Ornithine Restores Ureagenesis Capacity and Mitigates Hyperammonemia in Otc\textsuperscript{spf-ash} Mice\textsuperscript{1}

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ABSTRACT We showed that Otc\textsuperscript{spf-ash} mice, a model of ornithine transcarbamylase deficiency, were able to sustain ureagenesis at the same rate as control mice, despite reduced enzyme activity, when a complete mixture of amino acids was provided. An unbalanced amino acid mixture, however, resulted in reduced ureagenesis and hyperammonemia. To study the effect of ornithine supplementation [316 μmol/(kg h)] on urea and glutamine kinetics in conscious Otc\textsuperscript{spf-ash} mice under a glycine-alanine load [6.06 mmol/(kg h)], a multiple tracer infusion protocol ([15\textsuperscript{C}]urea, [5-15\textsuperscript{N}]glutamine, [2,3,3,4,4 \textsuperscript{D}]glutamine and [ring-\textsuperscript{D}\textsubscript{3}] phenylalanine) was conducted. Ornithine supplementation increased ureagenesis [3.18 ± 0.88 vs. 4.56 ± 0.51 mmol/(kg h), P < 0.001], reduced plasma ammonia concentration (1125 ± 62 vs. 193 ± 94 μmol/L, P < 0.001), and prevented acute hepatic enlargement (P < 0.006) in Otc\textsuperscript{spf-ash} mice. Ornithine supplementation also increased [95 ± 20 vs. 120 ± 16 μmol/(kg h), P < 0.001] the transfer of \textsuperscript{15}N from glutamine to urea, to values observed in the control mice [123 ± 17 μmol/(kg h)]. De novo amido-N glutamine flux was higher [1.57 ± 0.37 vs. 3.04 ± 0.86 mmol/(kg h); P < 0.001] in Otc\textsuperscript{spf-ash} mice, but ornithine supplementation had no effect (P < 0.56). The flux of glutamine carbon skeleton was affected by both genotype (P < 0.0001) and by ornithine (P = 0.036). In conclusion, ornithine supplementation restored ureagenesis, mitigated hyperammonemia, prevented liver enlargement, and normalized the transfer of \textsuperscript{15}N from glutamine to urea. These data strongly suggest that ornithine has the potential for the biochemical correction of OTCD in Otc\textsuperscript{spf-ash} mice.


KEY WORDS: • glutamine • spf-ash mouse • ornithine • OTCD • urea cycle disorders

Ornithine transcarbamylase deficiency (OTCD)\textsuperscript{4} is the most prevalent urea cycle disorder in humans, with an estimated incidence of 1:14,000 births (1). A mouse model of this deficiency, the sparse fur-abnormal skin and hair (Otc\textsuperscript{spf-ash}) mouse, was well-characterized at the gene (2), transcript (3), and protein levels (4). However, not until recently was urea production in this model for a urea cycle disorder studied in vivo (5). Otc\textsuperscript{spf-ash} mice are able to maintain a rate of ureagenesis comparable to that in control mice, despite only 5% residual enzyme activity (6), when a complete mixture of amino acids was infused, to impose a defined nitrogen load on the urea cycle (5). When an incomplete amino acid mixture was infused, however, Otc\textsuperscript{spf-ash} mice were unable to maintain ureagenesis and became hyperammonemic. The provision of urea cycle intermediates (UCI) was shown to prevent ammonia toxicity after a lethal dose of ammonia in rodents (7,8). Moreover, the normal response to a sudden nitrogen load is an increase in hepatic UCI, as shown in mice injected with ammonia; although liver ornithine increased within 5 min in control mice, this did not occur in Otc\textsuperscript{spf-ash} mice (9). The provision of these UCI seems to be of extrahepatic origin, and the inability of mutant mice to provide the liver with these metabolites might be a major factor in limiting ureogenic capacity, in addition to reduced activity of hepatic OTCD. For example, work in transgenic mice showed that restoring intestinal OTCD activity, and thus the endogenous supply of UCI, was enough to increase ureagenesis (10,11). The addition of ornithine was shown to increase urea production by isolated perfused liver preparations in both mutant and control mice (12). Furthermore, the inhibition of ornithine aminotransferase, which results in an increase in the concentration of ornithine in all tissues, was shown to protect against acute ammonia intoxication after an ammonia challenge (13).

In the present experiment, we employed minimally invasive techniques and a multiple tracer approach to test the hypothesis that ornithine, an intermediate of the urea cycle, restores ureagenesis and prevents hyperammonemia in conscious Otc\textsuperscript{spf-ash} mice under a defined nitrogen load.
MATERIALS AND METHODS

Animals and treatments. The experiments were performed on B6EiC3Sn a/A-Otc<sup>−/−</sup>/ mice originally obtained from The Jackson Laboratory. Mice were housed in a specific pathogen-free facility, caged in pairs (littermate control and Otc<sup>−/−</sup>), and had access to a 19.9% crude protein autoclaved pelleted feed (Harlan Teklad LM-485 Autoclavable Rat/Mouse Diet). Dietary proximate analysis was as follows: protein (199 g/kg), gross energy (16.9 MJ/kg), fat (57 g/kg), fiber (44 g/kg), and ash (65 g/kg). Autoclaved reverse osmosis water was available at all times. Mice were under a 12-h light cycle (0600 to 1800) in a temperature- (22 ± 2°C) and humidity- (55 ± 5%) controlled environment. All animal procedures were authorized by the University of Illinois Institutional Animal Care and Use Committee.

At 0630 on the day of the infusion, feed was removed and the mice were transferred to a new cage with paper towel bedding. Mice (age = 43.7 ± 2.34 d, mean ± SD) were weighed at 0930 and infusions started at 1030. The lateral tail vein catheterization procedure was described in detail elsewhere (14). In brief, intravascular catheters were made of polytetrafluoroethylene (0.15-mm i.d., 0.30-mm o.d., SUBL-120 Braintree Scientific) and silastic tubing (0.30-mm i.d., 0.64-mm o.d., Dow Corning). After the tail was warmed in warm water, a 27-gauge needle was inserted into the lateral tail vein. The needle was removed and the catheter introduced through the puncture and secured to the tail with cyanoacrylate glue. Mice were restrained by adhesive tape across the base of the tail during the infusion.

The control groups (GA; n = 9 paired Otc<sup>−/−</sup> and wild-type littersmates; age = 43.4 ± 2.3 d) were administered a continuous infusion of an equimolar glycine-alanine mixture (Sigma-Aldrich) at a rate of 6.06 mmol/(kg h), resulting in a nitrogen load of 85 mg N/(kg h). The ornithine-supplemented group (GAO; n = 9 paired littersmates; age = 43.9 ± 2.4 d) was additionally infused with ornithine [316 μmol/(kg h)]. The infusates also contained 25,000 IU/L of soybean haptarin (LEO Pharma) plus the tracers described below.

At ~4 h after feed removal, a priming dose of [13C<sub>15</sub>NO<sub>2</sub>]urea (60 μmol/kg), [15<sup>N</sup>-L]glutamine (250 μmol/kg), [2,3,3,4,4<sup>D</sup>]<sup>2</sup>L-glutamine (125 μmol/kg) and [ring-D<sub>3</sub>]<sup>2</sup>L-phenylalanine (45 μmol/kg) was given to the mice. The priming dose was followed immediately by a continuous infusion of [15<sup>N</sup>-L]urea ([90 μmol/(kg h)], [15<sup>N</sup>-L]glutamine [250 μmol/(kg h)], [2,3,3,4,4<sup>D</sup>]<sup>2</sup>L-glutamine [125 μmol/(kg h)] and [ring-D<sub>3</sub>]<sup>2</sup>L-phenylalanine [45 μmol/(kg h)]. After a 4-h infusion, mice were killed by decapitation and blood collected. The liver was excised immediately, the gall bladder removed, and liver weight recorded. Plasma was obtained after centrifugation at 1500 × g for 15 min at 4°C; ammonia was measured in fresh plasma; the remainder of the plasma was frozen at −20°C until analysis. Additionally, blood samples were collected and liver weight recorded from control (n = 9) and Otc<sup>−/−</sup> mice (n = 8) after 8 h of food deprivation to establish background and reference values.

Preliminary continuous infusions of [2,3,3,4,4<sup>D</sup>]<sup>2</sup>L-glutamine [250 μmol/(kg h)] and [ring-D<sub>3</sub>]<sup>2</sup>L-phenylalanine [45 μmol/(kg h)] were conducted in 6 control mice, together with glycine and alanine as described for the experimental infusions. Muscle (gastrocnemius) and plasma enrichments were determined, and isotopic equilibration between these two tissues was considered to indicate that plateau enrichment had been achieved (15).

Sample analysis. Plasma urea isotopic enrichment was determined by electron impact GCMS after the urea was derivatized to the glycine amide group and carbon skeleton, and phenylalanine entry rate was calculated from the isotopic dilution of the infused tracer at plateau enrichment, as

\[ MER = R \cdot \left( \frac{100}{E} - 1 \right), \]

where ER is the plasma entry rate (flux) of the metabolite M [mmol/(kg h)], R is the infusion rate [mmol/(kg h)] and E is the enrichment of M at plateau (mpe). Corrections were made to include the contribution of 13<sup>N</sup> urea to the urea ER.

The rate of protein catabolism was calculated by assuming a protein content of 4.3 g phenylalanine/100 g protein (17). Phenylalanine ER (PER) was also used to calculate glutamine de novo fluxes, assuming a protein content of 6.95 g glutamine/100 g protein (15) as follows:

\[ de_{novo,GER} = \text{GER} - \text{PER} \times (6.95/146)/(4.3/165), \]

where the de novo glutamine entry rate (GER; amido group or carbon skeleton) is the measured entry rate minus the contribution of proteolysis. The glutamine and phenylalanine content of whole-body protein (6.95 and 4.3 g/100 g protein) as well as their molecular weights (146 and 165) are considered in the second term of the equation.

Recycling of the glutamine amido group was calculated by difference between the carbon skeleton and the amido group de novo fluxes.

Data analysis. The experimental design was a completely randomized design with a 2 × 2 factorial arrangement of treatments. Data were analyzed using the proc mixed procedure of SAS (v. 9.1, SAS Institute). Fixed effects were genotype (control or Otc<sup>−/−</sup>), infusion treatment, and their interaction. Litter was the random effect of the model. If a significant interaction was obtained (P ≤ 0.05), the post hoc Tukey procedure for multiple pairwise comparisons was also applied.

RESULTS

The urea entry rate, as well as the transfer of 15<sup>N</sup> from infused [5,15<sup>N</sup>-L]glutamine to urea, increased (P < 0.001; Table 1) in Otc<sup>−/−</sup> mice administered ornithine supplementation, reaching values comparable to those in the control mice [4.56 vs. 4.44 mmol/(kg h) and 121 vs. 123 mmol/(kg h), respectively]. A smaller percentage of the infused 15<sup>N</sup> was incorporated into urea in the Otc<sup>−/−</sup> mice administered the GA treatment [42 vs. 52.5%, P < 0.001]. PUN did not differ between the control mice and the ornithine-supplemented Otc<sup>−/−</sup> mice; unsupplemented mutant mice, however, had lower PUN (Table 2). Plasma ammonia concentration in noninfused mice did not differ between controls and Otc<sup>−/−</sup> mice (121 and 89 μmol/L; P = 0.20). However, plasma ammonia concentration in infused mice was affected (P < 0.001) by genotype, treatment, and the interaction between genotype and treatment (Table 2). Plasma ammonia concentration doubled as result of the ornithine-supplemented infusion in Otc<sup>−/−</sup> mice, but was increased 12-fold in the unsupplemented mutants. After the 4-h infusion protocol, 2 unsupplemented mice Otc<sup>−/−</sup> mice were lethargic and had a reduction in body temperature. The plasma ammonia for these 2 mice was >2000 μmol/L. No increase in ammonia was detected in control mice in either treatment (P = 0.76).

Liver weight, as a proportion of body weight, was higher (P < 0.02) in noninfused Otc<sup>−/−</sup> mice than in controls (P = 0.47 vs.

ORNYTHINE AND UREAGENESIS IN Otc<sup>−/−</sup> MICE
TABLE 1

Urea entry rate and $^{15}$N transfer from [5,$^{15}$N] glutamine to urea in control and Otc$^{spf-ash}$ mice under a nitrogen load [86 mg N/(kg h)] and ornithine supplementation [316 $\mu$mol/(kg h)]

<table>
<thead>
<tr>
<th>Item</th>
<th>GA$^2$</th>
<th>GA$^3$</th>
<th>SEM</th>
<th>Genotype</th>
<th>TRT</th>
<th>Gen × TRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>Wild</td>
<td>Otc$^{spf-ash}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21.4</td>
<td>18.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea enrichment, mpe</td>
<td>2.13</td>
<td>3.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UER, mmol/(kg h)</td>
<td>4.30</td>
<td>3.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{15}$N-urea, mmol/(kg h)</td>
<td>131$^a$</td>
<td>95$^b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gin:Urea, %</td>
<td>56.9$^a$</td>
<td>42.0$^b$</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

1 Values are least-square means, n = 9. Means in a row with superscripts without a common letter differ, P < 0.05.
2 GA = equimolar solution of glycine and alanine [86 mg N/(kg h)].
3 GAO = equimolar solution of glycine and alanine [86 mg N/(kg h)] plus ornithine supplementation [316 $\mu$mol/(kg h)].

4.90%, respectively). In addition to reducing plasma ammonia concentration, ornithine supplementation protected (P = 0.14) the acute hepatic enlargement (~13%) that occurred in infused Otc$^{spf-ash}$ mice compared with noninfused mutant mice.

Results from the preliminary infusion of [2H$_2$] glutamine and [1H$_3$] phenylalanine showed that the unprimed infusion reached isotopic equilibrium by 2 h, without further increase (P > 0.37) in the enrichment ratio between muscle and plasma. The enrichment ratio between muscle and plasma was 0.82 and 0.88, for glutamine and phenylalanine, respectively.

A significant effect of treatment (P < 0.003) and a trend for the interaction (P = 0.06) were detected for the flux of phenylalanine. Unsupplemented Otc$^{spf-ash}$ mice had a lower phenylalanine flux (P < 0.02) than control and ornithine supplemented mutant mice (Table 3). This translated into lower protein degradation in unsupplemented Otc$^{spf-ash}$ mice (Table 3).

Control mice had lower de novo amido-glutamine flux than Otc$^{spf-ash}$ mice (P < 0.001; Table 3), but there was no effect (P = 0.56) due to ornithine supplementation. The de novo total flux of the glutamine carbon skeleton, however, tended (P = 0.07) to increase further in ornithine-supplemented Otc$^{spf-ash}$ mice. Supplemented Otc$^{spf-ash}$ mice had greater (P = 0.04) de novo total flux of the glutamine carbon skeleton than unsupplemented Otc$^{spf-ash}$ mice. The carbon skeleton glutamine flux was greater (P < 0.001) than the amido-glutamine flux, but the 2 were correlated (r = 0.95, P < 0.001). The ratio of these 2 fluxes was normalized in ornithine-supplemented Otc$^{spf-ash}$ mice (P = 0.14). A higher recycling of the glutamine amido group was observed in ornithine-supplemented Otc$^{spf-ash}$ mice (P < 0.01) than in controls and unsupplemented mutant mice (Table 3). Plasma phenylalanine and ornithine concentrations increased (P < 0.001) in ornithine-supplemented mice. Glutamine concentration, however, increased only in ornithine-supplemented Otc$^{spf-ash}$ mice (Table 2).

DISCUSSION

The single point mutation that is responsible for the Otc$^{spf-ash}$ mutation results in 5–10% vestigial enzyme activity in liver (6) and small intestine (4). The ornithine transcarbamylase present, however, is indistinguishable from the wild type (18). We showed previously that Otc$^{spf-ash}$ mice were able to maintain ureagenesis despite the reduced enzyme activity present. However, this was dependent on the amino acid mixture infused (5). Previous research showed that UCI increased after an NH$_4$Cl injection in wild-type mice, but not in Otc$^{spf-ash}$ mice (9,12). The ability of exogenous urea cycle intermediates to prevent ammonia toxicity after a lethal dose of

TABLE 2

Liver weight, as a percentage of body weight, and plasma ammonia, glutamine, phenylalanine, and ornithine concentrations in control and Otc$^{spf-ash}$ mice under a nitrogen load [86 mg N/(kg h)] and ornithine supplementation [316 $\mu$mol/(kg h)]

<table>
<thead>
<tr>
<th>Item</th>
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<th>SEM</th>
<th>Genotype</th>
<th>TRT</th>
<th>Gen × TRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea, mmol/L</td>
<td>9.2$^a$</td>
<td>5.3$^b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonia, mmol/L</td>
<td>118$^b$</td>
<td>1125$^a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gin, mmol/L</td>
<td>1108$^b$</td>
<td>773$^a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe, mmol/L</td>
<td>106.6$^a$</td>
<td>65.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orn, mmol/L</td>
<td>52$^a$</td>
<td>90$^b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver, %</td>
<td>4.9$^c$</td>
<td>6.2$^d$</td>
<td></td>
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</tr>
</tbody>
</table>

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3 GAO = equimolar solution of glycine and alanine [86 mg N/(kg h)] plus ornithine supplementation [316 $\mu$mol/(kg h)].
NH₄Cl was shown not only in mice (8), but also in other species (7). UCL were shown to increase urea synthesis rate and reduce orotic production in isolated hepatocytes (19) and isolated perfused liver (12) from mutant Otc⁽⁰⁻⁻⁾ mice. In the present experiment, ornithine infusion clearly restored ureagenesis and plasma urea concentration and mitigated hyperammonemia. However, plasma ammonia concentration was still increased in ornithine-supplemented Otc⁽⁰⁻⁻⁾ mice, compared with non-infused mice, whereas no increase was seen in control mice, regardless of ornithine supplementation.

The chronic liver enlargement (~11%) observed in Otc⁽⁰⁻⁻⁾ mice is consistent with previous reports in human patients (20,21). In addition, un-supplemented Otc⁽⁰⁻⁻⁾ mice exhibited an acute liver enlargement (P < 0.01).

The preliminary infusions of labeled glutamine and phenylalanine revealed that isotopic equilibration between plasma and muscle was reached after 2 h of continuous infusion. This contrast with data in humans in which no isotopic equilibration of glutamine was reached even after 11 h of continuous infusion, possibly due to the large intramuscular glutamine pool present in humans (15). Glutamine turnover measured under these conditions does not represent whole-body glutamine turnover but rather the interorgan flux of glutamine (15). The values obtained in the present experiment, 0.82 for glutamine and 0.88 for phenylalanine, reflect the intracellular dilution of the tracer by muscle protein breakdown.

The reduction in the phenylalanine flux in un-supplemented mutant mice indicated lower protein catabolism. This might have been caused by the high ammonia concentrations observed. Ammonia was shown to cause an osmotic influx of water into the lysosomes and inhibition of lysosomal cathepsins due to increase in pH, which results in a reduction in protein degradation in liver and muscle (22).

The carbon skeleton of glutamine turned over faster than in the amido group in mice; however, in un-supplemented Otc⁽⁰⁻⁻⁾ mice, the carbon flux skeleton was only 24% greater than the amido group flux, whereas in both ornithine-supplemented Otc⁽⁰⁻⁻⁾ mice and control mice, it was 50–65%. Glutamine flux derived from multiple labels previously demonstrated the different behavior between the carbon skeleton and the amido group (23). Minimal recycling of the carbon skeleton of glutamine occurs after deamination, thus yielding larger fluxes than the amido group (15). Further, the minimal recycling of the label into the amido group [(15), unpublished observations] indicates recycling of the amide nitrogen label released by the action of glutaminase back to glutamine.

In conclusion, ornithine supplemented Otc⁽⁰⁻⁻⁾ mice were able to dispose of a nitrogen load of amino acids by increasing ureagenesis and by recycling the amido group of glutamine, which together with the expansion of the glutamine pool, mitigated hyperammonemia. The expansion of the glutamine pool is an early signal of the failure of the urea cycle to detoxify ammonia; as a result, plasma ammonia and glutamine concentrations are highly correlated in human OTCD patients (24). The absence of hyperglutaminemia in un-supplemented Otc⁽⁰⁻⁻⁾ mice, the reduction in protein degradation, the acute hepatic enlargement, together with the high concentrations of plasma ammonia and hyperammonemic symptomatology in 2 mice, indicated not only decompensation, but also that Otc⁽⁰⁻⁻⁾ mice were being pushed beyond their metabolic flexibility. Ornithine supplementation was able to prevent the associated symptomatology by restoring ureagenesis, and thus offers the potential for the biochemical correction of OTCD in Otc⁽⁰⁻⁻⁾ mice.

**LITERATURE CITED**


