Reduced citrulline availability by OTC deficiency in mice is related to reduced nitric oxide production

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Luiking YC, Hallemeesch MM, van de Poll MC, Dejong CH, de Jonge WJ, Lamers WH, Deutz NE. Reduced citrulline availability by OTC deficiency in mice is related to reduced nitric oxide production. Am J Physiol Endocrinol Metab 295: E1315–E1322, 2008. First published August 12, 2008; doi:10.1152/ajpendo.00055.2008.—The amino acid arginine is the sole precursor for nitric oxide (NO) synthesis. We recently demonstrated that an acute reduction of circulating arginine does not compromise basal or LPS-inducible NO production in mice. In the present study, we investigated the importance of citrulline availability in ornithine transcarbamoylase-deficient spfash (OTCD) mice on NO production, using stable isotope techniques and C57BL6/J (wild-type) mice controls. Plasma amino acids and tracer-to-tracee ratios were measured by LC-MS. NO production was measured as the in vivo conversion of L-[guanido-15N2]arginine to L-[guanidino-15N]citrulline; de novo arginine production was measured as conversion of L-[ureido-13C-5,5,2H2]citrulline to L-[guanidino-13C-5,5-2H2]arginine. Protein metabolism was measured using L-[ring-2H5]phenylalanine and L-[ring-2H2]tyrosine. OTC deficiency caused a reduction of systemic citrulline concentration and production to 30–50% (P < 0.001), reduced de novo arginine production (P < 0.05), reduced whole-body NO production to 50% (P < 0.005), and increased net protein breakdown by a factor of 2–4 (P < 0.001). NO production was twofold higher in female than in male OTCD mice in agreement with the impaired ability of the small intestine to convert ornithine to citrulline, catalyzed by the enzyme ornithine transcarbamoylase (OTC; Ref. 48). Subsequently, ~83% of the citrulline released from the small intestine into the circulation is taken up by the kidney where it is largely (~75%) converted to arginine (45, 49). The importance of enteral citrulline production for arginine synthesis was previously demonstrated in rats by OTC inhibition (23), in mice with the spfash mutation causing reduced OTC activity (28), and in rats with short bowel syndrome in which both intestinal citrulline production and renal arginine production were reduced by 50% (10). Besides the intestinal-renal axis for citrulline and arginine production, an additional amount of citrulline comes from nonintestinal sources. The intracellular arginine-citrulline cycle related to NO production in endothelial cells seems a likely candidate, as suggested in both mice and human endothelial cell studies (3, 13, 39). Along these lines, it is conceivable that a reduction in the capacity to produce citrulline may reduce arginine de novo synthesis and availability, which, in turn, may affect NO synthesis (17).

NO has important cardiovascular, neuronal, and immunological functions. NO is produced from arginine by nitric oxide synthase (NOS), of which neuronal (NOS1), endothelial (NOS3), and inducible (iNOS or NOS2) isoforms exist (14, 24). Of these, NOS1 and NOS3 are expressed constitutively and are therefore often collectively termed cNOS. The NOS2 isform is inducible by various microbial products (including LPS) and inflammatory cytokines (32).

Sparse-fur mouse (spfash) expresses a mutant OTC gene, which has only 5–10% of the OTC activity of the wild-type variant (11, 37) and is an X-linked gene (34). In agreement with the impaired ability of the small intestine to convert glutamine to citrulline (Fig. 1), the spfash phenotype is characterized by elevated glutamine and ammonia, and reduced citrulline and arginine plasma levels (1, 50). Thus far the spfash mouse model of OTC deficiency was mainly used to explore the effects of gene therapy in urea cycle disorders (1). In a recent study by Marinì et al. (28), an interaction between spfash mutation and genetic background on ureagenesis, arginine metabolism, and NO production was observed with minor effects on ureagenesis but development of hyperammonemia in B6 mice (29). We used the spfash mutation as a tool to

The amino acid arginine serves multiple functions, including the production of nitric oxide (NO; Ref. 48). Sources of endogenous production of arginine are protein breakdown and synthesis from citrulline (de novo arginine synthesis; Ref. 48). The small intestine and kidney play a central role in this de novo arginine production (the “gut-kidney axis”; Refs. 3, 8). The enterocytes of the small intestine produce citrulline from glutamine and proline through a series of metabolic conversions. The final step in citrulline synthesis is the conversion of ornithine to citrulline, catalyzed by the enzyme ornithine transcarbamoylase (OTC; Ref. 48). Subsequently, ~83% of the citrulline released from the small intestine into the circulation is taken up by the kidney where it is largely (~75%) converted to arginine (45, 49). The importance of enteral citrulline production for arginine synthesis was previously demonstrated in rats by OTC inhibition (23), in mice with the spfash mutation causing reduced OTC activity (28), and in rats with short bowel syndrome in which both intestinal citrulline production and renal arginine production were reduced by 50% (10). Besides the intestinal-renal axis for citrulline and arginine production, an additional amount of citrulline comes from nonintestinal sources. The intracellular arginine-citrulline cycle related to NO production in endothelial cells seems a likely candidate, as suggested in both mice and human endothelial cell studies (3, 13, 39). Along these lines, it is conceivable that a reduction in the capacity to produce citrulline may reduce arginine de novo synthesis and availability, which, in turn, may affect NO synthesis (17).

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determine the importance of the capacity to synthesize citrulline for arginine biosynthesis and NO production in healthy and LPS-treated C57BL/6J mice. In addition, the importance of protein turnover for arginine availability was studied. Stable isotope techniques were applied to measure metabolic parameters in vivo (20).

MATERIALS AND METHODS

Animals. C57BL/6J (wild-type; controls) and OTC-deficient spf<sup>mut</sup> (OTC) mice (15–22 g, 2–3 mo old) were obtained from Jackson Laboratories, bred at the Dept. of Anatomy and Embryology (Academic Medical Centre) and transported to the Centralized Animal Facilities of Maastricht University. OTC-deficient spf<sup>mut</sup> males were homozygote (OTC is X-linked), and we also used homozygote OTC-deficient spf<sup>mut</sup> females. Genotyping was confirmed by using the method described by Kobayashi et al. (25), using the primers OTC int4 and ex4 (Eurogentec). Animals were allowed to adapt for at least 1 wk to their new housing. The mice were fed standard lab chow (Hope Pharms, Woerden, The Netherlands), which results in enzyme-deficiency-related hyperammonemia in OTC-deficient mice, and were subjected to a standard 12-h light-dark cycle (7:30 AM to 7:30 PM). Room temperature was maintained at 25°C. Experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (43) and approved by the Ethical Committee of Animal Research of Maastricht University.

Experimental protocol. Male and female C57BL/6J and OTC mice were randomly assigned to receive LPS (Escherichia coli O55: B5; 250 μg in 0.5 ml saline; Sigma, St. Louis, MO) or a correspond-

ing

Injection of arginine was done intraperitoneally, followed by a subcutaneous infusion of arginine (Hope Pharms, Woerden, The Netherlands), which results in enzyme-deficiency-related hyperammonemia in OTC-deficient mice, and were subjected to a standard 12-h light-dark cycle (7:30 AM to 7:30 PM). Room temperature was maintained at 25°C. Experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (43) and approved by the Ethical Committee of Animal Research of Maastricht University.

Experimental protocol. Male and female C57BL/6J and OTC mice were randomly assigned to receive LPS (Escherichia coli O55: B5; 250 μg in 0.5 ml saline; Sigma, St. Louis, MO) or a corresponding volume of saline. Drinking water was provided, but food was withheld after the injection of endotoxin or saline to avoid the effects of differences in food intake. LPS-treated mice were put under a heating lamp to ensure maintenance of body temperature. Food was withheld after the injection of endotoxin or saline to avoid the effects of differences in food intake. LPS-treated mice were put under a temperature controller (Technical Brecht, Germany) on ice. Thereafter, the animal was killed by cervical dislocation, while the animals were under anesthesia.

Blood was centrifuged to obtain plasma, because we have recently shown that plasma sampling is required in organ-balance metabolic studies using amino acid tracers that do not equilibrate well with blood-cell cytoplasm (19). For determination of amino-acid concentrations and tracer-to-tracee ratios (TTRs), 80 μl plasma were added to 7 mg dry sulfoalicylic acid, vortexed, frozen in liquid nitrogen, and stored at −80°C. Plasma amino acid concentrations were measured as described previously (40). Amino acid TTRs were measured using a fully automated liquid chromatography–mass spectrometry system, using 9-fluorenylmethylchlororofomate as amino-acid derivative (41). SUMAA is the SUM of the following amino acids: glutamate, asparagine, serine, glutamine, glycine, threonine, histidine, citrulline, alanine, taurine, arginine, tyrosine, valine, methionine, isoleucine, phenylalanine, tryptophan, leucine, ornithine, and lysine. For determination of plasma urea and ammonia, 20 μl plasma was added to 80 μl 1% trichloroacetic acid. Plasma urea and ammonia were determined using commercially available kits on a Cobas Mira S (Roche Diagnostica, Hoffman La Roche, Basel, Switzerland), as described previously (9). For plasma nitrate and nitrate analysis, 50 μl plasma was added to 100 μl acetonitrile (Biosolve, Valkenswaard, The Netherlands). Plasma nitrite and nitrate were analyzed as detailed previously (5).

Calculations. Plasma arginine, citrulline, phenylalanine, and tyrosine production rates (Q) were calculated from the arterial isotopic enrichment values of [15N2]arginine, [13C-2H2]citrulline, [2H5]phenylalanine, and [2H2]tyrosine, respectively, using the standard steady-state isotope dilution equation:

\[
Q = I / TTR
\]

(1)

where TTR is the tracer-to-tracee ratio, and I is the rate of infusion of the tracer. TTR was corrected for background enrichment, and, whenever multiple masses of one amino acid were enriched, contribution of isotopomers from lower masses to the measured TTR was accounted for as described by Vogt et al. (42).

Calculation of the plasma arginine-to-citrulline flux (NO production) was performed as follows:

\[
Q_{Arg \rightarrow Cit} = Q_{Cit} \times TTR_{Cit\rightarrow Arg}/TTR_{Arg\rightarrow Cit} (6),
\]

(2)

where \(Q_{Cit}\) is the plasma citrulline flux (mmol·10 g<sup>–1</sup>·min<sup>–1</sup>), estimated from the primed constant infusions of [15N2]arginine. TTR<sub>Cit→Arg</sub> and TTR<sub>Arg→Cit</sub> are the respective TTRs of [15N]citrulline and [15N2]arginine. The stable-isotope measurement of NO production is considered to represent NO production better and more directly than measurement of plasma nitrate, since the latter may be affected by other nitrate sources like dietary nitrate (26). However, we are also aware that our method may not represent the total NO production, based on the discrepancy found with urinary NO<sub>3</sub> production (2).

Calculation of the plasma citrulline-to-arginine flux (de novo arginine production) was performed as follows:

\[
Q_{Cit\rightarrow Arg} = Q_{Arg} \times TTR_{Arg\rightarrow Cit}/TTR_{Cit\rightarrow Arg} (49),
\]

(3)

where \(Q_{Arg}\) is the plasma arginine flux (mmol·10 g<sup>–1</sup>·min<sup>–1</sup>), estimated from the primed constant infusions of [15N2]arginine. TTR<sub>Arg→Cit</sub> and TTR<sub>Cit→Arg</sub> are the respective TTRs of [15N2]arginine and [13C-2H2]citrulline.

Plasma clearance (ml·10 g<sup>–1</sup>·min<sup>–1</sup>) of citrulline and arginine was calculated as \(Q_{Cit}/[cit\_A]/Q_{Arg}/[arg\_A]\), where \([cit\_A]\ and \([arg\_A]\ represent arterial citrulline and arginine concentrations.

Protein turnover was measured by using the phenylalanine model, which is a well-established method (38) with phenylalanine appearance from protein breakdown and phenylalanine disappearance to protein synthesis and conversion of phenylalanine to tyrosine. Plasma
phenylalanine to tyrosine flux (phenylalanine hydroxylation) was calculated as:

\[ Q_{\text{Phe} \rightarrow \text{Tyr}} = Q_{\text{Tyr}} \times \frac{\text{TTR}_{\text{Phe}(M+4)/\text{TTR}_{\text{Phe}(M+5)}}}{H20849} \]

where \( Q_{\text{Tyr}} \) is the plasma tyrosine production, estimated from infusion of \([^{2}H2]\text{Tyr}\).

The rates of whole body protein turnover (synthesis and breakdown) were obtained from the phenylalanine tracer data, with:

\[ \text{protein breakdown} = Q_{\text{Phe}} \]
\[ \text{protein synthesis} = \text{protein breakdown} - Q_{\text{Phe} \rightarrow \text{Tyr}} \]

where \( Q_{\text{Phe}} \) is the plasma phenylalanine production, estimated from infusion of \([^{2}H5]\text{Phe}\) tracer.

**Statistics.** All data are shown as means ± SE. Data were tested using two-way ANOVA to test basal metabolic differences related to main effects and interaction between genotype (control or OTCD) and sex (male or female); factors are genotype and sex. A Bonferroni correction was applied to correct for this subanalysis (only basal group) by considering a \( P \) value of <0.025 as significant for this subanalysis. A three-way ANOVA was used to test the LPS effects on metabolic parameters, accounting for differences in the response related to main effects and interaction between LPS (+ or −), genotype (control or OTCD), and sex (male or female); factors are LPS, genotype, and sex. To test metabolic differences due to genotype or LPS for males and females separately, a two-way ANOVA with Bonferroni correction was applied. Pearson’s correlation test was used. A \( P \) value of <0.05 was considered significant.

**RESULTS**

Effects of reduced citrulline availability on basal NO production. In male and female OTCD mice, circulating citrulline levels were reduced to 55–60\% \((P < 0.001; \text{Fig. 2})\) and arginine to 60–80\% of those in wild-type mice (Fig. 3; \( P < 0.001 \)). Circulating glutamine was higher in male OTCD mice but not in female mice \([P < 0.01 \text{ (interaction)}]\). Ornithine and sum of amino acids were not affected by OTCD. In addition, OTC deficiency caused an almost twofold increase in plasma ammonia (Table 1; \( P < 0.05 \)) but did not affect plasma urea levels.

Tracer-to-tracee ratios for the infused isotopes \( L-[\text{guanidino-}^{15}\text{N}_2]\text{arginine}, L-[\text{ureido-}^{13}\text{C,5,5-}^{2}\text{H}_2]\text{citrulline}, L-[\text{ring-}^{2}\text{H}_5]\text{phenylalanine}, \) and \( L-[\text{ring-}^{2}\text{H}_2]\text{tyrosine} \) are listed in Table 2.

Parameters of whole body protein metabolism are listed in Table 3. Net protein breakdown was increased in OTCD mice \((P < 0.001; \text{Fig. 3})\), which was mainly due to a higher protein breakdown in males and a lower protein synthesis in the

![Fig. 2. Systemic citrulline production (WB Ra CIT) and plasma citrulline levels ([CIT]) under baseline (control) conditions and upon LPS treatment in wild-type (WT) and OTC-deficient (OTCD) male and female mice. Number of animals: male mice-control \((n = 8 \text{ for WT}; n = 9 \text{ for OTCD})\), male mice-LPS \((n = 9 \text{ for WT}; n = 8 \text{ for OTCD})\), female mice-control \((n = 7 \text{ for WT}; n = 8 \text{ for OTCD})\), and female mice-LPS \((n = 5 \text{ for WT}; n = 7 \text{ for OTCD})\). Control: citrulline production and plasma citrulline were both higher in female mice than male mice \((P < 0.001)\); OTCD mice showed lower levels of citrulline production and plasma citrulline \((P < 0.001; \text{two-way ANOVA})\). LPS: citrulline production increased in males but not in females \((P < 0.05 \text{ for interaction})\); a genotype difference was observed in the response of plasma citrulline \((P < 0.05 \text{ for interaction}; \text{three-way ANOVA})\). Symbols used to clarify statistical effects: *basal genotype effect; †LPS effect (increase vs. control).](http://ajpendo.physiology.org/)

**E1317 ARGinine DEFICIENCY AND NO PRODUCTION**

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Control: net protein breakdown was higher in OTCD mice (P<0.001) than in male mice (P<0.001). Plasma arginine was lower in OTCD mice (P<0.001) and also higher in female than in male mice (P<0.001). Plasma arginine was lower in OTCD mice (P<0.001) and slightly higher in female mice (P<0.05). LPS: net protein breakdown increased in males but not in females (P<0.05 for interaction), while this increase was highest in OTCD mice (P<0.001) and also higher in female compared with wild-type mice (P<0.001; three-way ANOVA). Symbols used to clarify statistical effects: basal genotype effect; ↑ or ↓ for LPS effect (increase or decrease vs. control, respectively).

Females compared with wild-type mice (Table 3). The reduction of circulating citrulline in OTCD mice was accompanied by a similar reduction of systemic citrulline production to 30–50% of that seen in wild-type mice (P<0.001; Fig. 2). Circulating citrulline levels and citrulline production were significantly higher in female animals than in male animals (P<0.01). Plasma citrulline clearance, i.e., the amount of plasma cleared from citrulline per minute, was significantly higher in female animals than in male animals (P<0.01; ANOVA). Symbols used to clarify statistical effects: *basal genotype effect; ↑ or ↓ for LPS effect (increase or decrease vs. control, respectively).

Table 1. Arterial concentrations of substrates in male and female WT and OTC-deficient mice treated with saline or LPS

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
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<th>Female</th>
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<td>Nitrite</td>
<td>WT (n = 8)</td>
<td>OTCD (n = 9)</td>
<td>WT (n = 9)</td>
<td>OTCD (n = 8)</td>
<td>WT (n = 7)</td>
<td>OTCD (n = 8)</td>
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<tr>
<td>Nitrates</td>
<td>1.2±0.2</td>
<td>4.1±0.9</td>
<td>3.2±0.9</td>
<td>▲</td>
<td>6.8±1.3</td>
<td>▲</td>
<td>4.4±0.8</td>
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<td>11.9±2.7</td>
<td>▲</td>
<td>7.2±1.5</td>
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<tr>
<td>Nitrate</td>
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<td>28±4</td>
<td>116±13</td>
<td>▲</td>
<td>113±9</td>
<td>▲</td>
<td>29±2</td>
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<td>148±13</td>
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<td>▲</td>
<td>362±43</td>
<td>▲</td>
<td>ND</td>
<td>233±30</td>
<td>ND</td>
<td>521±104</td>
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<tr>
<td>Urea</td>
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<td>7.7±1.0</td>
<td>11.3±0.3</td>
<td>▲</td>
<td>9.4±0.5</td>
<td>▲</td>
<td>6.8±0.7</td>
<td>7.3±0.5</td>
<td>13.0±1.2</td>
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<td>11.3±0.9</td>
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<td>98±22</td>
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<td>2,923±97</td>
<td>▲</td>
<td>3,032±132</td>
<td>▲</td>
<td>2,609±50</td>
<td>2,233±137</td>
<td>3,295±139</td>
<td>▲</td>
<td>2,742±264</td>
</tr>
</tbody>
</table>

Values are means ± SE in μM and urea in mM. WT, wild-type; OTCD, OTC deficient; ND, not determined. Statistics (two-way ANOVA for basal differences; three-way ANOVA includes LPS effects): G: P<0.05 for genotype effect; L: P<0.05 for LPS effect; S: P<0.05 for sex effect; G×L, G×S, L×S: P<0.05 for interactive effect. Symbols used to clarify statistical effects: *basal genotype effect; ↑ or ↓ for LPS effect (increase vs. control).
lower ($P < 0.005$) in OTCD mice (0.22 ± 0.03 vs. 0.38 ± 0.03 ml·10 g body wt·$^{-1}$·min$^{-1}$ in male wild-type mice; 0.31 ± 0.03 and 0.35 ± 0.01 ml·10 g body wt·$^{-1}$·min$^{-1}$ in female OTCD and wild-type mice, respectively). The reduction of arginine concentration in OTCD mice was accompanied by a significant reduction of systemic arginine production (46 ± 5 and 31 ± 2 nmol·10 g body wt·$^{-1}$·min$^{-1}$ in male WT and OTCD mice, respectively; 49 ± 2 and 37 ± 4 nmol·10 g body wt·$^{-1}$·min$^{-1}$ in female WT and OTCD mice, respectively; $P < 0.005$) and by a significant reduction in de novo arginine production ($P < 0.05$; Fig. 3). This de novo arginine production was similar to citrulline production, higher in female than in male mice ($P < 0.001$). Plasma arginine clearance was not different between OTCD and wild-type mice.

Reduced arginine availability due to OTC deficiency was accompanied by a significant 50% reduction of basal NO production in both male and female mice ($P < 0.005$; Fig. 4). Basal NO production was in generally higher in female than in male mice ($P < 0.05$). The reduction of the NO production rate by the chronic reduction of circulating arginine in OTC-deficient mice did not correlate with plasma nitrite and nitrate, since these were not affected in OTCD mice (Table 1).

**Effects of reduced citrulline availability on LPS-induced NO production.** In response to treatment with bacterial endotoxin, the arterial concentrations of arginine, ornithine, glutamine, and ammonia and the total level of amino acids increased ~20–40% in wild-type and OTCD mice, both in males and females (Table 1; Fig. 3). Plasma nitrite concentration responded similarly (Table 1), while the increase in plasma nitrate concentration differed between male and female mice, both in wild-type and OTCD mice (Table 1). In contrast, the increase in plasma citrulline and urea concentration after LPS was significantly smaller in OTCD mice than in controls (Fig. 2; Table 1).

Whole body net protein breakdown increased almost two-fold in male OTCD mice in response to LPS (Fig. 3), which was mainly due to a reduction in protein synthesis. Protein metabolism was not changed after LPS in wild-type and female mice (Table 3). Whole body arginine production increased in OTCD mice in response to LPS (arginine production was 45 ± 1 and 39 ± 5 nmol·10 g body wt·$^{-1}$·min$^{-1}$ in OTCD males and females after LPS, respectively) but not in wild-type mice ($P < 0.05$ for interaction). Similarly, de novo arginine production increased only in OTCD mice in response to LPS ($P < 0.005$ for interaction; Fig. 3). A slight increase in whole body citrulline production after LPS was only present in male mice ($P < 0.05$ for interaction; Fig. 2). A significant reduction in plasma citrulline clearance was present with LPS in all groups ($P < 0.001$), while plasma arginine clearance tended to be reduced in all groups during LPS ($P = 0.06$).

LPS administration caused an increase in NO production, as measured by stable isotopes, that was more prominent in male than in female animals ($P < 0.05$ for interaction; Fig. 4) but not different between wild-type and OTCD mice. NO production was correlated with circulating arginine levels in OTCD and wild-type mice ($r = 0.7$; $P < 0.001$ for male mice; $r = 0.5$, $P < 0.05$ for female mice; Fig. 5). A significant correlation was also observed between NO production and whole body citrulline production ($r = 0.5$, $P < 0.005$ for male mice; $r = 0.7$, $P < 0.001$ for female mice; not shown). As this relationship was evident in both male and female mice, it indicates that the rate of NO production is dependent on the citrulline availability and the circulating concentration of arginine.

**DISCUSSION**

In the present study, the effects of a reduced capacity to synthesize citrulline with subsequent reduced citrulline availability were investigated on arginine metabolism and basal and LPS-induced NO production. Sparse fur and abnormal skin and hair (Spfash) mutant mice with deficient OTC activity were used as a model of citrulline deficiency, which was confirmed

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**Table 2. Tracer-to-tracee ratios of infused isotopes at steady state**

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<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
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<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>WT (n = 8)</td>
<td>WT (n = 9)</td>
</tr>
<tr>
<td>ARG2</td>
<td>0.34±0.049</td>
<td>0.44±0.015</td>
</tr>
<tr>
<td></td>
<td>0.35±0.055</td>
<td>0.33±0.015</td>
</tr>
<tr>
<td>CT3</td>
<td>0.16±0.012</td>
<td>0.55±0.049</td>
</tr>
<tr>
<td></td>
<td>0.13±0.008</td>
<td>0.42±0.046</td>
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<tr>
<td>PHE5</td>
<td>0.13±0.009</td>
<td>0.13±0.007</td>
</tr>
<tr>
<td></td>
<td>0.12±0.008</td>
<td>0.117±0.004</td>
</tr>
<tr>
<td>TYR2</td>
<td>0.16±0.011</td>
<td>0.31±0.025</td>
</tr>
<tr>
<td></td>
<td>0.15±0.010</td>
<td>0.304±0.024</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>WT (n = 7)</td>
<td>WT (n = 8)</td>
</tr>
<tr>
<td>ARG2</td>
<td>0.34±0.025</td>
<td>0.40±0.021</td>
</tr>
<tr>
<td></td>
<td>0.349±0.025</td>
<td>0.405±0.021</td>
</tr>
<tr>
<td>CT3</td>
<td>0.16±0.010</td>
<td>0.28±0.030</td>
</tr>
<tr>
<td></td>
<td>0.136±0.007</td>
<td>0.125±0.008</td>
</tr>
<tr>
<td>PHE5</td>
<td>0.137±0.008</td>
<td>0.22±0.011</td>
</tr>
<tr>
<td></td>
<td>0.137±0.007</td>
<td>0.222±0.011</td>
</tr>
<tr>
<td>TYR2</td>
<td>0.16±0.011</td>
<td>0.31±0.025</td>
</tr>
<tr>
<td></td>
<td>0.15±0.010</td>
<td>0.304±0.024</td>
</tr>
</tbody>
</table>

Values are means ± SE. ARG2, 1-[guanidino-$^{15}$N]arginine; CT3, 1-[ureido-$^{13}$C-5,5-$^{15}$N]citrulline; PHE5, 1-[ring-$^3$H]$^3$phenylalanine; TYR2, 1-[ring-$^3$H]$^3$tyrosine.

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**Table 3. Whole body protein metabolism in male and female WT and OTC-deficient mice treated with saline or LPS**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>WT (n = 8)</td>
<td>WT (n = 9)</td>
</tr>
<tr>
<td></td>
<td>OTC (n = 9)</td>
<td>OTC (n = 8)</td>
</tr>
<tr>
<td>WB PB</td>
<td>25±1</td>
<td>36±3*</td>
</tr>
<tr>
<td></td>
<td>27±1</td>
<td>42±2↑</td>
</tr>
<tr>
<td>WB PS</td>
<td>20±1</td>
<td>18±3*</td>
</tr>
<tr>
<td></td>
<td>20±1</td>
<td>8±2↓</td>
</tr>
<tr>
<td>WB Net PB</td>
<td>5±1</td>
<td>19±2*</td>
</tr>
<tr>
<td></td>
<td>7±1</td>
<td>34±2↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>WT (n = 7)</td>
<td>WT (n = 8)</td>
</tr>
<tr>
<td></td>
<td>OTC (n = 7)</td>
<td>OTC (n = 8)</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WB PB</td>
<td>41±2</td>
<td>40±4</td>
</tr>
<tr>
<td></td>
<td>37±5</td>
<td>37±3</td>
</tr>
<tr>
<td>WB PS</td>
<td>30±1</td>
<td>20±3*</td>
</tr>
<tr>
<td></td>
<td>27±3</td>
<td>17±5</td>
</tr>
<tr>
<td>WB Net PB</td>
<td>11±1</td>
<td>19±3*</td>
</tr>
<tr>
<td></td>
<td>10±2</td>
<td>20±5</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Values are means ± SE in nmol·10 g·$^{-1}$·min$^{-1}$. WB, protein breakdown; PS, protein synthesis. Statistics (2-way ANOVA for basal differences; 3-way ANOVA includes LPS effects): G: $P < 0.05$ for genotype effect; L: $P < 0.05$ for LPS effect; S: $P < 0.05$ for sex effect; $L \times G$, $L \times S$, $P < 0.05$ for interactive effect. Symbols used to clarify statistical effects: *basal genotype effect; ↑ or ↓ for LPS effect (increase or decrease vs. control, respectively).
by the significant reduction and turnover of citrulline in both male and female animals. Citrulline deficiency resulted in diminished de novo arginine production (50% in OTCD mice vs. ∼25% in wild-type mice). As a result, circulating arginine decreased by ∼20% in males and 40% in females. Basal NO production was 50% lower in OTCD mice. However, the NO response to LPS was similar in OTCD mice and wild-type mice, which is probably related to the change in net protein breakdown and therefore arginine availability.

Mice model. The primary aim of using the OTC-deficient spfash mice was to determine the importance of the capacity to synthesize citrulline for arginine biosynthesis and NO production in healthy and LPS-treated mice. Although we did not study physiological effects (e.g., hemodynamic effects) related to the observed metabolic changes in the OTC-deficient spfash mice, overt signs of disease described under normal conditions are limited to growth retardation, abnormal skin and hair, hyperammonemia, and impaired cognition (1, 27). No genotype differences in cytokine levels have been detected in response to LPS in OTC-deficient spfash mice (27). In line with previous observations (28, 29) in OTC-deficient spfash mice of the B6 genetic background, we did not observe reduced ureagenesis in our model. Moreover, more pronounced effects of the genotype defect were present in the male mice on plasma glutamine levels and de novo arginine production, related to the X-linked genetic defect.

The stable-isotope method used to measure NO production in this study yields different results than the NO production derived from plasma nitrate/nitrite measurements. This discrepancy was described before (26) and is in line with the observation by Beaumier et al. (2) who observed that only ∼30% of urinary nitrate production is derived from arginine. By calculating NO production under the condition of isotopic steady state, intracellular recycling of the tracer will have minor effects on isotopic calculations.

In this model of endotoxemia, plasma amino acid levels including plasma arginine were increased at 6 h after LPS injection, both in wild-type and OTCD mice. This is probably related to the LPS-induced increase in net protein breakdown (at least in the male OTCD mice), in line with our previous studies (4, 18), combined with the diminished arginine clearance that we observed during LPS in this study for both groups.

Correlation between citrulline and arginine availability and NO production. Citrulline production, citrulline plasma levels, de novo arginine production, arginine plasma levels, and NO production were all lower in OTCD mice in our study. Although net protein breakdown was increased in OTCD mice, this could not restore NO production under basal conditions, probably because plasma arginine concentrations were not restored. Increased net protein breakdown after LPS coincided with an increase in plasma arginine and elevated NO production. Combining all data in wild-type and OTC-deficient mice showed a positive relation between NO production and circulating arginine levels. Several other lines of evidence indicate that the endogenous production of the bulk of NO by NOS is dependent on the extracellular concentration of arginine and
cellular arginine transport (47). However, some intracellular
resynthesis of arginine from citrulline also occurs (7). The
CAT1 and CAT2B transporters, which are associated with NO
production by NOS1 and NOS3, and NOS2, respectively (30,
35), have a \( K_m \) of 100–150 \( \mu M \) (12). In view of the much
lower \( K_m \) of NO for arginine (2–20 \( \mu M \)), our in vivo data
underscore the hypothesis that if NOS enzyme concentration
is not made limiting, NO production in vivo is mainly determined
by the rate of arginine uptake into the cells. This seems in line
with lysine-induced suppression of arginine transport by CAT1
and subsequent inhibitory effects on endothelial NO produc-
tion with functional effects on blood flow (51). Acute reduction
of circulating arginine by intravenous arginine administration
did not compromise basal or LPS-induced NO production in
male Swiss mice (21), probably due to maintained tissue
arginine levels (21). In OTC deficiency, tissue arginine levels
are reduced (33). This suggests that intracellular arginine (i.e.,
intracellular arginine transport or production) is important
for NO production.

Intracellular (de novo) arginine regenerated from citrulline
has been linked specifically to NO production by NOS3 (22,
36), which is important under basal conditions (16). Although
we could not establish a direct correlation between de novo
arginine production and NO production (not shown), such a
direct correlation did exist between whole-body citrulline
production and NO production in the present study. However,
further mass studies are needed to identify the source of
citrulline. Although citrulline produced via the OTC pathway
is the major source, citrulline production via the NOS pathway
also contributes ~10% to whole body citrulline production.

Extracellular arginine, which is important for NOS2-medi-
ated NO production (7, 36), is increased by LPS due to
increased net protein breakdown and seems adequate for in-
creased NO production in OTCD mice as well. This fits with
the correlation observed between circulating arginine and NO
production. Uptregulation of CAT2 mRNA, as has been ob-
served for renal glomeruli in LPS-treated rats (35) and macro-
phages in culture (7), may facilitate arginine uptake and further
enhance NO synthesis under proinflammatory conditions.

Our present data thus indicate that basal NO synthesis is
probably affected by a local (intracellular) decrease of arginine
de novo synthesis in NO-synthesizing cells due to deficient
citrulline availability. NO production after LPS is unaffected
by deficient citrulline availability, probably due to increased
arginine availability from net protein breakdown.

Sex-related NO production. Although basal NO production
is higher in female than in male mice, NO production is
reduced by OTC deficiency in both sexes (an ~50% reduction
in basal NO production). The lower NO production in male
mice coincides with a lower whole-body citrulline and de novo
arginine production compared with female mice, which may be
explained by the X-linked OTC pathway (44) that affects
citrulline and de novo arginine production to a greater extent
in male than in female OTC-deficient mice. These findings of a
larger effect of OTC deficiency in male mice reveal that a
gene-dosage effect emerges when an enzyme that normally
exhibits a very high molecular specific enzyme activity incurs
a severe mutation.

The sex-dependent gene-dosage effect on NO synthesis was
no longer seen after LPS treatment since all males increased
NO production. The adaptation to LPS was sex dependent,
since female mice did not increase their net protein breakdown,
only slightly increased plasma arginine, while NO production
was not significantly increased.

In conclusion, reduced citrulline availability due to OTC
deficiency has major effects on basal NO production, probably
due to diminished de novo arginine production. In contrast,
OTC deficiency does not impair the increase in NO production
in response to endotoxemia, probably because circulating argi-
ine levels also increased in endotoxemic OTCD mice as a
result of increased net protein breakdown. The apparent dif-
ferences between sexes could be attributed to a gene-copy
effect of the X-linked OTC gene and provides a basis to sort
out the effects of sex hormones on arginine and NO metabo-
лизм. While our data clearly show that citrulline, de novo
arginine, and NO production decline when OTC activity is
impaired, it remains to be investigated whether OTC is subject
to regulation under pathological conditions. Citrulline supple-
mentation could be a potential therapeutic strategy to increase
(basal) NO production, but this requires further study.

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