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

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AND was higher in ICR Down was the main source of arginine during the postabsorptive state such as mitochondria, caveolas, and cytosol (8, 33). Further-
synthesis by the kidney (7). The metabolism of arginine (Fig. 1) is
exported into the blood, serving as the precursor for arginine
uric acid entry rate 479 and 327, respectively; SE 18
D4\textsuperscript{[14C]}citrulline, and L-[ring-D\textsuperscript{5}]phenylalanine to investigate the interac-
2.8, ICR: 9.0, ICR
D4\textsuperscript{[14C]}citrulline, and L-[ring-D\textsuperscript{5}]phenylalanine to investigate the interac-
tion between genetic background and spf-ash mutation on ureagen-
esis, arginine metabolism, and nitric oxide production. ICR\textsuperscript{[14C]}citrulline mouse
reduced ureagenesis (5.5 ± 0.3 mmol·kg\textsuperscript{-1}·h\textsuperscript{-1}) and developed
mild hyperammonemia (145 ± 19 μmol/l) when an unbalanced
nitrogen load was imposed; however, B6\textsuperscript{[14C]}citrulline mice became hyper-
ammonemic (671 ± 15 μmol/l) due to compromised ureagenesi-
s (3.4 ± 0.1 mmol·kg\textsuperscript{-1}·h\textsuperscript{-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\textsuperscript{-1}·h\textsuperscript{-1}). Argi-
nine entry rate was only reduced in B6\textsuperscript{[14C]}citrulline mice (B6\textsuperscript{[14C]}-ash, 332,
ICR\textsuperscript{[14C]}-ash: 453; SE 20.6 μmol·kg\textsuperscript{-1}·h\textsuperscript{-1}). Genetic background and
mutation had an effect on nitric oxide production (B6: 3.4, B6\textsuperscript{[14C]}-ash,
2.8, ICR: 9.0, ICR\textsuperscript{[14C]}-ash: 4.6, SE 0.7 μmol·kg\textsuperscript{-1}·h\textsuperscript{-1}). Protein
breakdown was the main source of arginine during the postabsorptive state
and was higher in ICR\textsuperscript{[14C]}citrulline than in B6\textsuperscript{[14C]}citrulline mice (phenylalanine
entry rate 479 and 327, respectively; SE 18 μmol·kg\textsuperscript{-1}·h\textsuperscript{-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 concen-
trations and frequent hyperammonemic 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, caveolas, 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 ureagenesi-
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 interac-
tions offer opportunities on a genetic level to modify the
metabolic consequences of specific mutations. The ultimate
effects on metabolism flux contribute to variable expressivity
and susceptibility (15). Metabolic analysis of mutations on
cogenetic 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
hypomorph mouse model for OTC deficiency (OTC\textsuperscript{[14C]}-ash) (21). Thus, it seems that increasing the supply of ornithine
was able to compensate for the reduced enzyme activity and main-
tain 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 ureagen-
esis, arginine metabolism, and NO production.

MATERIALS AND METHODS

Animals and Treatments

B6Eic3S3n a/–Otc\textsuperscript{[14C]}-ash mice (B6\textsuperscript{[14C]}-ash) were originally ob-
tained from The Jackson Laboratory (Bar Harbor, ME). The mutation
was bred into ICR mice (ICR\textsuperscript{[14C]}-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
mg/kg), gross energy (16.9 MJ/kg), fat (45 mg/kg), fiber (60 mg/kg),
and ash (70 mg/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 envi-
ronment. All animal procedures were authorized by the Baylor Col-
lege 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<sup>spf-ash</sup> 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<sup>spf-ash</sup> and ICR<sup>spf-ash</sup> 5 per group) were also infused with increasing rates of ornithine (0, 80, 150, 300, and 600 mmol/kg) following a continuous infusion of [¹³C¹⁸O]urea (66 mmol/kg), L-¹⁵N<sub>2</sub>arginine (23.2 mmol/kg), L-[5,5-D<sub>2</sub>]ornithine (7.7 mmol/kg), L-[ureido-¹³C<sub>4</sub>, 4,4,5,5-D<sub>4</sub>]citrulline (7 mmol/kg), and L-[ring-D<sub>3]</sub>phenylalanine (10 mmol/kg) was given to the mice. The priming dose was followed immediately by a continuous infusion of [¹³C¹⁸O]urea (100 mmol·kg⁻¹·h⁻¹), L-[guanido-¹⁵N<sub>2</sub>]arginine (23.2 mmol·kg⁻¹·h⁻¹), L-[5,5-D<sub>2</sub>]ornithine (7.7 mmol·kg⁻¹·h⁻¹), L-[ureido-¹³C<sub>4</sub>, 4,4,5,5-D<sub>4</sub>]citrulline (7 mmol·kg⁻¹·h⁻¹), and L-[ring-D<sub>3</sub>]phenylalanine (10 mmol·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 isotypic 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 sample was derivatized with 25 µl of a 1:1 mixture of N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA; Sigma) and acetonitrile, at 80°C for 20 min in the tightly capped V-vials. The analysis was performed in a 5973 Agilent GC MSD (Agilent Technologies, Santa Clara, CA) in SIM mode, 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% w/v solution), centrifuged, and the supernatant was passed over a cation exchange column (Dowex AG 50W-8X, 100 –200 mesh 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-mercaptoethanol, 10 µg liver protein extracts were loaded for Western blot assay. Total protein was fractionated in 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 hOTC (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

\[ R_{AM} = i_y \times \left( \frac{100}{E_M} - 1 \right) \]

where \( R_{AM} \) is the plasma entry rate (flux) of the metabolite \( M \) (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)), \( i_y \) is the infusion rate (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \)), and \( E_M \) is the enrichment of \( M \) at plateau (mpe).

The rate of conversion of arginine to citrulline \( (Rc_{Citrulline} \rightarrow \text{Arginine}) \), citrulline to ornithine \( (Rc_{Citrulline} \rightarrow \text{Ornithine}) \), and ornithine to citrulline \( (Rc_{Ornithine} \rightarrow \text{Citrulline}) \) were determined as follows (30a)

\[ R_{Citrulline \rightarrow \text{Arginine}} = Ra \times \left( \frac{E_{\text{Citrulline}}}{E_{\text{Arginine}}} \right) \left( \frac{Ra_{\text{Arginine}}}{i_{\text{arginine}} + Ra_{\text{Arginine}}} \right) \]

where \( Ra \times \text{ and } Ra \) are the plasma fluxes of the precursor and product, respectively, determined from the steady-state enrichments of the infused tracers \( [^{15}\text{N}_2] \text{arginine}, [5,5\text{ D}_2] \text{ornithine}, [13\text{C},4,4,5,5\text{ D}_4] \text{citrulline}; \) \( E_{\text{Arginine}} \) and \( E_{\text{Citrulline}} \), the respective plasma enrichments of the precursors and products \( (^{15}\text{N}_2) \text{arginine}/[13\text{C},4,4,5,5\text{ D}_4] \text{citrulline}, [13\text{C},4,4,5,5\text{ D}_4] \text{arginine}, [13\text{C},4,4,5,5\text{ D}_4] \text{citrulline}/[4,4,5,5,5\text{ D}_4] \text{ornithine}, \) and \( [5,5\text{ D}_2] \text{ornithine}/[5,5\text{ D}_2] \text{citrulline} \) and \( i_{\text{arginine}} \) 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 \([^{6-13}\text{C}_5,5,4,4\text{ D}_4] \text{arginine} (\text{from the infused citrulline}) \) and \([5,5,4,4\text{ D}_4] \text{ornithine} \).

During the deamination of ornithine to L-glutamate-γ-semialdehyde by Ornithine AminoTransferase (OAT), one deuterium is lost from the isotopic label, the conversion of arginine to ornithine was estimated by the ratio between \([^{6-13}\text{C}_5,5,4,4\text{ D}_4] \text{arginine} \) (from the infused citrulline) and \([5,5,4,4\text{ D}_4] \text{ornithine} \).

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

\[ R_{\text{Arginine}} = 1.29 \times Ra_{\text{phenylalanine}} \]

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 \( (R_{\text{Arginine, MI}}) \) was calculated as follows

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

**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)

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

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

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 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 B6spf-ash mice. Breakpoint analysis showed that supplementation with \(~100 \mu\text{mol ornithine} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}\) 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 ICRspf-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 \textit{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 production was higher in the alanine-glycine treatment than in the saline control [4.92 and 2.70 (SE 0.08) mmol·kg\(^{-1}\)·h\(^{-1}\), respectively], and the difference in the amount of urea produced represented 73% of the nitrogen load infused. However, ureagenesis was not depressed in B6\textit{spf-ash} mice, although two mice developed signs of hyperammonemia. No effect of nitrogen load was detected for any of the other entry rates measured, and thus results were pooled to show the effect of genetic background and \textit{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 B6\textit{spf-ash} mice (Table 1). The entry rate of arginine, however, was only reduced in B6\textit{spf-ash} mice. A significant reduction (\(P < 0.001\)) in ornithine 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 (\(P < 0.029\)). Similarly, a higher entry rate of phenylalanine was found in ICR mice (\(P < 0.001\)) and in animals carrying the \textit{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 \(\mu\)mol·kg\(^{-1}\)·h\(^{-1}\)) representing 1.94% of the total plasma arginine entry rate of these animals, whereas in B6 and ICR\textit{spf-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 \(\mu\)mol·kg\(^{-1}\)·h\(^{-1}\)), but it represented a higher percentage (89%) of the citrulline entry rate. Likewise, a larger quantity (23.4 \(\mu\)mol·kg\(^{-1}\)·h\(^{-1}\)) and greater proportion (16.5%) of
the plasmatic citrulline was converted into ornithine in ICR wild-type control mice 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 protein breakdown in all the mice studied, but spf-ash mutant mice derived a larger proportion from this source (Table 2). However, not all the arginine released from protein breakdown entered the circulation; an important proportion (up to ~37%) was metabolized intracellularly.

**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 ureagenesis when infused with the alanine-glycine mixture.

![Diagram](https://example.com/diagram.png)

**Fig. 5.** Arginine originating from protein breakdown and from de novo synthesis from citrulline in spf-ash mice from 2 different genetic backgrounds (B6 and ICR). RaM, denotes the plasma entry rate of metabolite M; X→Y, interconversion of metabolite X into Y; ArgMI, arginine released from protein and metabolized intracellularly. Values are expressed in μmol·kg⁻¹·h⁻¹ unless noted. Significance was calculated using preplanned orthogonal contrasts, means with superscript differ (P < 0.05).

**Table 1.** Plasma entry rate and interconversions of urea cycle intermediates in WT and spf-ash mutant mice of 2 different genetic backgrounds (B6 or ICR)

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>ICR</th>
<th>SE</th>
<th>Mut†</th>
<th>Back†</th>
<th>Int†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma entry rate, μmol·kg⁻¹·h⁻¹</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>RaArg</td>
<td>421*</td>
<td>332†</td>
<td>455*</td>
<td>5.6</td>
<td>0.001</td>
<td>0.001</td>
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<tr>
<td>RaCit</td>
<td>118*</td>
<td>43†</td>
<td>138*</td>
<td>4.6*</td>
<td>0.001</td>
<td>0.001</td>
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<tr>
<td>RaOrn</td>
<td>266†</td>
<td>372*</td>
<td>464*</td>
<td>6.0</td>
<td>0.001</td>
<td>0.001</td>
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<tr>
<td>RaCit/RaArg</td>
<td>804†</td>
<td>747†</td>
<td>1057†</td>
<td>3.3</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>RaOrn/RaCit</td>
<td>301†</td>
<td>327†</td>
<td>392†</td>
<td>1.2</td>
<td>0.001</td>
<td>0.001</td>
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<tr>
<td><strong>Interconversion, μmol·kg⁻¹·h⁻¹</strong></td>
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<td></td>
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<tr>
<td>RaArg-RaCit</td>
<td>3.4†</td>
<td>2.8†</td>
<td>9.0*</td>
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<td>RaCit-RaArg</td>
<td>59.5†</td>
<td>53.7†</td>
<td>85.0*</td>
<td>4.6</td>
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<td>RaOrn-RaArg</td>
<td>89.7†</td>
<td>33.3§</td>
<td>126.0*</td>
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<td>RaCit-RaOrn</td>
<td>12.6†</td>
<td>5.4†</td>
<td>23.4*</td>
<td>1.2</td>
<td>0.001</td>
<td>0.001</td>
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<tr>
<td>RaOrn-RaCit</td>
<td>46.2†</td>
<td>28.1†</td>
<td>112.7*</td>
<td>5.3</td>
<td>0.001</td>
<td>0.001</td>
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<tr>
<td><strong>Entry rate fate, %</strong></td>
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<tr>
<td>RaArg-RaCit</td>
<td>0.79†</td>
<td>0.85§</td>
<td>1.94*</td>
<td>0.96*</td>
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<td>RaCit-RaArg</td>
<td>75.3†</td>
<td>76.5†</td>
<td>88.9*</td>
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<td>12.4†</td>
<td>16.5*</td>
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<td>0.001</td>
<td>0.138</td>
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<tr>
<td>RaOrn-RaCit</td>
<td>17.3†</td>
<td>7.5§</td>
<td>24.0*</td>
<td>10.4</td>
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</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. RaX/Y, plasma entry rate of arginine, citrulline, ornithine, phenylalanine; UCI, urea cycle intermediates; RaCit→Cit, interconversion rate between metabolite X and Y; RaCit→RaCit, percentage of the entry rate of metabolite X converted into metabolite Y. WT, wild-type.
**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</th>
<th>µmol·kg⁻¹·h⁻¹</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>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R_{C_{u}u}=\alpha_{AR}</td>
<td>421.0*</td>
<td>352†</td>
<td>455*</td>
<td>453*</td>
<td>20.6</td>
<td>0.032</td>
<td>0.001</td>
</tr>
<tr>
<td>R_{P_{Prot}}=\alpha_{AR}</td>
<td>89.7†</td>
<td>53.3§</td>
<td>126.0*</td>
<td>61.6‡</td>
<td>5.8</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>R_{P_{Prot}}=\alpha_{RcCit}</td>
<td>389‡</td>
<td>506†</td>
<td>4211‡</td>
<td>618§</td>
<td>23.2</td>
<td>0.003</td>
<td>0.001</td>
</tr>
<tr>
<td>R_{ARG-MT}</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>
</tr>
<tr>
<td>Origin of R_{C_{u}u}</td>
<td>57§</td>
<td>123‡</td>
<td>177**</td>
<td>226*</td>
<td>12.7</td>
<td>0.001</td>
<td>0.001</td>
</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. \( R_{C_{u}u} \): plasma entry rate of arginine; \( R_{P_{Prot}}=\alpha_{AR} \), arginine released from protein breakdown; \( R_{P_{Prot}}=\alpha_{RcCit} \), contribution of citrulline to the plasma entry rate of arginine; \( R_{ARG-MT} \), arginine released from protein breakdown and metabolized intracellularly; \( R_{C_{u}u} \), 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. The entry rates 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 (34), although this concept has been recently questioned in humans (32). In their seminal work, Windmueller and Spaeth (34) showed that 83% of circulating citrulline was taken up by the kidney, which exported 75% of this citrulline as arginine. Their results offer a glimpse of two little known processes: the utilization of citrulline by endothelial cells and macrophages to generate arginine and support the local production of NO synthesis has been shown in the past (11, 35). This implies that the conversion of \[^{15}\text{N}_2\]arginine to \[^{15}\text{N}\]citrulline fails to take this source of arginine into account, thereby providing a minimal estimate for NO synthesis.

NO Production

NO production, determined by the conversion of \[^{15}\text{N}_2\]arginine to \[^{15}\text{N}\]citrulline, represented a small proportion (up to 1.9%) of the total arginine flux. These findings agree with previous reports utilizing an identical protocol conducted both in humans (4) and mice (10). Despite the reduced amount of arginine utilized for the synthesis of NO, it seems that NO production might be responsive to arginine supplementation, a 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

Ornithine 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, \( I \) 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 $\gamma$-glutamate oxidized in the C5 position by the action of OAT. Because OAT and OTC are both present in enterocytic mitochondria, the channeling of $\gamma$-glutamate to citrulline was expected. However, up to 24% (113 $\mu$mol $\cdot$ kg$^{-1}$ $\cdot$ 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 nitric 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.

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