Interaction between murine spf-ash mutation and genetic background yields different metabolic phenotypes

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Submitted 13 August 2007; accepted in final form 8 October 2007

Marini JC, Erez A, Castillo L, Lee B. Interaction between murine spf-ash mutation and genetic background yields different metabolic phenotypes. Am J Physiol Endocrinol Metab 293:E1764–E1771, 2007. First published October 9, 2007; doi:10.1152/ajpendo.00525.2007.—The spf-ash mutation in mice results in reduced hepatic and intestinal ornithine transcarbamylase. However, a reduction in enzyme activity only translates in reduced ureagenesis and hyperammonemia when an unbalanced nitrogen load is imposed. Six-week-old wild-type control and spf-ash mutant male mice from different genetic backgrounds (B6 and ICR) were infused intravenously with [13C14N]urea, [15N2]arginine, [1-5,5-D2]ornithine, [1,6-13C]4,4,5,5-D4citrulline, and [1-ring-D5]phenylalanine to investigate the interaction between genetic background and spf-ash mutation on ureagenesis, arginine metabolism, and nitric oxide production. ICR(spf-ash) mice maintained ureagenesis (5.5 ± 0.3 mmol·kg−1·h−1) and developed mild hyperammonemia (145 ± 19 μmol/l) when an unbalanced nitrogen load was imposed; however, B6(spf-ash) mice became hyperammonemic (671 ± 15 μmol/l) due to compromised ureagenesis (3.4 ± 0.1 mmol·kg−1·h−1). Ornithine supplementation restored ureagenesis and mitigated hyperammonemia. A reduction in citrulline entry rate was observed due to the mutation in both genetic backgrounds (wild-type: 128, spf-ash: 60; SE 4.0 μmol·kg−1·h−1). Arginine entry rate was only reduced in B6(spf-ash) mice (B6(spf-ash): 332, ICR(spf-ash): 453; SE 20.6 μmol·kg−1·h−1). Genetic background and mutation had an effect on nitric oxide production (B6: 3.4, B6(spf-ash): 2.8, ICR: 9.0, ICR(spf-ash): 4.6, SE 0.7 μmol·kg−1·h−1). Protein breakdown was the main source of arginine during the postabsorptive state and was higher in ICR(spf-ash) than in B6(spf-ash) mice (phenylalanine entry rate 479 and 327, respectively; SE 18 μmol·kg−1·h−1). Our results highlight the importance of the interaction between mutation and genetic background on ureagenesis, arginine metabolism, and nitric oxide production. These observations help explain the wide phenotypic variation of ornithine transcarbamylase deficiency in the human population.

arginine; nitric oxide; urea cycle

ORNITHINE TRANSCARBAMYLASE (OTC) deficiency is the most common urea cycle disorder in humans (3). The reduction in ureagenesis capacity results in high plasma ammonia concentrations and frequent hyperammonemnic crises that can result in coma and even death (1). OTC is also expressed in enterocytes where it functions in the synthesis of citrulline, which is then exported into the blood, serving as the precursor for arginine synthesis by the kidney (7). The metabolism of arginine (Fig. 1) is highly compartmentalized, not only involving different organs, but different intracellular compartments and subcompartments, such as mitochondria, caveolae, and cytosol (8, 33). Furthermore, different isoforms of some of the enzymes involved [e.g., arginase, nitric oxide (NO) synthase] exist or different tissue-specific subcellular localization of the same isoform may occur (6). This compartmentalization of arginine metabolism reflects a complex regulation due to the multiple functions of this amino acid. It functions as a precursor for NO, creatine, and agmatine synthesis, in addition to its role in ureagenesis and protein synthesis. Thus, the reduction in OTC activity may not only reduce ureagenesis, but also impair the functional role of arginine in OTC-deficient patients thereby compromising NO synthesis.

Moreover, these complex biochemical and cellular interactions offer opportunities on a genetic level to modify the metabolic consequences of specific mutations. The ultimate effects on metabolite flux contribute to variable expressivity and susceptibility (15). Metabolic analysis of mutations on cogenic mouse strains offers a powerful approach to identify these genetic determinants as well as the associated range of phenotypic expression.

We showed previously that ornithine supplementation was able to restore ureagenesis and mitigate hyperammonemia in a hypomorphic mouse model for OTC deficiency (OTC(spf-ash)) (21). Thus, it seems that increasing the supply of ornithine was able to compensate for the reduced enzyme activity and maintain ureagenesis. Because the de novo production of ornithine is not affected by the reduction in OTC activity caused by the spf-ash mutation, we hypothesized that mouse genetic background might interact with the mutation to produce different phenotypical presentations of the disorder. Thus, the present study was designed to investigate whether the spf-ash mutation interacts with the genetic background of mice to affect the metabolic phenotype of the disorder in relationship to ureagenesis, arginine metabolism, and NO production.

MATERIALS AND METHODS

Animals and Treatments

B6EiC3Sn a/A-Ot(spf-ash)J mice (B6(spf-ash)) were originally obtained from The Jackson Laboratory (Bar Harbor, ME). The mutation was bred into ICR mice (ICR(spf-ash)) and backcrossed for at least 10 generations. Mice were housed in a SPF facility and had access to a 20% crude protein autoclaved pelleted feed (LabDiet, PicoLab Rodent Diet 20). Dietary proximate analysis was as follows: protein (200 g/kg), gross energy (16.9 MJ/kg), fat (45 g/kg), fiber (60 g/kg), and ash (70 g/kg). Autoclaved reverse osmosis water was available at all times. Mice were under a 12-h light cycle (0600 to 1800) in a 5%)-controlled environment. All animal procedures were authorized by the Baylor College of Medicine Institutional Animal Care and Use Committee.

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Ornithine titration. To determine the supplementation rate of ornithine needed to restore ureagenesis and reduce hyperammonemia in *spf-ash* mutant mice of two different backgrounds, 6-wk-old mice were utilized in a completed randomized experimental design. At 0700 the day of the infusion, feed was removed and mice were transferred to a new cage with paper towel bedding. Mice were weighed at 0930 and infusions started at 1030. The lateral tail vein catheterization procedure has been described in detail elsewhere (20). Mice were restrained by adhesive tape across the base of the tail during the infusion.

Mice were continuously infused for 4 h with an equimolar glycine-alanine mixture (Sigma, St. Louis, MO) at a rate of 6.06 mmol·kg⁻¹·h⁻¹, resulting in a nitrogen load of 85 mg N·kg⁻¹·h⁻¹. We previously showed that mutant B6*spf-ash* mice developed hyperammonemia and a reduced rate of ureagenesis with this protocol (21); however, there were no differences between control wild-type and mutant *spf-ash* mice if the nitrogen load was provided by a complete mixture of amino acids (22). Mutant mice (B6*spf-ash* and ICR*spf-ash*, 5 per group) were also infused with increasing rates of ornithine (0, 80, 160, 240, and 320 µmol·kg⁻¹·h⁻¹). Wild-type control mice (B6 and ICR, 5 per group) were also infused with the glycine-alanine mixture but received no ornithine supplementation and served as controls. A priming dose of [1³C₁⁸O]urea (60 µmol/kg) followed immediately by a continuous infusion of [1³C₁⁸O]urea (90 µmol·kg⁻¹·h⁻¹) was conducted to determine the entry rate of urea. Preliminary work showed that urea isotopic plateau enrichment is reached within 1 h utilizing this infusion protocol (19). After a 4-h infusion, mice were euthanized by decapitation and blood was collected. Because of the concern of incomplete mixing of the tracer in blood, which would lead to a higher enrichment when blood is collected by decapitation, a short interval (15–20 s) between the end of the infusion and decapitation was allowed. Plasma was obtained after centrifugation at 1,500 g for 15 min at 4°C; ammonia was measured in fresh plasma, and the rest of the plasma was frozen at −20°C until analysis.

Arginine metabolism and NO production. To study the effect of genetic background (B6 or ICR), mutation (wild-type or *spf-ash* mutant), and nitrogen load (saline or alanine-glycine at a rate of 6.06 mmol·kg⁻¹·h⁻¹) on arginine metabolism and NO production, 6-wk-old mice (10 per group) were utilized in a 2 × 2 × 2 factorial arrangement of treatments. A schedule similar to the one outlined previously was followed. Approximately 3 h after feed removal, a priming dose of [1³C₁⁸O]urea (66 µmol/kg), l-[¹⁵N₂]arginine (23.2 µmol/kg), l-[5,5 D₂]ornithine (7.7 µmol/kg), l-[ureido-¹³C, 4,4,5,5,D₄]citrulline (7 µmol/kg), and l-[ring-D₃]phenylalanine (10 µmol/kg) was given to the mice. The priming dose was followed immediately by a continuous infusion of [1³C₁⁸O]urea (100 µmol·kg⁻¹·h⁻¹), l-[guanido-¹⁵N₂] arginine (23.2 µmol·kg⁻¹·h⁻¹), l-[5,5 D₂]ornithine (7.7 µmol·kg⁻¹·h⁻¹), l-[ureido-¹³C,4,4,5,5,D₄] citrulline (7 µmol·kg⁻¹·h⁻¹), and l-[ring-D₃]phenylalanine (10 µmol·kg⁻¹·h⁻¹) for 4 h. Our preliminary studies and published literature (10) showed that isotopic steady state is reached for these amino acids within 30 min of a primed continuous infusion. At the end of the infusion, mice were euthanized by decapitation and blood, small intestine, and liver were collected. Tissue samples were immediately frozen in liquid nitrogen. Plasma and tissue samples were stored at −80°C. Plasma samples were deproteinized and derivatized within 10 days of collection.

Sample Analysis

Plasma urea isotopic enrichment was determined by EI GCMS after the urea was derivatized to the tert-Butyldimethylsilyl (t-BDMS) derivative. Plasma (20 µl) protein was precipitated with ice-cold acetone (100 µl) and the supernatant containing the urea was obtained after centrifugation at 1,500 g for 15 min at 4°C. The supernatant was evaporated under a gentle stream of nitrogen gas at 80°C and the isotope ratio was determined by NCI GCMS monitoring m/z ions 546, 548, and 550.

Plasma citrulline, arginine, and phenylalanine enrichments were determined as their dansyl derivatives by LCMS utilizing a TQS Quantum Ultra System (Thermo Finnigan, San Jose, CA) and monitoring m/z ions 231, 232, and 234.

For the determination of ornithine enrichments, 5 µl of plasma were deproteinized with ice-cold sulfosalicylic acid (10% wt/vol solution), centrifuged, and the supernatant was passed over a cation exchange column (Dowex AG 50W-8X, 100–200 mesh H⁺ form; Bio-Rad Laboratories, Richmond, CA). Ornithine was eluted with 2 mol/kg L-[5,5-D₂]ornithine and the isotope ratio was determined by NCI GCMS monitoring m/z ions 546, 548, and 550.

Plasma citrulline, arginine, and phenylalanine enrichments were determined as their dansyl derivatives by LCMS utilizing a TQS Quantum Ultra System (Thermo Finnigan, San Jose, CA) and monitoring m/z ions 408, 409, 410, and 413 for arginine, 409, 410, 411, and 414 for citrulline, and 399 and 404 for phenylalanine.

Ammonia was determined in fresh plasma samples, by reductive amination of 2-oxoglutarate and oxidation of NADPH, employing a commercial kit (Sigma).

Tissue samples were homogenized in lysis buffer (0.0625 M Tris-HCl, pH 7.4, 2% SDS) for 1 min, followed by incubation at 95°C for 30 min. The protein concentration of the lysate was measured with Micro BCA reagent (Pierce, Rockford, IL). After treatment with 5% β-mecaptopethanol, 10 µg liver protein extracts were loaded for Western blot assay. Total protein was fractionated on a 12% polyacrylamide gel (ISC BioExpress, Kaysville, UT), transferred to Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA), probed with a nonspecific polyclonal antibody against HOC (provided by Mendel Tuchman, Children’s National Medical Center, Washington, DC) and immunoreactive OTC was detected by enhanced chemiluminescence.
(Amersham Biosciences, Piscataway, NJ) (23). The density of each band was normalized utilizing α-tubulin (Sigma 1:10,000) and quantified using an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA).

Calculations

The entry rate of urea, ornithine, arginine, citrulline, and phenylalanine was calculated from the isotopic dilution of the infused tracer at plateau enrichment, as

\[ R_{M} = i_{M} \times \frac{100}{E_{M}} \]

where \( R_{M} \) is the plasma entry rate (flux) of the metabolite \( M \) (μmol·kg\(^{-1}\)·h\(^{-1}\)), \( i_{M} \) is the infusion rate (μmol·kg\(^{-1}\)·h\(^{-1}\)) and \( E_{M} \) is the enrichment of \( M \) at plateau (nape).

The rate of conversion of arginine to citrulline (\( R_{Citrulline} \)), citrulline to arginine (\( R_{Arginine} \)), and ornithine to citrulline (\( R_{Citrulline} \)) were determined as follows:

\[ R_{Citrulline} = R_{Citrulline}  \times \frac{E_{Citrulline}}{E_{Arginine}} \times \frac{E_{Arginine}}{E_{Citrulline}} = i_{Citrulline} + R_{Citrulline} \]

where \( R_{Citrulline} \) and \( R_{Arginine} \) are the plasma fluxes of the precursor and product, respectively, determined from the steady-state enrichments of the infused tracers [%\(^{15}\)N\(_{2}\)arginine, [5,5 D\(_{2}\)]ornithine, [13C,4,4,5,5,D\(_{4}\)]citrulline]; \( E_{Citrulline} \) and \( E_{Arginine} \), the respective plasma enrichments of the precursors and products (%\(^{15}\)N\(_{2}\)arginine/\(^{15}\)N\(_{2}\)citrulline, [13C,4,4,5,5,D\(_{4}\)]arginine/\(^{15}\)N\(_{2}\)citrulline, [5,5 D\(_{2}\)]ornithine/\(^{15}\)N\(_{2}\)citrulline, and [5,5,4,4,D\(_{4}\)]citrulline) and \( i_{X} \) is the rate of infusion of the labeled precursor. Because the conversion of arginine to ornithine implies the loss of the guanido group, and hence the isotopic label, the conversion of arginine to ornithine was estimated by the ratio between [6-\(^{13}\)C,4,4,5,5,D\(_{4}\)]arginine (from the infused citrulline) and [5,5,4,4,D\(_{4}\)]ornithine.

During the deamination of ornithine to L-glutamate-γ-semialdehyde by Ornithine AminoTransferase (OAT), one deuterium is lost from ornithine to citrulline (\( R_{Citrulline} \)), citrulline to arginine (\( R_{Arginine} \)), and ornithine to citrulline (\( R_{Citrulline} \)).

The net rate of arginine released from body protein breakdown (\( R_{Prot} \)) was calculated based on phenylalanine entry rate (\( R_{Prot} \)) and by assuming an arginine/phenylalanine molar ratio of 1.29 (12).

\[ R_{Prot} = 1.29 \times R_{Prot} \]

During feed deprivation, arginine entering the plasma either originates from citrulline or protein degradation. The net rate of arginine originating from protein degradation and metabolized intracellularly (\( R_{Prot-MI} \)) was calculated as follows:

\[ R_{Prot-MI} = R_{Prot-MI} - (R_{Arginine} - R_{Citrulline} - Arginine) \]

Data Analysis

The following broken-line model was fitted to urea production and plasma ammonia data from the ornithine titration study utilizing Nonlinear Regression Analysis Program (NLREG; Sherrod 2003)

\[ Y = a + b \times O , \text{ for } O < O_{\text{breakpoint}} \]

\[ Y = a + b \times O_{\text{breakpoint}} \text{ for } O > O_{\text{breakpoint}} \]

where \( Y \) is the observed urea production or plasma ammonia concentration at a given ornithine supplementation rate \( O \); \( a \), the intercept; \( b \), the slope of the function; \( O_{\text{breakpoint}} \), is the breakpoint of the function (minimal supplementation rate of ornithine to achieve plateau urea production or plasma ammonia concentration).

The experimental design for the arginine metabolism and NO production study was a completely randomized design with a 2 × 2 factorial arrangement of treatments. Data were analyzed statistically with the proc mixed procedure of SAS (v. 9.1, SAS, Cary, NC). Fixed effects were genetic background (B6 or ICR), mutation (wild-type or mutant), and nitrogen load (saline or alanine-glycine) and their interactions. If a significant interaction was obtained (\( P \leq 0.05 \)), the post hoc Tukey procedure for multiple pairwise comparisons was also applied. Preplanned orthogonal contrasts were conducted to compare the effect of genetic background on the spf-ash mutation.

RESULTS

Ornithine Titration

The addition of ornithine to the alanine-glycine mixture infused increased (\( P < 0.001 \)) urea production and reduced (\( P < 0.001 \)) plasma ammonia in B6/\( spf-ash \) mice. Breakpoint analysis showed that supplementation with ~100 μmol ornithine·kg\(^{-1}\)·h\(^{-1}\) was needed to prevent the reduction in ureagenesis and to mitigate hyperammonemia caused by the nitrogen load imposed (Figs. 2 and 3). However, the infusion of alanine-glycine did not cause hyperammonemia nor reduce ureagenesis in ICR/\( spf-ash \) mice and thus ornithine supplementation had no effect in these animals.

Arginine Metabolism and NO Production

In the arginine metabolism and NO production study, the entry rate of urea was affected by genetic background [B6 and...
ICR, 3.55 and 4.06 (SE 0.08) mmol·kg⁻¹·h⁻¹, respectively; 
P < 0.001], mutation [control wild-type and mutant spf-ash, 
3.66 and 3.96 (SE 0.08) mmol·kg⁻¹·h⁻¹, respectively; 
P < 0.016], and nitrogen load (P < 0.001). As expected, urea produc-
tion was higher in the alanine-glycine treatment than in the 
saline control [4.92 and 2.70 (SE 0.08) mmol·kg⁻¹·h⁻¹, re-
spectively], and the difference in the amount of urea produced 
represented 73% of the nitrogen load infused. However, ure-
agenesis was not depressed in B6spf-ash mice, although two 
mice developed signs of hyperammonemia. No effect of nitro-
gen load was detected for any of the other entry rates mea-
sured, and thus results were pooled to show the effect of 
genetic background and spf-ash mutation.

Western blot analysis showed a comparable reduction in 
OTC abundance in mutant mice, from both backgrounds, in 
hepatic and intestinal tissue (Fig. 4). Citrulline entry rate 
was reduced (P < 0.001) in mutant animals, but a greater 
reduction (P < 0.001) was observed in B6spf-ash mice (Table 
1). The entry rate of arginine, however, was only reduced in 
B6spf-ash mice. A significant reduction (P < 0.001) in 
orithine entry rate due to genetic background was detected in 
B6 mice compared with ICR mice. The plasma entry rate 
of urea cycle intermediates (UCI) was greater in ICR mice 
(P < 0.001) and in wild-type animals (P < 0.029). Simi-
larly, a higher entry rate of phenylalanine was found in ICR 
mice (P < 0.001) and in animals carrying the spf-ash 
mutation (P < 0.003).

The conversion of arginine to citrulline, a proxy for NO 
production, was higher in ICR wild-type control mice (9 
μmol·kg⁻¹·h⁻¹) representing 1.94% of the total plasma argi-
nine entry rate of these animals, whereas in B6 and ICRspf-ash 
mice it was ~0.9% (Table 1). The conversion of citrulline into 
arginine was not only quantitatively higher in ICR wild-type 
control mice (126 μmol·kg⁻¹·h⁻¹), but it represented a higher 
percentage (89%) of the citrulline entry rate. Likewise, a larger 
quantity (23.4 μmol·kg⁻¹·h⁻¹) and greater proportion (16.5%) of

<table>
<thead>
<tr>
<th>Tissue</th>
<th>B6</th>
<th>ICR</th>
<th>WT</th>
<th>spf-ash</th>
<th>SE</th>
<th>Gen</th>
<th>Back</th>
<th>Int.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.56³</td>
<td>0.45³³</td>
<td>0.99³</td>
<td>0.35³</td>
<td>0.09</td>
<td>0.001</td>
<td>0.004</td>
<td>0.027</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.62³</td>
<td>0.32³</td>
<td>2.07³</td>
<td>0.27³</td>
<td>0.08</td>
<td>0.001</td>
<td>0.038</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Fig. 3. Plasma ammonia concentration in spf-ash mutant mice from 
2 different genetic backgrounds supplemented intravenously with 
orithine (line graph) and in wild-type control mice from the same 
background (bar graph). Mice were infused intravenously with an 
alanine-glycine mixture (6.06 mmol·kg⁻¹·h⁻¹). Broken-line anal-
ysis yielded a breakpoint value for the function of 100.8 
μmol·kg⁻¹·h⁻¹. Symbols (*) B6; ( ) ICR are means ± SE (n = 5).

Fig. 4. Ornithine transcarbamylase abundance in liver 
and small intestinal tissue of wild-type control and 
spf-ash mutant mice from 2 different genetic back-
grounds. α-Tubulin was utilized to normalize the 
intensity of the ornithine transcarbamylase (OTC) 
bands. Values († arbitrary units) in the same row with 
different superscript letters are statistically different at 
P < 0.05.
the plasmatic citrulline was converted into ornithine in ICR wild-type control mice more than in mutant mice from both backgrounds and B6 wild-type controls. Plasmatic ornithine also made a greater contribution (112.7 μmol·kg\(^{-1}·h^{-1}\); 24% of the ornithine entry rate) to the synthesis of citrulline in ICR wild-type control mice.

The entry rates and interconversion of the UCI in mutant spf-ash mice from both genetic backgrounds, as well as the arginine originating from protein breakdown, are shown in Fig. 5. Most of the circulating arginine was derived from arginine originating from protein breakdown and from de novo synthesis from citrulline in ICR wild-type control mice.

However, was not observed when the multitracer protocol was followed and it could be explained in part by the “supplementation” due to the infusion of tracers (38 μmol UCI·kg\(^{-1}·h^{-1}\)). Nevertheless, two B6\(^{spf-ash}\) mice developed clear signs of hyperammonemia, but without a reduction in ureagenesis when infused with the alanine-glycine mixture.

DISCUSSION

The combination of mouse genetic models together with stable isotope multitracer protocols allows for the dissection of complex metabolic pathways in vivo in conscious animals. The present study shows that the spf-ash mutation interacted with the genetic background of mice in most of the variables analyzed. A similar reduction in OTC enzyme abundance in both B6 and ICR resulted in reduced citrulline entry rate. ICR mice, however, were able to compensate for the reduced OTC enzyme activity and displayed arginine entry rates similar to control animals.

**Ornithine Titration**

In agreement with our previous report, supplementation with ornithine prevented hyperammonemia and restored ureagenesis in B6\(^{spf-ash}\) mice (21). However, the alanine-glycine protocol failed to reduce ureagenesis and to cause hyperammonemia in ICR\(^{spf-ash}\) mice and thus ornithine supplementation had no effect in these animals. The reduction in ureagenesis, however, was not observed when the multitracer protocol was followed and it could be explained in part by the “supplementation” due to the infusion of tracers (38 μmol UCI·kg\(^{-1}·h^{-1}\)). Nevertheless, two B6\(^{spf-ash}\) mice developed clear signs of hyperammonemia, but without a reduction in ureagenesis when infused with the alanine-glycine mixture.
**Table 2. Origin of plasma arginine in WT and spf-ash mutant mice of 2 different genetic backgrounds (B6 or ICR)**

<table>
<thead>
<tr>
<th>Origin of RaArg, μmol·kg⁻¹·h⁻¹</th>
<th>WT</th>
<th>spf-ash</th>
<th>WT</th>
<th>spf-ash</th>
<th>SE</th>
<th>Mut†</th>
<th>Back†</th>
<th>Int†</th>
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</thead>
<tbody>
<tr>
<td>RaArg</td>
<td>421.0*</td>
<td>332†</td>
<td>455*</td>
<td>453*</td>
<td>20.6</td>
<td>0.032</td>
<td>0.001</td>
<td>0.038</td>
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<tr>
<td>R_Cu→Arg</td>
<td>89.7†</td>
<td>33.3§</td>
<td>126.0*</td>
<td>61.6‡</td>
<td>5.8</td>
<td>0.000</td>
<td>0.001</td>
<td>0.497</td>
</tr>
<tr>
<td>R_Prot→RaArg</td>
<td>389‡</td>
<td>506†</td>
<td>421‡</td>
<td>618†</td>
<td>23.2</td>
<td>0.003</td>
<td>0.001</td>
<td>0.094</td>
</tr>
<tr>
<td>R_Cu→RaArg</td>
<td>332†</td>
<td>298†</td>
<td>329†</td>
<td>391†</td>
<td>17.1</td>
<td>0.369</td>
<td>0.007</td>
<td>0.004</td>
</tr>
<tr>
<td>R_Arg-MI</td>
<td>57§</td>
<td>123‡</td>
<td>177‡</td>
<td>226‡</td>
<td>12.7</td>
<td>0.011</td>
<td>0.001</td>
<td>0.495</td>
</tr>
<tr>
<td>Origin of R_cCit, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R_Cu→cCit</td>
<td>21.1†</td>
<td>10.0§</td>
<td>27.3*</td>
<td>13.2‡</td>
<td>0.7</td>
<td>0.001</td>
<td>0.001</td>
<td>0.043</td>
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<tr>
<td>R_Prot→cCit</td>
<td>78.9‡</td>
<td>90.0*</td>
<td>72.7§</td>
<td>86.8‡</td>
<td>0.7</td>
<td>0.001</td>
<td>0.001</td>
<td>0.043</td>
</tr>
<tr>
<td>R_Arg-MI</td>
<td>14.0‡</td>
<td>29.3†</td>
<td>34.3†</td>
<td>36.9*</td>
<td>1.7</td>
<td>0.001</td>
<td>0.001</td>
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</tr>
</tbody>
</table>

Results are means ± SE (n = 20). Mut†, effect of the mutation; Back†, effect of the genetic background; Int†, interaction between the 2 main effects.

*†‡§Values in the same row with different superscript letters are statistically different at P < 0.05. ¹RaArg, plasma entry rate of arginine; ²R_Prot→RaArg, arginine released from protein breakdown; ³R_Cu→RaArg, contribution of citrulline to the plasma entry rate of arginine; ⁴R_Arg-MI, arginine released from protein breakdown and metabolized intracellularly; ⁵R_cCit, percentage of arginine released from protein breakdown that was metabolized intracellularly.

### OTC Abundance and Its Effect on Citrulline and Arginine Entry Rates

A reduction in OTC enzyme abundance was observed in mutant mice, from both backgrounds, which is consistent with the lower OTC activity described previously in spf-ash mice (27). This decrease in activity has been shown to result from a reduction in enzyme abundance, and not in enzyme-specific activity (26). The reduction in OTC abundance in mutant mice resulted in a decrease in the entry rate of citrulline, which was more pronounced in B6 spf-ash mice. This was not reproduced for arginine entry rate for which ICR spf-ash mice had rates similar to wild-type control animals; in contrast, arginine entry rate was diminished by ~22% in B6 spf-ash mice. This entry rate for arginine and citrulline in the present experiment were similar to the ones reported by others utilizing more invasive procedures in an unconscious mouse model (2, 10).

Between 75 and 89% of the citrulline flux was accounted for by its conversion to arginine. It has been established in rodents that the liver takes very little, if any, of the circulating citrulline (2, 14). This phenomenon which has been dubbed “arginine paradox” (14).

### Contribution of Protein Arginine to Ra

ICR spf-ash mice had a higher phenylalanine entry rate, indicating greater protein breakdown, and thus more arginine was released from protein in these animals which contributed to the higher arginine entry rate observed. Protein breakdown was the main source of circulating arginine during the postabsorptive state in wild-type control and spf-ash mice of both backgrounds. However, a large proportion (up to 37%) of the arginine released by endogenous protein is further metabolized intracellularly before it reaches the general circulation. Liver releases very little, if any, arginine into the general circulation and as a consequence it does not contribute to the plasma arginine entry rate (5, 34). However, it is clear from the high urea entry rate that a very active arginine synthesis takes place in the hepatocytes. In the present experiment, the urea entry rate (~4.9 mmol) was ~10-fold higher than the arginine entry rate showing the high compartmentalization of urea metabolism.

### Ornithine Entry Rate and Conversion to Citrulline

Oxidative rate of appearance, the third UCI studied, was reduced only in B6 wild-type control mice. Ornithine entry rate should not be affected, at least directly, by the reduction in OTC activity due to the spf-ash mutation. Ornithine has been shown in mice to be the first UCI to increase in the liver after a sudden nitrogen load, expanding the amount of intermediates and supporting the need for ammonia detoxification through urea synthesis (17). Similarly, in sheep, the portal infusion of ammonia increased the extraction rate of ornithine, but not of the other UCI (24). Thus, it seems that ornithine is crucial in the expansion of hepatic UCI to sustain ureagenesis and 2) that it originates in extrahepatic tissue. However, the infusion and sampling protocol employed in the present report measured whole body entry rates and were unable to determine the intestinal release and liver uptake of ornithine.
The de novo synthesis of ornithine takes place in mitochondria. The carbon skeleton provided by l-glutamate γ-semialdehyde, which can originate from glutamate (32a), or from the oxidation of proline (34a), is transaminated in the C5 position by the action of OAT. Because OAT and OTC are both present in enterocytic mitochondria, the channeling of l-glutamate γ-semialdehyde to citrulline was expected. However, up to 24% (113 μmol·kg^{-1}·h^{-1}) of the circulating ornithine was converted into citrulline by ICR wild-type control mice, indicating a significant entry of plasmatic ornithine into intestinal mitochondria. Furthermore, this implies that the contribution of circulating ornithine to citrulline production accounted for ~80% of the citrulline carbon skeleton. These findings contrast with in vitro data, in which glutamine was the local precursor for ornithine synthesis in piglet enterocytes (36), but it is supported by recent in vivo data in newborn piglets (31). It thus seems that the interorgan exchange of ornithine might be crucial to support citrulline production by the small intestine.

The increase in the supply of UCI either by exogenous supplementation (9, 28) or by increasing endogenous ornithine concentration, through the inhibition of OAT (30), has been shown to prevent hyperammonemia after a nitrogen load in rodents. Likewise, the ectopic (i.e., intestinal) correction of OTC deficiency in mice by means of gene therapy has proven to correct the phenotypic and metabolic features of this inborn error (13). Therefore, the availability of UCI interacts with the mutation to yield different phenotypic expressions of the OTC deficiency.

Relevance of the Present Findings in Relationship to OTC Deficiency in the Human Population

A recent update in OTC deficiency in humans has reported 341 distinct mutations (37). Because OTC deficiency is an X-linked trait, the phenotypic expression in females is highly variable due to random inactivation of an X-chromosome, and a large proportion do not show any symptoms throughout their life (18). However, the wide phenotypic heterogeneity, even in patients within the same family, is poorly understood. A combination of genetic and environmental variables, such as infection, have been suspected to be the cause for such heterogeneity (37). Genetic background is an important determinant of the cumulative metabolite flux through a specific biochemical pathway in vivo and is the best correlate with human phenotype (15, 16).

The early diagnosis, improved care, and overall better prognosis of OTC deficiency patients and UCDs in general have underscored clinical findings that point to alternative pathophysiological processes related to dysregulation of arginine metabolism and/or NO production. For example, a reduced NO production (25) and an increased prevalence of essential hypertension (29) have been reported in some patients. The translation of these animal studies to the context of human UCD would allow us to begin to correlate specific clinical consequences with nitrix oxide dysregulation in situations where genetic determinant, i.e., urea cycle mutation, is paramount. A limitation of studying the contribution of NO dysregulation to common disease processes is limited by the major impact of environment in the general population. In urea cycle patients a highly penetrant genetic mutation affecting NO flux could be more easily correlated with specific clinical effects.

The present study highlights the importance of the interaction between mutation and genetic background on ureagenesis and NO production. Supplementation of OTC disorder patients with UCI might prove helpful in not only increasing their ureogenic potential but in sustaining NO production. The relative efficacy of UCI supplementation to affect NO production could potentially be more easily evaluated in the urea cycle disorder patient.

ACKNOWLEDGMENTS

We thank I. Cajo and M. Wu for technical assistance. The polyclonal hOTC antibody was a gift from H. Morizono and M. Tuchman.

GRANTS

The work was supported by USDA Grant 6250-51000-044 and National Institutes of Health Grants K01-RR-024173 and RO1-DK-54450.

REFERENCES


