A multitracer stable isotope quantification of the effects of arginine intake on whole body arginine metabolism in neonatal piglets

Kristine L. Urschel,1 Mahroukh Rafii,2 Paul Pencharz,1,2,3,4 and Ronald O. Ball1,2,4

1Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta; 2The Research Institute, The Hospital for Sick Children, Toronto; and Departments of 3Paediatrics and 4Nutritional Science, University of Toronto, Toronto, Ontario, Canada

Submitted 10 May 2007; accepted in final form 20 June 2007

Urschel KL, Rafii M, Pencharz PB, Ball RO. A multitracer stable isotope quantification of the effects of arginine intake on whole body arginine metabolism in neonatal piglets. Am J Physiol Endocrinol Metab 293: E811–E818, 2007. First published June 26, 2007; doi:10.1152/ajpendo.00290.2007.—We have previously shown that deficient arginine intake increased the rate of endogenous arginine synthesis from proline. In this paper, we report in vivo quantification of the effects of arginine intake on total endogenous arginine synthesis, on the rates of conversion between arginine, citrulline, ornithine, and proline, and on nitric oxide synthesis. Male piglets, with gastric catheters for diet and isotope infusion and femoral vein catheters for blood sampling, received a complete diet for 2 days and then either a generous (+Arg; 1.80 g·kg⁻¹·day⁻¹; n = 5) or deficient (−Arg; 0.20 g·kg⁻¹·day⁻¹; n = 5) arginine diet for 5 days. On day 7, piglets received a primed, constant infusion of [guanido-¹⁵N₂]arginine, [ureido-¹³C,5,5-²H₂]citrulline, [U-¹³C]ornithine, and [¹⁵N⁺¹³C]proline in an integrated study of the metabolism of arginine and its precursors. Arginine synthesis (µmol·kg⁻¹·h⁻¹) from both proline (+Arg: 42, −Arg: 74; pooled SE: 5) and citrulline (+Arg: 67, −Arg: 120; pooled SE: 15) was higher in piglets receiving the −Arg diet (P < 0.05); and for both diets proline accounted for ~60% of total endogenous arginine synthesis. The conversion of proline to citrulline (+Arg: 39, −Arg: 67, pooled SE: 6) was similar to the proline-to-arginine conversion, confirming that citrulline formation limits arginine synthesis from proline in piglets. Nitric oxide synthesis (µmol·kg⁻¹·h⁻¹), measured by the rate conversion of [guanido-¹⁵N₂]arginine to [ureido-¹³C]citrulline, was greater in piglets receiving the +Arg diet (105) than in those receiving the −Arg diet (46, pooled SE: 10; P < 0.05). This multi-isotope method successfully allowed many aspects of arginine metabolism to be quantified simultaneously in vivo.

Although the carbon backbone of citrulline may come from either proline or glutamine/glutamate via pyrroline-5-carboxylate (P5C) or from arginine that is converted to ornithine via the action of arginase (EC no. 3.5.3.1), all the carbon that appears in endogenously synthesized arginine must come from citrulline. Therefore, the conversion of citrulline to arginine is the best measure of total endogenous arginine synthesis, and this method has been successfully used to measure whole body arginine synthesis in adult humans (9). In previous studies, we used the proline-to-arginine conversion as a measure of whole body arginine synthesis in neonatal piglets (27–29, 31); however, how much of the total endogenous arginine synthesis in piglets that arises from proline is currently unknown. By comparing the rate of conversion of proline to arginine to the rate of the citrulline-to-arginine conversion, the relative importance of proline as an arginine precursor can be assessed.

In a previous study, we found that the addition of either arginine or citrulline to an arginine-deficient basal diet resulted in similar improvements in whole body arginine status (29), but ornithine and proline did not. These findings indicated that it was citrulline formation that was limiting to endogenous arginine synthesis; however, rates of conversion were not measured in the previous study (29). The fractional net conversions of proline to metabolites such as citrulline and ornithine have been measured (3, 28, 31); however, because neither citrulline nor ornithine fluxes were measured, the molar rates of conversions between metabolites could not be calculated. If the molar rates of conversion between the major intermediates in the proline-to-arginine synthetic pathway are known, then it will be possible to identify the contribution of each to whole body arginine synthesis.

Although piglets receiving a generous intake of arginine tended to have higher plasma nitrate plus nitrite concentrations than those receiving a deficient arginine diet (31), the effect of arginine intake on the rate of nitric oxide synthesis has not been measured in neonates. In adult men, arginine intake did not have an effect on nitric oxide synthesis (2, 13); however, the dietary regulation of arginine synthesis is very different in neonates compared with adults (9, 31). Therefore, the response of nitric oxide synthesis to different arginine intakes may also be different in neonates compared with adults. The effect of arginine intake on whole body nitric oxide synthesis in neonates is of particular interest, because low plasma arginine concentrations (40) and fluxes (11) have been described in infants suffering from persistent pulmonary hypertension of the neonate (PPHN) (11) and necrotizing enterocolitis (NEC) (40). For both PPHN and NEC, arginine supplementation has been shown to reduce the incidence (1, 15) of the disease or reduce the severity of the symptoms (20); for both pathologies, the role of arginine as a nitric oxide precursor has been suggested as a reason for the effectiveness of arginine supplementation.

The major objectives in this study were to determine the effect of arginine intake in enterally fed piglets on 1) the portion of total endogenous arginine synthesis originating from proline, 2) the rates of conversion between the various intermediates in the arginine synthetic pathway, and 3) the rate of nitric oxide synthesis. The present study was designed to address these objectives by using the simultaneous infusion of four isotopes: [guanido-¹⁵N₂]arginine, [ureido-¹³C,5,5-²H₂]citrulline,

MATERIALS AND METHODS

Animals and surgical procedures. All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee for the University of Alberta. Ten intact male Landrace × Large White × Duroc piglets (1.4–1.8 kg; Hypor, Regina, SK, Canada) were obtained from the University of Alberta Swine Research and Technology Centre at 1–2 days of age. Piglets were removed from the sow and immediately underwent surgical procedures to implant a gastric catheter for diet and isotope infusion and a femoral vein catheter for blood sampling. The femoral vein catheter was advanced to the vena cava to allow for the sampling of mixed venous blood. Preparative procedures, surgical procedures, postsurgical care, and piglet housing were as previously described (28, 30).

Diets and treatment groups. The complete elemental diet was designed to meet all nutrient requirements of piglets (22) and was continuously infused enterally via the gastric catheter by use of pressure-sensitive infusion pumps. Diet composition (28, 29), preparation (28, 29), and piglet adaptation to the enteral diet (28, 29) were all as previously described.

Piglets received the complete diet until the morning of day 3 and then were randomly assigned (n = 5/diet) to either a generous (+Arg: 1.8 g·kg⁻¹·day⁻¹) or a deficient (−Arg: 0.2 g·kg⁻¹·day⁻¹) intake of arginine for the remainder of the trial. The amino acid compositions of these diets were identical to the basal and +Arg diets that were recently described (29). These diets were chosen to represent diets that would result in minimal and maximal rates of arginine synthesis (31) without creating adverse metabolic effects (7). Diets were made isonitrogenous by adjusting the concentrations of alanine and glycine (29).

Blood sampling. Blood samples (2 ml) were collected every 24 h, beginning immediately prior to the allocation to test diets on day 3 and continuing until the end of the trial. The daily blood samples were used for the determination of plasma ammonia, urea nitrogen, and nitric oxide (Product DINO-250; Bio Assay Systems, Hayward, CA) concentrations were determined every 24 h during test diet infusion (day 3–day 7) using spectrophotometric assays.

For the plasma samples taken during the day 7 constant infusion, the isotopic enrichment of each of the infused isotopomers and its product amino acids were measured using liquid chromatography-tandem mass spectrometry. To prepare the samples, 25 μl of plasma was deproteinized by adding 500 μl of methanol, vortexed for 30 s, and then centrifuged at 9,000 g for 10 min at 37°C. The supernatant was dried under nitrogen at 37°C and then derivatized with 100 μl of butanol and 50 μl of 0.5% HCl. The derivatized samples were analyzed on an API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX) operated in positive ionization mode with a TurboIonSpray ionization probe source (operated at 5,800 V and 600°C), coupled to an Agilent 1100 HPLC system (Agilent Technologies, Canada, Canada) and a 4.6 mm × 150 mm C18 column (Hypersil ODS, 3 μm). The detection of unlabeled proline, ornithine, citrulline, and arginine were made by employing their characteristic transitions of butylated parent-to-daughter ions at m/z 172.2 to 70, 189.3 to 70, 232.3 to 70, and 231.3 to 70, respectively. Similar characteristic transitions were applied to their enriched isotope distributions. Prior to entering the triple quadrupole mass spectrometer, the individual amino acids were separated using a Dionex Acclaim organic acid column (5-μm 120A, 4.0 × 250 mm; Dionex Canada, Oakville ON, Canada) and were eluted over 15 min with a binary liquid chromatography gradient (0–30% acetonitrile containing 0.1% formic acid) at a flow rate of 700 μl/min. The retention times were 3.75, 5.93, 8.06, and 9.14 min for ornithine, arginine, citrulline, and proline, respectively.

Calculations. For each isotopeomer of each amino acid studied, the isotopic enrichment was determined using a previously described formula (32). For the infused and product isotopeomers, the isotopic plateau was defined as having a slope not different from zero and included at least four points for the infused isotopeomers and at least three points for the product isotopeomers.
The enrichment values were then used to calculate the whole body flux for each of the infused amino acids using the following formula (25):

\[
Q (\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}) = \text{isotope infusion rate} \times \left[ \frac{\text{enrichment}_{\text{isotope}}}{\text{enrichment}_{\text{plasma}}} - 1 \right]
\]

Enrichment values were also used to calculate the conversion rate of each infused amino acids to its product amino acids \((Q_{\text{precursor} \rightarrow \text{product}})\) using previously described formulas (25):

\[
Q_{\text{precursor} \rightarrow \text{product}} (\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}) = \left( \frac{\text{enrichment}_{\text{product amino acid}}}{\text{enrichment}_{\text{precursor amino acid}}} \right) \times Q_{\text{product amino acid}} \times \left( \frac{\text{isotope infusion rate}_{\text{precursor amino acid}}}{\text{isotope infusion rate}_{\text{precursor amino acid}}} + Q_{\text{precursor amino acid}} \right)
\]

The \(Q_{\text{arginine} \rightarrow \text{citrulline}}\) was used as a measure of nitric oxide synthesis, because for every mole of citrulline formed from arginine, a mole of nitric oxide was also formed. This approach to measuring nitric oxide synthesis has been used previously in both humans (8) and pigs (5). The portion of arginine flux that was used for nitric oxide synthesis was calculated using

\[
\%\text{arginine flux to NO} = \left( \frac{Q_{\text{arginine} \rightarrow \text{citrulline}}}{Q_{\text{arginine}}} \right) \times 100\%
\]

The relative importance of proline as an arginine precursor was calculated by comparing the \(Q_{\text{proline} \rightarrow \text{arginine}}\) with the \(Q_{\text{citrulline} \rightarrow \text{arginine}}\), using the following formula:

\[
\%\text{total endogenous arginine synthesis from proline} = \left( \frac{Q_{\text{proline} \rightarrow \text{arginine}}}{Q_{\text{citrulline} \rightarrow \text{arginine}}} \right) \times 100\%
\]

The amount of endogenously synthesized arginine that remained in the plasma as arginine was referred to as net arginine synthesis and was calculated by

\[
\text{net arginine synthesis (} \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}) = Q_{\text{citrulline} \rightarrow \text{arginine}} - Q_{\text{citrulline} \rightarrow \text{ornithine}}
\]

**Statistical analysis.** Unless specifically noted, all data were analyzed using the mixed model of SAS version 9.1 (SAS Institute, Cary, NC), and data were considered statistically significant if \(P < 0.05\), and trends were considered at \(0.05 < P < 0.10\). When the fixed effects were significant \((P < 0.05)\), least square means were compared using the pdiff test.

The dependent variables plasma ammonia, urea, and nitric oxide concentrations were analyzed using repeated-measures analysis where the fixed effect was diet and the random variables were piglet nested within diet and day. The Kenward-Roger option was used to estimate the denominator degrees of freedom. The variance-covariance matrix was chosen for each analysis on the basis of the lowest value for Schwarz’s Bayesian criterion. All remaining variables were analyzed using diet as the fixed effect and piglet nested within diet as the random variable.

**RESULTS**

**Piglet performance.** All piglets remained healthy and active for the duration of the 7-day trial. There were no differences in piglet weight at the initiation of test diet infusion (pooled mean: 1.88 kg, pooled SE: 0.05 kg). Piglets receiving the −Arg diet gained significantly more weight (0.82 kg) than piglets receiving the +Arg diet (0.64 kg, pooled SE: 0.04 kg, \(P < 0.01\)), resulting in a trend \((P = 0.06)\) for a higher body weight (kg) in the −Arg piglets at the end of the experiment (+Arg: 2.51, −Arg: 2.71, pooled SE: 0.07). A similar effect of arginine intake on body weight has been recently described in enterally fed neonatal piglets (31) and was attributed to an increase in extracellular fluid volume due to the dietary arginine deficiency and subsequent large increase in plasma glutamine concentrations, which has previously been associated with an increase in total body water gain (18). Although body composition was not measured in this study, the explanation offered by Wilkinson et al. (31) for differences in weight gain due to arginine intake seems to be a plausible explanation for the present findings: the −Arg piglets in the present study had a 507% higher plasma glutamine concentration than the +Arg piglets (Table 1), presumably due to the role of glutamine as an ammonia scavenger, as has been previously observed and discussed (29, 31). In the study of House et al. (18), piglets receiving 10% of their dietary amino acids as glutamine (plasma glutamine ~300 μmol/l), gained ~250 g more water than those piglets receiving a glutamine-free diet. This may explain the differences in final body mass and body weight gain between the two groups in the present study.

**Plasma amino acid concentrations.** Plasma arginine and ornithine concentrations were 968 and 221% greater, respectively, in the piglets receiving the +Arg than in piglets receiving the −Arg diet \((P < 0.05; \text{Table 1})\). With the exception of glutamine, the plasma concentrations of other amino acids related to arginine metabolism were not affected by arginine intake (Table 1).

**Plasma ammonia, urea, and nitrate + nitrite concentrations.** We (29) have recently discussed the use of plasma ammonia and urea concentrations as indicators of whole body arginine status, although a similar relationship between arginine status and plasma nitrate + nitrite (a measure of nitric oxide) concentrations has not been previously established. Diet \((P = 0.0003)\) and day \((P < 0.0001)\), but not their interaction \((P > 0.05)\), had a significant effect on plasma ammonia concentrations. From day 5 onward, piglets in the −Arg group had higher plasma ammonia concentrations than those in the +Arg group (Table 2). Plasma urea concentrations were affected by diet \((P = 0.002)\), day \((P = 0.003)\), and their interaction \((P = 0.03)\) (Table 2). Although the plasma urea concentration in the +Arg piglets remained unchanged throughout the 5-day period, the urea concentrations in the −Arg piglets increased until day 5 and then remained constant, and higher than in the +Arg piglets, for the remainder of the trial. Plasma nitrate + nitrite concentration remained unchanged regardless of diet, day, or the interaction (Table 2).

**Table 1. Day 7 plasma concentrations (μmol/l) of amino acids related to arginine metabolism in enterally fed neonatal piglets**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>+Arg</th>
<th>−Arg</th>
<th>Pooled SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>267</td>
<td>25</td>
<td>22*</td>
</tr>
<tr>
<td>Aspartate</td>
<td>9</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Citrulline</td>
<td>120</td>
<td>118</td>
<td>16</td>
</tr>
<tr>
<td>Glutamate</td>
<td>83</td>
<td>77</td>
<td>6</td>
</tr>
<tr>
<td>Glutamine</td>
<td>69</td>
<td>419</td>
<td>35*</td>
</tr>
<tr>
<td>Ornithine</td>
<td>132</td>
<td>41</td>
<td>6*</td>
</tr>
<tr>
<td>Proline</td>
<td>466</td>
<td>474</td>
<td>54</td>
</tr>
</tbody>
</table>

Values are least square means; \(n = 5\). *Denotes a significant dietary effect \((P < 0.05)\).

AJP-Endocrinol Metab • VOL 293 • SEPTEMBER 2007 • www.ajpendo.org
Plateau isotope enrichments. All infused isotopomers reached a stable isotopic plateau by at least 4.5 h into the infusion, and all product isotopomers reached a stable isotopic plateau by at least 5 h into the infusion. The mean enrichment and coefficient of variation (CV) for each isotopomer at plateau are given in Table 3. Piglets receiving the +Arg diet had significantly lower plasma [guanido-15N2]arginine, [guanido-13C; 5,5-2H2]arginine, [1,2,3,4,5-13C5]arginine, [1,2,3,4,5-13C5]citrulline, [15N,1,2,3,4,5-13C5]citrulline, enrichments and significantly greater plasma [ureido-13C;5,5-2H2]citrulline enrichments than those receiving the amino acid isotopomers examined (significantly lower plasma [guanido-15N2]arginine, [guanido-13C;5,5-2H2]arginine, [1,2,3,4,5-13C5]citrulline, [15N,1,2,3,4,5-13C5]citrulline, [15N;1,2,3,4,5-13C5]ornithine* 0.96 (6.3) 2.22 § (4.6) 0.07 (1.1) [15N;1,2,3,4,5-13C5]proline† 0.73 (6.2) 0.60 (11.4) 0.07 (4.3) [1,2,3,4,5-13C5]arginine† 2.27 (5.5) 4.88 § (4.8) 0.23 (1.4) [15N,1,2,3,4,5-13C5]arginine 119 114 12 [15N;U-13C5]proline† 0.76 (8.9) 1.46§ (9.5) 0.10 (1.8) [U-13C5]ornithine† 2.27 (5.5) 4.88§ (4.8) 0.23 (1.4) [15N;U-13C5]ornithine 142 112 17 [5-2H]proline† 0.15 (2.1) 0.12 (2.4) 0.05 (0.3) [U-13C5]proline† 0.73 (6.2) 0.60 (11.4) 0.07 (4.3) [15N;U-13C5]proline† 3.07 (3.7) 3.68 (5.5) 0.28 (2.1) Values are least square means; *n = 4 or *n = 5. Values in parentheses are the values for the CV of the plateau enrichment. §Significant dietary effect for mean enrichment (P < 0.05); †trend for significant dietary effect (0.05 < P < 0.10). There was no effect of diet on the CV for any of the amino acid isotopomer enrichments for these precursor and product amino acids.

Intragastric amino acid fluxes. Because the isotopes were administered intragastrically, the fluxes were influenced by both first-pass splanchnic metabolism and the metabolism by peripheral tissues. Arginine and ornithine fluxes were 130 and 137% greater (P < 0.05), respectively, in the piglets receiving the +Arg diet than in those receiving the −Arg diet (Table 4; see the schematic summary of the arginine, citrulline, ornithine, and proline kinetics in the supplemental figure in the online version of this article.). Neither proline flux nor citrulline flux was different between treatment groups (Table 4).

Arginine conversion to nitric oxide. The rate of arginine-to-citrulline conversion, a measure of nitric oxide synthesis,

Table 4. Whole body fluxes of the amino acids (μmol·kg("superscript-1")·h("superscript-1") and Qprecursor→product (μmol·kg("superscript-1")·h("superscript-1")) in enterally fed neonatal piglets after enteral tracer infusion

### Table 2. Plasma ammonia (μmol/l), urea (mmol/l), and nitrate + nitrite (μmol/l) concentrations in enterally fed neonatal piglets

<table>
<thead>
<tr>
<th>Day</th>
<th>+Arg</th>
<th>−Arg</th>
<th>Pooled SE</th>
<th>+Arg</th>
<th>−Arg</th>
<th>Pooled SE</th>
<th>+Arg</th>
<th>−Arg</th>
<th>Pooled SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>33</td>
<td>44</td>
<td>9</td>
<td>0.60</td>
<td>0.59*</td>
<td>0.25</td>
<td>61</td>
<td>69</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>46</td>
<td>9</td>
<td>0.90</td>
<td>1.59†</td>
<td>0.25</td>
<td>62</td>
<td>96</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>101b</td>
<td>86b</td>
<td>10</td>
<td>0.90</td>
<td>2.30*</td>
<td>0.25</td>
<td>62</td>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>120</td>
<td>99</td>
<td>10</td>
<td>1.06</td>
<td>2.85§</td>
<td>0.25</td>
<td>69</td>
<td>89</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>122</td>
<td>129</td>
<td>10</td>
<td>0.78</td>
<td>2.55‡</td>
<td>0.25</td>
<td>63</td>
<td>66</td>
<td>20</td>
</tr>
</tbody>
</table>

* Values are least square means; n = 5. *Significant dietary effect (P < 0.05). **Significant dietary effect (0.05 < P < 0.10). There was no effect of diet on the CV for any of the amino acid isotopomers examined.
was 228% greater in piglets receiving the +Arg vs. the −Arg diet (P < 0.05; Table 4). However, regardless of arginine intake, the portion of arginine flux converted to nitric oxide was not different (+Arg: 13.1%, −Arg: 12.7%, pooled SE: 2.2%; P > 0.05).

Arginine synthesis from precursor amino acids. Arginine synthesis from both proline and citrulline was greater in the piglets in the −Arg group than in those in the +Arg group (P < 0.05), but ornithine conversion to arginine was not affected by arginine intake (P > 0.05; Table 4). The portion of total endogenous arginine synthesis (Qcitrulline→arginine) that was accounted for by proline was similar for both diets (+Arg: 63%, −Arg: 56%, pooled SE: 5%, P > 0.05).

Net arginine synthesis. Piglets receiving the −Arg (41.1 μmol·kg⁻¹·h⁻¹) diet had a greater rate of net arginine synthesis than those receiving the +Arg diet (−3.5 μmol·kg⁻¹·h⁻¹, pooled SE: 9.1 μmol·kg⁻¹·h⁻¹, P < 0.05).

Rates of conversions between other amino acids. The rate of proline conversion to citrulline was greater in piglets receiving the −Arg diet (P < 0.05); these values were strikingly similar to the rates of proline conversion to arginine for both diets (Table 4). Piglets receiving the +Arg diet had a higher rate of ornithine conversion to proline than those receiving the −Arg diet (P < 0.05; Table 4). All other amino acid conversion rates were not affected by arginine intake (P > 0.05; Table 4).

**DISCUSSION**

The present study is unique because all isotopic measurements were made simultaneously and within each piglet; therefore, this study was an extremely comprehensive description of whole body arginine metabolism in neonatal piglets. Previously unanswered or ambiguous questions with regard to arginine metabolism in the neonate could therefore be answered. Using this isotope infusion methodology, this study confirmed that total endogenous arginine synthesis was greater when a deficient dietary intake of arginine was provided than when a generous intake was provided. Proline was the primary arginine precursor, and that the limitation in the rate of endogenous arginine synthesis from proline was citrulline formation. In addition, this study provides the first experimental evidence in neonates that arginine intake affects the rate of whole body nitric oxide synthesis.

**Total endogenous arginine synthesis in enterally fed piglets.** Although previous research showed that the rate of proline conversion to arginine was greater in piglets receiving an arginine-deficient diet than in those receiving a generous arginine diet (29, 31), this alone did not provide sufficient evidence to unequivocally conclude that whole body endogenous arginine synthesis was increased during arginine deficiency. In that previous research (29, 31), it was possible that the greater rate of proline-to-arginine conversion in the arginine-deficient piglets was offset by a decrease in the rate of conversion of other precursors to arginine. Therefore, it was necessary to measure the rate of total endogenous arginine synthesis, using the rate of citrulline-to-arginine conversion, to determine the true effect of arginine intake on arginine synthesis. Total endogenous arginine synthesis was greater in piglets receiving the −Arg diet than in those receiving the +Arg diet (Table 4). This is in clear contrast to both adult humans and rats, where there was no observed increase in the citrulline-to-arginine conversion in response to an arginine-free diet (9, 14). The regulation of arginine synthesis in adult mammals is clearly different than in neonatal mammals; therefore, the results of research on arginine metabolism in adults cannot be extrapolated to neonates.

In addition to having a greater rate of total endogenous arginine synthesis, piglets receiving the −Arg diet also had a greater rate of net arginine synthesis (41.1 μmol·kg⁻¹·h⁻¹) than the +Arg piglets (−3.5 μmol·kg⁻¹·h⁻¹). Net arginine synthesis is the amount of endogenously synthesized arginine that remained as arginine and was calculated by subtracting the rate of citrulline-to-ornithine conversion from the rate of total endogenous arginine synthesis. In piglets receiving the −Arg diet, two-thirds of the newly synthesized arginine was available for use in metabolic functions such as protein, creatine, polyamine, and nitric oxide synthesis. Piglets receiving the +Arg diet, on the other hand, had no net arginine synthesis. This clearly demonstrates for the first time that, although there is a basal rate of arginine synthesis when the metabolic arginine requirement (38) is being fully met through dietary intake, all of this newly synthesized arginine is converted to ornithine. The ornithine is largely destined for oxidation in piglets (30), as is the case in humans (12).

**Relative contribution of proline to total endogenous arginine synthesis.** Regardless of arginine intake, proline was the major precursor for arginine synthesis, with the proline-to-arginine conversion accounting for −60% of total endogenous arginine synthesis; therefore, other arginine precursors must account for the remaining 40% of endogenous arginine synthesis and need to be identified in vivo. In vitro enterocyte work in suckling piglets showed that, although the major precursor for arginine was proline (33, 37), glutamine addition to the incubation medium also increased the arginine content of the cell extracts (36, 37). In piglets receiving either a generous or a deficient arginine diet, there was no detectable radioactivity in arginine following an intragastric infusion of [3,4-³H]glutamate (31). Glutamate has shown to be extensively catabolized during first-pass splanchnic metabolism (24); therefore, only a very small portion (~5%) of the infused isotope may have reached the general circulation. Therefore, if circulating glutamate/glutaminase was a precursor for arginine synthesis, by either the intestine or other tissues, this contribution would not have been measurable using an intragastric tracer. The importance of circulating glutamine and glutamate for whole body arginine synthesis has not been measured in enterally fed neonatal piglets by use of intravenously infused isotopes, but an arteriovenous study in 2-wk-old suckling piglets found that there was a significant intestinal uptake of glutamine and release of arginine (34).

Another possibility for the source of the remaining 40% of endogenous arginine synthesis is the recycling of dietary arginine. Arginine was extensively converted to ornithine in enterally fed piglets, particularly in those receiving a generous amount of arginine (28), and some of this ornithine may have been recycled back to arginine. A study using rat liver perfusions and isolated hepatocytes revealed that extramitochondrial arginine was converted extensively to ornithine, presumably via mitochondrial arginase II, and subsequently citrulline, in a series of reactions that appeared to be fairly tightly channeled (23). Although the liver was not a site of endogenous arginine synthesis in piglets (28), arginase II is located in many tissues, including the kidney and intestine (19); therefore, the results
from the rat liver perfusion and hepatocyte study (23) likely apply to other tissues. Dietary arginine recycling may have been especially important in piglets receiving the +Arg diet where all newly synthesized arginine was shown to be converted to ornithine.

Isotopic evidence that citrulline formation limits endogenous arginine synthesis from proline. All of the citrulline that was formed from proline was converted to arginine, regardless of arginine intake (Table 4), providing strong, direct evidence to confirm that the formation of citrulline limits arginine synthesis in enterally fed piglets (29). Wu et al. (37) proposed that low enterocyte mitochondrial N-acetylglutamate concentrations, an essential cofactor of carbamoylphosphate synthetase I (EC no. 6.3.4.16), may limit citrulline formation in vitro (37). The results from the present study (Table 4), our previous study that investigated effective arginine precursors (29), and the piglet enterocyte work of Wu et al. (37) taken together provide compelling evidence that citrulline formation limits endogenous arginine synthesis in enterally fed piglets and raises the interesting question as to why N-acetylglutamate formation is inadequate to support this critical function in week-old piglets.

Metabolism of intermediates in the proline-to-arginine pathway. Only 27 and 60% of the ornithine formed from proline for the piglets receiving the +Arg and −Arg diets, respectively, was converted to citrulline and subsequently arginine (Table 4). Although proline conversion to ornithine was similar for both diets in the present study, 73 and 40% of this ornithine in piglets receiving the +Arg and −Arg diets, respectively (Table 4), was metabolized by other pathways: either oxidation to carbon dioxide via the citric acid cycle, conversion back to proline, conversion to other amino acids such as glutamate and glutamine, or the synthesis of polyamines. In the present study, piglets in the +Arg group had a rate of ornithine conversion to proline that was 227% greater than the rate in the −Arg group, and the equilibrium of the ornithine aminotransferase (EC no. 2.6.1.13) reaction was clearly shifted toward proline vs. ornithine formation in the +Arg group (Table 4). Ornithine oxidation was not measured in the present study; however, ornithine oxidation was proportional to ornithine flux in our previous study in piglets (30); therefore, the piglets in the +Arg group likely had a greater rate of ornithine oxidation than the piglets in the −Arg group. Together, these findings, in combination with the fact that there was a greater extent of metabolism of proline-derived ornithine to metabolites other than citrulline in the +Arg vs. −Arg piglets (Table 4), indicate that, when arginine intake is adequate or in excess of the requirements, ornithine is directed away from arginine synthesis and toward the formation of other metabolic products.

Arginine as a nitric oxide precursor. The rate of [ureido-15N]citrulline formation from [guanido-15N2]arginine has been used in mice (17), growing pigs (5), and humans (8) to measure nitric oxide synthesis. However, the present study is the first to use this isotopic technique in neonatal animals to study the effect of arginine intake on whole body nitric oxide synthesis. Piglets receiving the +Arg diet synthesized twice as much nitric oxide as piglets receiving the −Arg diet (Table 4). The results from the present study are in agreement with previous work in growing pigs recovering from endotoxemia, where pigs receiving an arginine supplement had a greater rate of nitric oxide synthesis than those receiving an isocaloric alanine supplement (6). However, other studies in healthy men (2, 13) and mice (17) did not find an effect of arginine intake (2, 13) or circulating arginine concentration (17) on nitric oxide synthesis. Therefore, nitric oxide synthesis appears to be sensitive to arginine intake or status only under certain physiological conditions (i.e., infancy or pathological states), but not in healthy adults.

In a previous study in enterally fed piglets, there was a statistical trend for higher plasma nitrate + nitrite (nitric oxide) concentrations in piglets receiving a generous vs. a deficient arginine diet (31); however, this was not confirmed in the present study (Table 2). Urinary nitrate excretion was not measured in this study; however, human infants with a greater rate of nitric oxide synthesis also had a greater amount of urinary nitrate excretion (8). Similarly, in rats, nitrate excretion increased with increasing arginine intake (35). Therefore, the piglets in the +Arg group, which had a greater rate of nitric oxide synthesis, may also have had a greater rate of nitrate excretion than the −Arg group; this could result in no net effect of arginine intake on plasma nitrate + nitrite concentrations.

Regardless of arginine intake, nitric oxide synthesis accounted for ~13% of whole body arginine flux in enterally fed neonatal piglets (Table 4). Both whole body arginine flux and nitric oxide synthesis were lower in piglets receiving the −Arg vs. the +Arg diet (Table 4), whereas the portion of arginine flux converted to nitric oxide was unaffected by diet; this provides clear and important evidence that nitric oxide synthesis was driven by arginine availability.

Nitric oxide synthesis accounted for 1.2% of plasma arginine flux in healthy adult humans (8), 0.15–0.47% of plasma arginine flux in neonates during and following PPHN (11), and 1.1 to 6.1% of arginine flux in mice (16, 17). In pigs recovering from endotoxemia, ~15% of arginine flux was used for nitric oxide synthesis, and this portion was independent of arginine intake (6). Therefore, a value of 13% of arginine flux used for nitric oxide synthesis in the piglets of the present study is substantially higher than most of the values obtained from previous studies. Endotoxemia results in a large increase in nitric oxide synthesis (5, 26), and this may explain why the pigs in the previous study (6) used a large portion of their arginine flux for nitric oxide production. However, the piglets in the present study were healthy, and therefore an alternate explanation for the high rate of nitric oxide synthesis is necessary.

An important distinction between the present and previous studies is that, in the previous studies examining nitric oxide synthesis (6, 8, 11, 13, 17), the arginine isotope was administered intravenously, whereas in the present study it was delivered intragastrically and therefore took into account the arginine that was converted to nitric oxide during first-pass splanchnic metabolism. In adult men, when a [guanido-15N2]arginine isotope was given either intravenously or intragastrically, the amount of 15NO3 excreted in the urine was approximately threefold greater when the isotope was given...
intra-gastrically (10), indicating that ∼75% of arginine conversion to nitric oxide may occur during first-pass splanchnic metabolism. This showed that the splanchnic region is an important site of nitric oxide synthesis from dietary arginine. If the arginine isotope in the present study had been administered intravenously, we believe that the portion of arginine flux accounted for by nitric oxide synthesis in the enterally fed piglets of the present study would have been similar to previous estimates in mice and humans (6, 8, 11, 13, 17). To verify that first-pass intestinal or splanchnic metabolism made a major contribution to whole body nitric oxide synthesis, additional research using intraportal or intravenous arginine infusions, to isolate the effects of first-pass intestinal and splanchnic metabolism, respectively, are required.

Although nitric oxide synthesis was not measured using an intravenously infused arginine isotope in the present study, in a previous study using identical diets and methods (28), an intravenously administered [4,5-3H]arginine isotope was used, and it was found that, in piglets receiving the +Arg and −Arg diets, respectively, 57 and 8% of the citrulline flux was derived from arginine. Using these fractional net conversions (28) and the citrulline fluxes from the present study (Table 4), the rate of arginine-to-citrulline conversion was 63 μmol·kg⁻¹·h⁻¹ (7.7% of arginine flux) in piglets receiving a generous arginine diet and 10 μmol·kg⁻¹·h⁻¹ (2.8% of arginine flux) in piglets receiving the deficient arginine diet. Because the isotope used was [4,5-3H]arginine, the conversion from arginine to citrulline was not equivalent to nitric oxide synthesis alone but is equivalent to citrulline derived from arginine metabolized by both the arginase and nitric oxide synthase (EC no. 1.14.13.39) pathways. The arginase pathway was likely responsible for a large portion of this citrulline formation, because the intravenous conversion of [4,5-3H]arginine to [4,5-3H]ornithine was 556 μmol·kg⁻¹·h⁻¹ for the +Arg diet and 141 μmol·kg⁻¹·h⁻¹ for the −Arg diet (28). This provides additional support for our proposal that if the [guanido-15N2]arginine isotope of the present study had been infused intravenously, then the portion of arginine flux accounted for by nitric oxide synthesis in our piglets would have been much closer to the portion measured in other healthy mammals (8, 16, 17).

Intestinal nitric oxide synthesis could be one explanation for the beneficial effects of supplemental arginine in human infants predisposed to or suffering from NEC and PPHN (1, 20). Our results also suggest that arginine supplements should be administered orally or intragastrically, particularly in the case of NEC, to have the greatest possible effect on nitric oxide synthesis.

Summary. Regardless of arginine intake, proline conversion to arginine accounted for ∼60% of endogenous arginine synthesis, and citrulline formation limited the rate of arginine synthesis from proline. Although piglets receiving the −Arg diet had a rate of endogenous arginine synthesis 79% greater than piglets receiving the +Arg diet, their rate of nitric oxide synthesis was only 44% of the rate in piglets receiving the +Arg diet. The unique multi-isotope methodology employed in the present study allowed us to simultaneously study many aspects of arginine metabolism in neonatal piglets and will be a useful experimental tool for future studies in both piglets and human neonates.

ACKNOWLEDGMENTS

K. Urschel’s current address: Middleburg Agricultural Research and Extension Center, Virginia Polytechnic Institute and State University, 5527 Sullivan’s Mill Road, Middleburg, VA 20117.

GRANTS

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada, Alberta Pork Producers Development Corporation, and the Canadian Institutes for Health Research (Fund FRN 12928). The amino acids were generously donated by Degussa AG, Hanau-Wolfgang, Germany. K. Urschel was supported by a Natural Sciences and Engineering Research Council of Canada PGSD Scholarship.

DISCLOSURE

R. Ball receives partial salary support from Alberta Pork.

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