tbody>
</table>

Values are means ± SE. †AII\textsuperscript{−/−} 17 and WT 19 observations.

Fig. 1. Arginase (Arg) I and II abundance in intestinal mucosa, kidney, and liver of wild-type (WT) and Arg II knockout (AII\textsuperscript{−/−}) mice. Note the different loading for Arg II in WT mice.
small intestine for the synthesis of citrulline was not different from zero (−3.1 ± 1.7 μmol·kg⁻¹·h⁻¹; P = 0.098; Table 3).

Arginine Recycling

The utilization of dietary arginine for citrulline synthesis can also be inferred from the recycling of the arginine tracer. The arginine tracer used generated [¹³C₅]arginine through the conversion of arginine to ornithine, ornithine to citrulline, and citrulline to arginine. The [¹³C₅][¹³C₆] arginine percentage was greater (P < 0.001) in WT mice than in AII⁻/⁻ animals (59 ± 4 and 15 ± 2%, respectively).

Contribution of Dietary Glutamine to the Synthesis of Citrulline

There was a greater recovery of the glutamine tracer infused intragastrically in plasma citrulline in AII⁻/⁻ mice (P < 0.001), but no changes for ornithine were detected (P = 0.206; Table 3). The total recovery of the tracer in these two amino acids was <1.5 ± 0.2% of the tracer infused. The contribution of dietary glutamine to the synthesis of citrulline was greater (P < 0.001) in AII⁻/⁻ mice than in WT animals (Table 3) and resulted in a greater contribution to the Ra of citrulline (3.2 ± 0.2 vs. 1.0 ± 0.1% Ra citrulline, P < 0.001). No differences (P = 0.207) in the contribution of dietary glutamine to circulating ornithine were observed.

There was no statistical difference (P = 0.091) in the rate at which the glutamine tracer was incorporated into citrulline through plasma ornithine between the two genotypes (Table 3). However, when the citrulline enrichment that was due to the use of ornithine originated from the glutamine tracer was calculated, AII⁻/⁻ mice had a higher enrichment than their WT counterparts (P = 0.044; Table 3). There was no difference (P = 0.092) between the two genotypes on the utilization of circulating ornithine derived from dietary glutamine for citrulline synthesis (Table 3); however, there was a greater utilization of glutamine for citrulline synthesis by the enterocytes due to the lack of arginase II enzyme (P < 0.001; Table 3).

Contribution of Dietary Proline to the Synthesis of Citrulline

Similar to what was observed for the glutamine tracer, there was a greater recovery of the proline tracer infused intragastrically in plasma citrulline in AII⁻/⁻ mice (P < 0.001), but no changes for ornithine were detected (P = 0.220; Table 3). The total recovery of the tracer in these two amino acids was <4.5 ± 2% of the tracer infused. The contribution of dietary proline to the synthesis of citrulline was greater (P < 0.001; Table 3) in AII⁻/⁻ mice than in WT animals and resulted in a greater contribution to the Ra of citrulline (15.9 ± 0.4 vs. 9.9 ± 0.2% Ra citrulline, P < 0.001). No difference (P = 0.224) in the contribution of dietary proline to circulating ornithine was observed in absolute terms (Table 3); however, dietary proline made a greater contribution in the WT mice, representing 6.4 ± 0.3% (AII⁻/⁻ = 4.8 ± 0.4%) of the entry rate of ornithine.

No differences were found in the rate at which the proline tracer was incorporated into citrulline through plasma ornithine (P = 0.879; Table 3) or the resulting plasma citrulline enrichment due to this source (P = 0.330). There was no difference (P = 0.873) between the two genotypes on the utilization of circulating ornithine derived from dietary proline for citrulline synthesis (Table 3); however, there was a greater utilization of proline by the small intestine for citrulline synthesis in AII⁻/⁻ mice (Table 3).

Dietary arginine was the main precursor for citrulline synthesis in WT mice (Fig. 2), followed by proline and glutamine. Note that only dietary precursors were studied, and thus plasma precursors or precursors generated from the turnover of protein within the enterocyte are not included. The lack of arginase II in AII⁻/⁻ mice resulted in a reduction in the utilization of dietary arginine but in a greater contribution of dietary glutamine and proline. For AII⁻/⁻ mice, proline was the main dietary precursor for citrulline synthesis.
Effect of Ornithine Supplementation on Citrulline Synthesis and Precursors Utilized

Ornithine supplementation increased \( P < 0.011 \) the rate of appearance of citrulline in \( \text{AII}^{-/-} \) mice but had no effect in the WT animals \( (P = 0.372; \text{Table 4}) \). The phenylalanine \( R_a \) was not affected by ornithine supplementation \( (P = 0.577) \) or mouse genotype \( (P = 0.251) \). A greater recovery of the proline tracer infused intragastrically in plasma citrulline was found in \( \text{AII}^{-/-} \) mice than in their WT counterparts \( (P < 0.001; \text{Table 4}) \), but no effect of ornithine supplementation was detected. This translated into a greater utilization of dietary proline for citrulline synthesis in the \( \text{AII}^{-/-} \) mice (Table 4). Ornithine supplementation made a greater contribution to the synthesis of citrulline in \( \text{AII}^{-/-} \) mice \( (P < 0.001) \), and that was dose dependent \( (P < 0.001; \text{Table 4}) \). The recovery of supplemental ornithine in citrulline represented 51 ± 4 and 40 ± 4% of the tracer infused in \( \text{AII}^{-/-} \) mice (ORN1 and ORN2, respectively) and 31 ± 2 and 27 ± 3% in WT mice (ORN1 and ORN2, respectively).

Ornithine supplementation reduced the contribution of proline to the synthesis of citrulline from 21 ± 1 to 16 ± 1% of the citrulline appearance rate in \( \text{AII}^{-/-} \) mice (control vs. supplemented, respectively, \( P < 0.001 \)), but had no effect in WT mice \( (\sim 12 \pm 1\% R_a \text{ citrulline}) \). The contribution of supplemental ornithine to the synthesis of citrulline represented 19 ± 28 ± 1% of the citrulline appearance rate in \( \text{AII}^{-/-} \) mice (ORN1 and ORN2, respectively; \( P < 0.001 \)) and 10 ± 1 and 18 ± 0% in WT mice (ORN1 and ORN2, respectively, \( P < 0.001 \)).

**DISCUSSION**

Consistent with previous reports, arginase II was present in both kidney and small intestinal mucosa in mice, whereas arginase I was detected only in the liver (5). The lack of arginase II in the small intestine of the knockout mice reduced the first-pass extraction of arginine. The arginine first-pass extraction observed in C57BL/6J WT mice was similar to our previous observation in Institute of Cancer Research (ICR) mice (12) on an identical diet but higher than those reported in other species, such as rats (33%; Ref. 21), piglets (40–50%; Refs. 2 and 20), and humans (33%; Ref. 4). This reduction in the first-pass extraction of arginine in \( \text{AII}^{-/-} \) mice increased the amount of dietary arginine reaching the peripheral circulation and resulted in a higher entry rate and a higher plasma arginine concentration, which is typical of this genotype (18). The lack of difference between \( \text{AII}^{-/-} \) and WT mice in ornithine entry rate and plasma ornithine concentration suggests that arginase I was able to compensate for the absence of arginase II (22).

The reduction \( (\sim 10\%) \) in the entry rate of citrulline observed in \( \text{AII}^{-/-} \) mice was clearly due to a reduction in ornithine availability for citrulline synthesis, because ornithine supplementation was able to restore the synthesis of citrulline to a level similar to WT mice. Furthermore, \( \text{AII}^{-/-} \) mice derived more citrulline from plasma ornithine than WT animals, which seems to be a compensatory mechanism to overcome (at least partially) the lack of ornithine generated by the action of arginase in the small intestine. Likewise, \( \text{AII}^{-/-} \) mice were able to utilize a larger amount of the supplemental ornithine infused intragastrically than WT mice.

Dietary arginine was the main dietary precursor for citrulline synthesis in WT mice, contributing the precursor for 33% of the entry rate of citrulline, which agrees with our previous work in ICR mice (11, 12). The lack of \( \text{AII}^{-/-} \) in the small intestine, and thus the inability to derive ornithine from argi-

Table 4. Rate of appearance of citrulline and phenylalanine and contribution of proline and ornithine to citrulline synthesis in \( \text{AII}^{-/-} \) and WT mice supplemented with ornithine (0, 46, and 92 \( \mu \text{mol·kg}^{-1}·\text{h}^{-1} \); control, ORN1, and ORN2, respectively)  

<table>
<thead>
<tr>
<th></th>
<th>( \text{AII}^{-/-} )</th>
<th></th>
<th></th>
<th>( \text{WT} )</th>
<th></th>
<th></th>
<th>Gen</th>
<th>TRT</th>
<th>Gen × TRT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>ORN1</td>
<td>ORN2</td>
<td>Control</td>
<td>ORN1</td>
<td>ORN2</td>
<td>Gen</td>
<td>TRT</td>
<td>Gen × TRT</td>
</tr>
<tr>
<td>Rate of appearance, ( \mu \text{mol·kg}^{-1}·\text{h}^{-1} )</td>
<td>Citrulline</td>
<td>115 ± 13</td>
<td>127 ± 7</td>
<td>132 ± 10</td>
<td>131 ± 14</td>
<td>137 ± 19</td>
<td>133 ± 14</td>
<td>&lt;0.134</td>
<td>&lt;0.113</td>
</tr>
<tr>
<td></td>
<td>Phenylalanine</td>
<td>479 ± 66</td>
<td>440 ± 75</td>
<td>460 ± 97</td>
<td>502 ± 83</td>
<td>471 ± 60</td>
<td>509 ± 84</td>
<td>&lt;0.251</td>
<td>&lt;0.577</td>
</tr>
<tr>
<td>Tracer recovery as citrulline, ( \mu \text{mol·kg}^{-1}·\text{h}^{-1} )</td>
<td>Proline</td>
<td>7.1 ± 0.6</td>
<td>6.6 ± 0.3</td>
<td>6.1 ± 0.4</td>
<td>4.8 ± 0.3</td>
<td>4.6 ± 0.6</td>
<td>4.6 ± 0.3</td>
<td>&lt;0.001</td>
<td>&lt;0.099</td>
</tr>
<tr>
<td></td>
<td>Ornithine</td>
<td>24.1 ± 1.1</td>
<td>37.2 ± 3.0</td>
<td>13.8 ± 1.0</td>
<td>24.2 ± 3.0</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dietary contribution to citrulline synthesis, ( \mu \text{mol·kg}^{-1}·\text{h}^{-1} )</td>
<td>Proline</td>
<td>23.4 ± 1.8</td>
<td>21.8 ± 1.0</td>
<td>20.3 ± 1.2</td>
<td>15.9 ± 0.9</td>
<td>15.2 ± 1.8</td>
<td>15.1 ± 1.0</td>
<td>&lt;0.001</td>
<td>&lt;0.102</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 9 \) observations. ORN1 and -2, levels of ornithine supplementation; Gen, genotype effect; TRT, ornithine supplementation effect; Gen × TRT, interaction between the 2 main effects. \( ab \), Preplanned orthogonal contrast, control vs. supplemented (ORN1 and ORN 2), differs \( P < 0.01 \).
nine locally by the enterocyte, reduced the total contribution of dietary arginine to 10% of the circulating citrulline. The precursor-intermediate-product model, employed to determine the utilization of dietary precursors, indicated that no dietary arginine was utilized by the small intestine for ornithine production in AII−/− mice, consistent with the lack of arginase II shown by Western analysis in the intestinal mucosa of these animals. This model also shows that dietary arginine contributed the enteral precursor for 25.1 μmol·kg−1·h−1 of citrulline in the WT mice, which closely agrees with the ~10% first-pass extraction difference (~30 μmol·kg−1·h−1) between the two genotypes. This suggests that the ornithine generated by action of mitochondrial arginase II is channeled mostly to the mitochondrial OTC for the synthesis of citrulline.

The contribution to the synthesis of citrulline of both dietary glutamine and proline was greater in AII−/− than in WT mice; the increased utilization of these two dispensable amino acids to the synthesis of citrulline in AII−/− mice was unlikely to have an impact on their availability for other metabolic processes because it represented a small proportion of the amount infused intragastrically (<1% for proline and <0.5% for glutamine). The utilization of dietary glutamine and proline for citrulline synthesis occurred directly in the small intestine rather than through the transport and utilization by the enterocyte of the ornithine generated from these two amino acids somewhere else in the body. Thus the reduction in ornithine availability, due to the lack of arginase in enterocytic mitochondria, resulted in an increase in the de novo synthesis of ornithine through OAT. Consistent with these observations, ornithine supplementation reduced the contribution of dietary proline to the synthesis of citrulline. These findings support our previous conclusions (11, 12) that “preformed” ornithine (either plasma ornithine or ornithine from arginine hydrolyzed within the enterocytes) is the preferred precursor for the synthesis of citrulline rather than “de novo” ornithine production from glutamine or proline by action of OAT. However, the synthesis of ornithine through the de novo pathway can be increased when preformed ornithine availability is reduced, either because arginine is absent from the diet (12) or because arginase II is not present.

The third enzyme that utilizes ornithine, ODC, has a crucial role for the synthesis of polyamines during the maturation and development of the small intestine during the 3rd week of life (16). However, the activity of ODC declines markedly at weaning and remains low throughout adulthood. This enzyme is cytosolic and probably utilizes extracellular ornithine rather than ornithine generated by mitochondrial arginase. Furthermore, it has been shown that ornithine generated from the hydrolysis of arginine by arginase appears not to be necessary for the production of polyamines because double arginase I and arginase II knockout mice have polyamine levels similar to WT mice (6, 18).

In conclusion, our results indicate that the main role of the arginase isoform present in the small intestinal mucosa is to provide ornithine for citrulline synthesis and that the absence of arginase activity limits the availability of ornithine-reducing citrulline production. Because arginine synthesis is the only known fate for citrulline, the utilization of arginine for citrulline production seems to be counterintuitive. Furthermore, the lack of arginase II results in compensatory mechanisms such as a greater utilization of extracellular ornithine (plasma ornithine and supplemental ornithine) and the upregulation of the de novo ornithine synthesis pathways. This newly defined role of enteral arginine needs to be taken into account if arginine becomes a target for pharmacological intervention, as has been proposed for essential hypertension (1) or atherosclerosis (17), among other conditions.

GRANTS

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DISCLOSURES

The authors have no disclosures to report.

REFERENCES


