Acute metabolic decompensation due to influenza in a mouse model of ornithine transcarbamylase deficiency

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ABSTRACT
The urea cycle functions to incorporate ammonia, generated by normal metabolism, into urea. Urea cycle disorders (UCDs) are caused by loss of function in any of the enzymes responsible for ureagenesis, and are characterized by life-threatening episodes of acute metabolic decompensation with hyperammonemia (HA). A prospective analysis of interim HA events in a cohort of individuals with ornithine transcarbamylase (OTC) deficiency, the most common UCD, revealed that intercurrent infection was the most common precipitant of acute HA and was associated with markers of increased morbidity when compared with other precipitants. To further understand these clinical observations, we developed a model system of metabolic decompensation with HA triggered by viral infection (PR8 influenza) using spf-ash mice, a model of OTC deficiency. Both wild-type (WT) and spf-ash mice displayed similar cytokine profiles and lung viral titers in response to PR8 influenza infection. During infection, spf-ash mice displayed an increase in liver transaminases, suggesting a hepatic sensitivity to the inflammatory response and an altered hepatic immune response. Despite having no visible pathological changes by histology, WT and spf-ash mice had reduced CPS1 and OTC enzyme activities, and, unlike WT, spf-ash mice failed to increase ureagenesis. Depression of urea cycle function was seen in liver amino acid analysis, with reductions seen in aspartate, ornithine and arginine during infection. In conclusion, we function was seen in liver amino acid analysis, with reductions seen in aspartate, ornithine and arginine during infection. In conclusion, we

INTRODUCTION
The urea cycle (UC; supplementary material Fig. S1) is present only in the liver and serves two purposes: (1) the de novo biosynthesis and degradation of arginine, and (2) the incorporation of excess nitrogen into urea. UC disorders (UCDs) are caused by loss of function in any of the enzymes responsible for ureagenesis, and are characterized by life-threatening episodes of acute metabolic decompensation with hyperammonemia (HA). The incidence of these disorders has been estimated at 1 in 30,000 live births (Summar and Tuchman, 2001). UCD can be considered in two groups. In proximal (mitochondrial) UCD [N-acetylglutamate synthetase (NAGS), carbamoyl phosphate synthetase 1 (CPS1) and ornithine transcarbamylase (OTC) deficiencies], ammonia disposal is severely compromised. In distal (cytosolic) UCD [argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG1) deficiencies], ammonia disposal is not as severely impaired and characteristic amino acid metabolites accumulate.

Acute metabolic decompensation with HA in UCDs is precipitated by dietary non-adherence, enhanced protein catabolism due to protein and/or caloric over-restriction, or intercurrent infection (Summar et al., 2008). Intercurrent infection, particularly with respiratory viruses, is the most common trigger of HA, accounting for >34% of episodes in a cohort of UCD subjects (Summar et al., 2008; Tuchman et al., 2008). Although it has not been systematically examined, intercurrent infection is suspected to result in greater morbidity versus other precipitants, based on anecdotal clinical experience (Singh et al., 2005). For UCDs, infection results in elevated plasma ammonia of longer duration when compared to other precipitants, resulting in prolonged hospital stays and increased utilization of medical resources. The current paradigm for acute HA treatment centers on addressing the increased whole-body protein catabolism brought on by protein and/or caloric insufficiency that can occur in dietary over-restriction or intercurrent infection. Reversing catabolism by drastically increasing parenteral caloric intake, regardless of the precipitant, has been the mainstay of acute HA crisis treatment (Singh et al., 2005). However, the pathophysiological processes behind different HA precipitants might be distinct, raising the possibility of targeted therapies that could alter the hospital course of UCD patients.

Given the well-recognized propensity of individuals with UCDs to experience severe and at times fatal HA, a translational approach was undertaken to develop a model of acute HA due to infection in UCD and describe the mechanisms of acute metabolic decompensation. To explore the etiology, clinical characteristics and morbidity associated with HA events, the longitudinal database of the Urea Cycle Disorders Consortium (UCDC) was queried to assess the severity of HA experienced by patients with the most common UCD, OTC deficiency. Infection was noted to be the most common precipitant of acute HA, with indicators of increased morbidity being present compared with other precipitants. To

Received 28 May 2013; Accepted 18 November 2013
TRANSLATIONAL IMPACT

Clinical issue
The urea cycle functions to incorporate ammonia, generated by normal metabolism, into urea. Urea cycle disorders (UCDs), which fall under the category of inborn errors of metabolism, are caused by loss-of-function mutations in any of the enzymes responsible for ureagenesis, and are characterized by potentially life-threatening episodes of acute metabolic decompensation with hyperammonemia (HA). Acute HA in UCD can be precipitated by any factor that affects metabolic balance, such as: dietary indiscretion, enhanced protein catabolism due to dietary over-restriction, or infection. Intercurrent infection is the most common precipitant of acute HA, with respiratory viruses being a leading cause. The aim of this study was to explore perturbations in UC function as experienced by UCD patients during infection.

Results
In a prospective analysis of a cohort of patients with ornithine transcarbamylase deficiency (OTCD), the most common UCD, the authors identified infection as the most common identifiable cause of acute decompensation with HA, and this was associated with markers of increased morbidity. To further understand these clinical observations, the authors developed a model system of metabolic decompensation with HA triggered by viral infection [influenza A/Puerto Rico/8/34 (PR8) virus] using spf-ash mice, an animal model of OTCD. In response to infection with PR8, spf-ash mice displayed an altered hepatic immune response. Unlike wild-type (WT) mice, spf-ash mice also displayed elevated liver transaminases, suggesting increased hepatic sensitivity to infection. Despite having no visible pathological changes detectable by histology, WT and spf-ash mice showed reduced activities of the first two enzymes of the urea cycle: carbamoyl phosphate synthetase 1 and ornithine transcarbamylase. In addition to these enzyme perturbations, spf-ash mice showed increased hyperammonia and, in contrast to WT mice, failed to increase ureagenesis during infection. Liver amino acid analysis revealed further perturbations in urea cycle function, with reductions seen in the intermediates aspartate, ornithine and arginine during infection.

Implications and future directions
These results provide insights into the metabolic perturbations triggered by influenza infection in the presence of UCD, including a reduction in levels of urea cycle intermediates. The findings could be important for the development of new approaches for the management of acute HA in UCDs. Regardless of the nature of the acute HA precipitant, the current medical management strategy is the same: to abolish protein intake and supplement with high caloric intake. These measures are not always successful. The experimental model used here could provide a platform for evaluating the efficacy of urea cycle intermediates alone or in combination with immune modifiers. This model could also be adapted to explore acute decompensation and the efficacy of treatments in other types of inborn errors of metabolism.

Development of a model of acute HA due to viral illness
Because of the distinct epidemiological parameters of infection in UCD (Fig. 1), we next sought to develop an in vivo model of acute HA due to infection. Two well-characterized experimental systems – the spf-ash mouse and the mouse-adapted PR8 influenza virus – were combined to create a murine model of viral infection in the setting of a UCD. Spf-ash and WT mice were infected on Day 0 using an infection aerosolization apparatus and euthanized on Day 5 (supplementary material Fig. S2A,B). spf-ash and WT challenged with PR8 were observed to have ruffled fur and decreased social and grooming behaviors beginning at Day 2-3 of the infection protocol (supplementary material Fig. S2A, gray shading). Protein intake, assessed by weighing the food daily, was recorded during the course of infection and expressed on a g/kg body weight/day basis (Fig. 2A). spf-ash mice had a lower body weight and protein intake (Day 1, 45.2 g/kg/day) compared with WT (136.6 g/kg/day) and had much lower body weights at baseline, typically weighing 20% less than sex- and age-matched littermates. Both groups decreased their protein intake over the course of the infection, with WT demonstrating a greater decrease (81.6%) versus spf-ash (72.8%) by Day 5. WT animals also lost a greater percentage of body weight at Day 5, likely reflecting greater body stores at baseline (Fig. 2A); controls were 97.4% of
starting body weight, whereas spf-ash were 101.3% of starting body weight \((P=0.01)\). From Day 2-5, with onset of sickness behaviors, both WT and spf-ash lost weight at similar rates.

**spf-ash and WT mice have similar lung infectious parameters**

Although spf-ash and WT animals both displayed physiological and behavioral characteristics suggestive of viral infection, we assessed viral titers and cytokine profiles in the lungs to confirm that infectious parameters were similar between the strains. Viral titers (Fig. 2B) were measured on Day 5 for lung homogenates using serial dilutions and measuring infectivity in Madin-Darby canine kidney (MDCK) epithelial cells. Whole lungs from infected WT and spf-ash mice displayed no differences in viral titers on Day 5 of infection \((P=0.77)\). Lung cytokine profiles were determined using a fluorescent-bead-based multiplex assay (Table 1). Both WT and spf-ash showed a robust response to viral infection with significant increases in a number of colony-stimulating, chemotactic and inflammatory cytokines [Infection (Inf), \(P<0.05\)]. No significant genotype differences were seen [Genotype (Gen), \(P>0.05\)]. With the exception of lower GM-CSF concentrations at Day 5 in spf-ash [Inf \(\times\) Gen, \(P=0.031\)], all remaining infection \(\times\) genotype interactions were not statistically significant [Inf \(\times\) Gen, \(P>0.05\)]. Thus, WT and spf-ash displayed similar infectious and inflammatory parameters during PR8 influenza infection.

**Hepatic response during PR8 infection**

It is well documented that humans and certain mouse strains can develop elevated serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) during respiratory viral infection (Polakos et al., 2006). However, whether these signs of hepatic insult translate into alterations in UC function has not been studied. Given the similar parameters of PR8 infection in both WT and spf-ash, we performed further biochemical studies to evaluate hepatic metabolic function (Fig. 3). spf-ash mice displayed greater hepatocyte sensitivity to infection, with increases in serum AST 3× over control animals (Fig. 3A, \(P=0.007\)). Serum ALT was on average 2× higher than controls, although this finding was not significant (Fig. 3A, \(P=0.25\)). Furthermore, the mean AST:ALT ratio in the infected spf-ash was 5.4 (Fig. 3A, \(P=0.01\)), suggesting an exogenous insult as the cause of the hepatitis. Acute metabolic decompensation due to intercurrent illness in patients with UCD is characterized by HA. Consistent with the previous studies in these mice, plasma ammonia was 3 to 4× greater at baseline in spf-ash mice compared with WT. However, during PR8 infection, plasma ammonia increased over 100 \(\mu\)g/dl in some animals \((P=0.04)\), whereas WT levels remained unchanged from baseline (Fig. 3B).

With significant elevations of markers of hepatic damage, liver histology was investigated in infected spf-ash and controls. Significant inflammation, necrosis and apoptosis were absent in all the liver samples (supplementary material Fig. S3). Although the increase in liver transaminases and ammonia seen in the plasma were suggestive of Reye syndrome in the spf-ash mice, macrovesicular [hematoxylin and eosin (H&E) stain, supplementary material Fig. S3A] and microvesicular (oil red O stain with baking, supplementary material Fig. S3C) steatosis were absent.

Analysis of viral load by qRT-PCR (data not shown) and measurement of viral titer (Fig. 3C) showed that no PR8 virus was
present in spf-ash livers. Despite a lack of virus detection in unperfused livers, spf-ash livers showed signs of hepatic sensitivity and damage (Fig. 3A). Profiling of the hepatic response to lung PR8 infection in WT B6 mice using mRNA expression arrays showed significant increases in the acute phase response and antiviral response pathways (our unpublished data). To examine the hepatic response to PR8 infection, we profiled a select panel of expressed response pathways (our unpublished data). To examine the hepatic response to lung PR8 infection in WT B6 mice using mRNA expression arrays showed significant increases in the acute phase response and antiviral response genes in WT and spf-ash mice during infection (Fig. 3D). Both WT and spf-ash showed a robust increase in serum amyloid A1 (SAA1) and lipocalin 2 (LCN2), markers of the acute-phase response (P<0.05 for both). However, when profiling the hepatic antiviral immune response, some discrepancies emerged. spf-ash showed elevations in TLR3, TLR7, MDA5 and RIG-I at baseline and, unlike WT, failed to show a clear differentiation in levels of expression with infection. Overall, these data suggest that spf-ash have an abnormal hepatic immune response to infection and, in some instances, increased activation of antiviral pathways at baseline.

**CPS1 and OTC enzyme activities are reduced in WT and spf-ash during infection**

In the setting of biochemical abnormalities indicating hepatitis with increased HA, we hypothesized that perturbations in hepatic nitrogen metabolism might be exacerbated during infection. Previous studies have indicated altered mitochondrial UC enzyme function in the setting of influenza infection (Pierson et al., 1976) but did not account for protein intake, which we find varies between WT and spf-ash animals. Because the expressions of UC enzymes are responsive to dietary signals (Snodgrass, 2004), all animals were housed individually and matched for protein intake on a g/kg body weight/day basis during the 5 days of infection to correct for protein-intake differences (Fig. 2A). Protein matching resulted in a factorial design (supplementary material Fig. S4A,B). Under this caloric-restriction regimen, weight loss in all experimental groups was similar on Day 5 (supplementary material Fig. S4C). To assess the depression of mitochondrial UC function, CPS1 and OTC activities were measured in liver homogenates (Fig. 4A). During infection, CPS1 activity decreased by 40% (P=0.001) in WT and 28% (P=0.018) in spf-ash, whereas OTC activity decreased by 7% (P=0.005) in WT and 21% (P=0.015) in spf-ash (Fig. 4A). These results suggest that reduction of CPS1 and OTC enzyme activities are part of the normal hepatic physiology of PR8 infection, which might not be tolerated by a compromised UC in spf-ash.

To further characterize the mechanism of decreased enzyme activity, CPS1 and OTC were quantified for protein expression differences (Fig. 4B). Surprisingly, CPS1 protein levels were threefold higher in spf-ash compared with WT, and infection had no significant effect on CPS1 protein levels in either the WT (P=0.787) or spf-ash (P=0.804) animals. The amount of OTC tended (P=0.086) to be reduced in WT littermates during infection, whereas OTC protein levels remained unchanged in the spf-ash mice.

**Table 1. Lung cytokines produced during acute infection with PR8**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>WT</th>
<th>Control</th>
<th>Infected</th>
<th>spf-ash</th>
<th>Control</th>
<th>Infected</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFa</td>
<td></td>
<td>76.2 (16.3)</td>
<td>308.1 (33.2)</td>
<td>73.9 (8.5)</td>
<td>294.6 (91.1)</td>
<td>&lt;0.0001</td>
<td>0.786 0.846</td>
</tr>
<tr>
<td>IFNγ</td>
<td></td>
<td>96.5 (29.1)</td>
<td>1713.0 (1294.4)</td>
<td>68.7 (6.9)</td>
<td>1520.3 (1865.4)</td>
<td>0.029</td>
<td>0.862 0.896</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td>1284.9 (321.1)</td>
<td>14034.6 (4586.1)</td>
<td>552.6 (453.2)</td>
<td>7269.5 (5056.9)</td>
<td>&lt;0.0001</td>
<td>0.059 0.119</td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td>275.6 (99.8)</td>
<td>190.8 (83.8)</td>
<td>208.1 (68.7)</td>
<td>167.6 (104.9)</td>
<td>0.183</td>
<td>0.327 0.626</td>
</tr>
<tr>
<td>IL-12p40</td>
<td></td>
<td>265.0 (66.7)</td>
<td>288.0 (88.9)</td>
<td>172.6 (47.7)</td>
<td>313.3 (153.7)</td>
<td>0.151</td>
<td>0.541 0.292</td>
</tr>
<tr>
<td>IL-17</td>
<td></td>
<td>179.6 (87.1)</td>
<td>82.9 (14.4)</td>
<td>94.1 (13.0)</td>
<td>91.6 (44.5)</td>
<td>0.054</td>
<td>0.124 0.065</td>
</tr>
<tr>
<td>MIP-1α</td>
<td></td>
<td>481.7 (55.1)</td>
<td>4848.8 (727.1)</td>
<td>411.8 (108.1)</td>
<td>4589.9 (1772.5)</td>
<td>&lt;0.0001</td>
<td>0.773 0.869</td>
</tr>
<tr>
<td>RANTES</td>
<td></td>
<td>1058.8 (448.9)</td>
<td>9175.1 (5408.0)</td>
<td>891.0 (267.7)</td>
<td>7086.3 (3016.3)</td>
<td>&lt;0.0001</td>
<td>0.454 0.522</td>
</tr>
<tr>
<td>KC</td>
<td></td>
<td>845.2 (434.7)</td>
<td>21446.1 (6871.1)</td>
<td>661.8 (156.2)</td>
<td>13785.9 (6007.1)</td>
<td>0.0001</td>
<td>0.115 0.131</td>
</tr>
<tr>
<td>MCP-1</td>
<td></td>
<td>530.9 (388.6)</td>
<td>25234.8 (4222.9)</td>
<td>419.4 (96.0)</td>
<td>18670.0 (5359.4)</td>
<td>&lt;0.0001</td>
<td>0.094 0.104</td>
</tr>
<tr>
<td>G-CSF</td>
<td></td>
<td>315.5 (97.1)</td>
<td>22859.6 (10797.4)</td>
<td>255.0 (28.8)</td>
<td>15528.5 (8778.7)</td>
<td>&lt;0.0001</td>
<td>0.308 0.316</td>
</tr>
<tr>
<td>GM-CSF</td>
<td></td>
<td>100.6 (142.7)</td>
<td>1370.1 (188.6)</td>
<td>238.8 (199.1)</td>
<td>925.3 (304.5)</td>
<td>&lt;0.0001</td>
<td>0.223 0.031</td>
</tr>
<tr>
<td>M-CSF</td>
<td></td>
<td>585.5 (160.3)</td>
<td>1075.2 (608.1)</td>
<td>322.9 (24.9)</td>
<td>775.5 (296.7)</td>
<td>0.012</td>
<td>0.103 0.909</td>
</tr>
</tbody>
</table>

Values for WT and spf-ash are shown as mean pg/g tissue, with s.d. in parentheses. WT and spf-ash mice were infected with PR8 influenza by aerosolization and sacrificed on Day 5 (n=4/group). Cytokine determinations were made in lung homogenates using a multiplex assay. Inf, infection; Gen, genotype. Univariate analysis with P<0.05 (shaded cells).
Given the increased immunoreactive CPS1 in the spf-ash liver extracts, we hypothesized that the hepatocytes had an increase in either protein content or number as a compensation for OTC deficiency. To determine whether there was an increase in mitochondrial number, we examined livers from WT and spf-ash by electron microscopy on Day 5 of infection (Fig. 4C). WT and spf-ash displayed occasional mitophagy and, on average, similar numbers of mitochondria of normal morphology.

**Altered nitrogen disposal in spf-ash during infection**

Given the demonstration of mitochondrial UC dysfunction by enzymology, stable isotopic tracing was employed to assess alterations in ureagenesis. Using a standard published protocol, enrichment of plasma $^{15}$N-urea was determined following an intraperitoneal (IP) injection of a single dose of $^{15}$N-ammonium chloride (4 mmol/kg body weight; $^{15}$NH$_4$Cl) on Day 5 (Fig. 4D). The end product of ammonia disposal, plasma $[^{15}$N]urea, was enriched in WT mice on Day 5 of infection ($P=0.03$). Conversely, plasma $[^{15}$N]urea enrichment was not only lower in spf-ash mice during the uninfected state but there was also a failure to increase incorporation of $^{15}$NH$_3$ into urea during the infected state ($P<0.01$).

Given this failure to increase ureagenesis, we next examined free amino acids in liver homogenates to evaluate UC intermediates (Table 2). spf-ash had elevated ornithine (Gen, $P=0.01$), aspartate (Gen, $P=0.024$) and arginine (Gen, $P<0.01$) in the absence of infection. However, a depression of these levels was observed during infection, suggesting a strong genotype × infection interaction (ornithine Gen × Inf, $P=0.018$, aspartate Gen × Inf, $P=0.015$ and arginine Gen × Inf, $P<0.01$). Thus, during infection, perturbations in UCD function can be seen in spf-ash at the enzyme and metabolite levels.

**DISCUSSION**

Life-threatening acute HA is a significant source of morbidity and mortality for patients with UCD, who experience 2.2-2.8 episodes per year on average, depending upon the underlying enzymatic defect (Summar et al., 2008). Prospective analyses of the OTC cohort presented here revealed that infectious precipitants led to increased hospitalization rates (Fig. 1C) and length of hospital stay (Fig. 1D). Consistent with our findings, a recent paper found that inborn errors of metabolism in general are an independent risk factor for hospitalization due to respiratory illnesses such as respiratory
Syncytial virus (RSV) infection (Kristensen et al., 2012). In addition to increased hospitalization rates and length of stay, infection-associated HA was also accompanied by increased utilization of IV ammonia scavengers (Fig. 1E). Because IV ammonia scavengers are usually reserved for patients with hyperammonemic encephalopathy, this surrogate marker of severity suggests increased morbidity when this patient population is exposed to infectious precipitants. In light of these parameters suggestive of increased morbidity, we aimed to investigate the pathophysiology underlying acute HA induced by infection.

We hypothesized that infection would lead to activation of the immune system with concomitant perturbations in UC function. The factorial design (supplementary material Fig. S4A) adopted in the present study allowed the effects of infection to be assessed in isolation of those related to dietary insufficiency, another common precipitant of HA. In this respect, although dietary insufficiency is present in both conditions, our results clearly indicate that there are distinct metabolic sequelae due to infection.

Although spf-ash and WT mice displayed similar markers of lung inflammation (Table 1), the hepatic immune response in spf-ash showed some key differences (Fig. 3D). Although virus was not detected in WT livers (Fig. 2B), mRNA elevations in TLR3, TLR7, MDA5, RIG-I and IRF7 were seen in response to infection. Activators of these pathways include the pathogen-associated molecular patterns (PAMPs) dsRNA and ssRNA, which can be seen during viral infection (Seki and Brenner, 2008). In addition to PAMPs, these pathways can be activated in response to dying host cells by damage-associated molecular patterns (DAMPs; e.g. dsRNA, ssRNA, mtDNA). Our findings in WT lead us to suggest that the liver is either reacting to undetectable levels of PR8 virus in the blood and/or liver sinusoidal spaces, or tissue damage from the lung. Interestingly, spf-ash display elevations in AST, ALT and markers of innate immune activation in the uninfected state (Fig. 3D). These data suggest that spf-ash might be experiencing baseline liver damage with innate immune activation via DAMPs. The baseline immune activation might also account for the hepatic sensitivity seen with infection (AST and ALT, Fig. 3A) and remains to be explored.

A previous study examining the effect of influenza infection on CPS1 and OTC activities in WT B6 mice showed appreciable reductions in CPS1 (12%) and OTC (17%) enzyme activities (Pierson et al., 1976). In the present study, we observed a greater reduction in CPS1 activity likely due to the intake-matching strategy applied (Fig. 4). These findings imply that a reduction in CPS1 and OTC activities is part of the normal physiological response to PR8 infection and might compound the pre-existing OTC deficiency in spf-ash. Interestingly, despite lower enzyme activity, immunoreactive CPS1 was actually increased in spf-ash mice with normal mitochondrial number and morphology (Fig. 4B,C). Indeed, spf-ash liver contains 33% more mitochondrial protein per gram of liver when compared with WT (Cohen et al., 1989). Nonetheless, despite greater CPS1 protein, spf-ash CPS1 enzyme activity was 15% lower in spf-ash mice. Our results suggest that, even in the uninfected state, a proportion of the CPS1 pool is inactive or subactive in spf-ash mice. This in vitro reduction in CPS1 activity could be related to altered post-translational modifications such as lysine acylation or acetylation (Duke-Sylvester et al., 2011; Nakagawa et al., 2009), which further suggests an avenue of investigation.

In our model, spf-ash mice seem to have lower ureagenesis during dietary restriction (Fig. 4D); however, these findings were not significant. More importantly, unlike WT, spf-ash failed to increase ureagenesis during infection (Fig. 4D) and experienced HA (Fig. 3B). This failure of increased ureagenesis was due to a depression in the pool of UC intermediates during infection.
acid-oxidation defects. explore acute decompensation due to infection in other types of precipitant. In addition, this model system could be adapted during acute metabolic decompensation in UCD due to a common

describing biochemical perturbations and the efficacy of treatments into their efficacy. Overall, this model might serve as a platform for infection while supplementing these amino acids will provide insight

ornithine in our model, formal studies on HA and ureagenesis during therapeutic standpoint, given the depletion of hepatic arginine and
decomposition due to infection in other types of

Brenner, 2008; Tiao et al., 1995).

This efficacy of ornithine seems to be related to an increase in OTC

ornithine aminotransferase might also be effective (Li et al., 1999).

parenteral nitrogen loading (Marini et al., 2006a). Inhibition of

supplementation restored ureagenesis and mitigated HA during

nitrogen load can sustain ureagenesis in the setting of a hypomorphic OTC enzyme. As further evidence, supplementation with UC intermediates can also prevent ammonia toxicity after a lethal dose of ammonia (Ben-Ari et al., 2010; Matsuda et al., 1996) or an unbalanced nitrogen load (Marini et al., 2006b). When challenged with an unbalanced nitrogen load, profound HA and decreased ureagenesis follows. These results suggest that the prevision of UC intermediates in the balanced nitrogen load can sustain ureagenesis in the setting of a hypomorphic OTC enzyme. As far as evidence, supplementation with UC intermediates can also prevent ammonia toxicity after a lethal dose of ammonia (Ben-Ari et al., 2010; Matsuda et al., 1996) or an unbalanced nitrogen load (Marini et al., 2006b). Of particular interest is ornithine. In B6 mice, an IP challenge with ammonium chloride results in elevations in hepatic ornithine within 5 minutes of injection, suggesting its importance in the incorporation of ammonia (Saheki et al., 1997). In spf-ash primary hepatocytes, ornithine increased ureagenesis and reduced orotic acid production (Moscioni et al., 2006). In the spf-ash mouse, ornithine supplementation restored ureagenesis and mitigated HA during parenteral nitrogen loading (Marini et al., 2006a). Inhibition of ornithine aminotransferase might also be effective (Li et al., 1999).

This efficacy of ornithine seems to be related to an increase in OTC and CPS1 activities, and a decrease in carbamoyl phosphate degradation (Ben-Ari et al., 2010; Neill et al., 2009; Seki and Brenner, 2008; Tiao et al., 1995).

In conclusion, we have developed a model of acute metabolic decompensation due to infection in the spf-ash mouse. From a therapeutic standpoint, given the depletion of hepatic arginine and ornithine in our model, formal studies on HA and ureagenesis during infection while supplementing these amino acids will provide insight into their efficacy. Overall, this model might serve as a platform for describing biochemical perturbations and the efficacy of treatments during acute metabolic decompensation in UCD due to a common precipitant. In addition, this model system could be adapted to explore acute decompensation due to infection in other types of inborn errors of metabolism such as organic acidemias and fatty-acid-oxidation defects.

### MATERIALS AND METHODS

#### Infection with A/PR/8/34 (PR8)

The experiments outlined were performed on B6 × B6EiC3Sn Und−/− mouse. Influenza A/PR/8/34 (PR8) virus was propagated in MDCK cells (cell line 104.1; ATCC) and titered in MDCK cells in 96-well plates by plaque assay using the method of Cullen et al. (1998). Mice were infected by intranasal instillation of a 10% (vol/vol) virus inoculum (previously quantitated on MDCK cells) as previously described (Fernandez-Sesma et al., 2006). 4- to 6-week-old spf-ash and littermate control mice were exposed to an infective dose of PR8 of 500 TCID50 in an aerosolization chamber (Glas-Col, Terre Haute, IN) (Moltedo et al., 2009). Mice were sacrificed on Day 0 and Day 5 of infection by 5% isoflurane inhalation with cervical dislocation. Plasma, serum and tissues were separated from whole blood and stored at −80°C until use. All animal care and procedures were carried out according to the criteria outlined in the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985) and were authorized by the Animal Care and Use Committees of the National Human Genome Research Institute and the Institutional Animal Care and Use Committee of the Mount Sinai School of Medicine.

#### Viral lung titer

Viral titers were determined using a published assay based on the infection of MDCK cells (Oh et al., 2000). The inverse of the dilution at which 50% of the wells showed cytopathic effect was recorded as the 50% tissue culture infectious dose (TCID50).

#### Lung cytokines

On Day 5 of infection, lungs were removed and immediately homogenized with a TissueRuptor (Qiagen, Valencia, CA) hand-held rotor-stator homogenizer in PBS. Cytokine detection in clarified tissue lysates was performed using a mouse cytokine panel (Millipore, Billerica, MA) with detection using a multiplex platform (Luminex, Austin, TX). Cytokine amounts were normalized to lung weight in grams.

#### Histology

H&E staining was performed on sectioned paraffin-embedded lung and liver tissue by the Department of Pathology, Mount Sinai Medical Center. Detection of apoptotic cells was performed using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA).

#### Biochemical studies

Plasma ammonia was determined using a glutamate-dehydrogenase-based assay according to manufacturer instructions (Sigma-Aldrich). Serum aspartate aminotransferase, alanine aminotransferase and urea determination were performed in the Center for Comparative Medicine and Surgery, Mount Sinai School of Medicine. Liver amino acids were quantified by ion exchange chromatography using a Biochrom 30 Amino Acid Analyzer (Biochrom, Cambridge, UK). Amino acid concentrations were calculated as µmol/100 gram of tissue and expressed as ratios relative to controls.

#### Measurement of ureagenesis

Mice were administered a dose of nitrogen as (15N)-labeled ammonium chloride according to experiments described previously (Cunningham et al., 2009; Plata-Salaman, 1998). After a 3-hour fast, mice received 4 mmol/kg body weight of 15NH4Cl (Cambridge Isotope Laboratories, Andover, MA).

### Table 2. Liver amino acids during acute infection

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>WT Control</th>
<th>Infected</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>291.4 (47.3)</td>
<td>419.7 (6.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ala</td>
<td>377.8 (69.1)</td>
<td>386.2 (108.0)</td>
<td>0.292</td>
</tr>
<tr>
<td>Gly</td>
<td>273.7 (37.5)</td>
<td>269.7 (35.3)</td>
<td>0.727</td>
</tr>
<tr>
<td>Glu</td>
<td>123.4 (12.5)</td>
<td>130.8 (42.9)</td>
<td>0.124</td>
</tr>
<tr>
<td>Orn</td>
<td>50.2 (8.6)</td>
<td>55.5 (5.1)</td>
<td>0.02</td>
</tr>
<tr>
<td>Cit</td>
<td>5.8 (0.8)</td>
<td>4.1 (0.5)</td>
<td>0.015</td>
</tr>
<tr>
<td>Asp</td>
<td>356.4 (21.8)</td>
<td>288.5 (32.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Arg</td>
<td>1.8 (0.3)</td>
<td>1.1 (0.2)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values for WT and spf-ash are shown as mean µmol/100 g wet weight, with s.d. in parentheses. For liver amino acids, livers were homogenized in sulfosalicylic acid and were determined by an amino acid analyzer (n=4/group). Inf, infection; Gen, genotype. Univariate analysis with P<0.05 (shaded cells).
by IP injection. Heparinized blood was collected by retro orbital bleeding 20 minutes after injection, and the plasma analyzed for the % of $^{15}$N isotope enrichment of urea by gas chromatography/mass spectrometry (GC-MS) (Galloway et al., 2000).

**OTC and CPS1 enzyme assays**

OTC enzyme activity was measured using a published colorimetric assay that detects the formation of L-citrulline (Pastralanidis et al., 1981). CPS1 enzymatic activity was performed using lysates prepared as in the OTC assay according to a published assay (Chan et al., 2009).

**qRT-PCR**

Lever tissue was thawed and homogenized in RIPA buffer on ice. DNA and RNA was extracted from homogenized liver tissue or cell pellets using a kit (Qiagen). For RNA, 1 μg was reverse transcribed to cDNA using a modified MMLV-reverse transcriptase (iScript, Bio-Rad, Hercules, CA). Real-time quantitative PCR reactions were carried out in 50 μl using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) or TaqMan systems (Applied Biosciences, Carlsbad, CA). Reactions were cycled and quantitated with an ABI 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA).

**Western blot analysis**

For western blot analysis, 30 μg of protein was loaded on 4-20% Tris-glycine polyacrylamide gels. The gels were transferred to polyvinilidene difluoride membrane using the iBlot Dry Blotting System (Life Technologies, Grand Island, NY). The membranes were blocked and probed with primary antibodies according to the manufacturers’ suggested dilutions: CPS1 (Abcam, Cambridge, MA), OTC (Novus Biologicals, Littleton, CO) and β-actin (Sigma-Aldrich, St Louis, MO). Incubation was done with appropriate secondary antibodies. Image analyses were performed using an Odyssey Imager (Li-Cor, Lincoln, NE).

**Electron microscopy**

Mouse livers (1 mm$^3$) were fixed overnight at 4°C in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and washed with cacodylate buffer three times, washed with water and placed in 1% saturated uranyl acetate in 50% methanol and then with lead citrate. The tissues were fixed with 2% OsO$_4$ for 2 hours, washed again with 0.1 M cacodylate buffer (pH 7.4) and washed with cacodylate buffer three times, washed with water and placed in 1% uranyl acetate for 1 hour. The tissues were subsequently sequentially dehydrated in ethanol and propylene oxide and embedded in EMBed 812 resin (Electron Microscopy Sciences, Hatfield, PA). Thin sections, approx. 80 nm, were obtained by utilizing the Leica ultracut-UCT ultramicrotome (Leica, Deerfield, IL) and placed onto 300 mesh copper grids and stained with 2% aqueous uranyl acetate and 1% aqueous lead citrate.

**Statistical analyses**

For the Rare Disease Clinical Research Network-sponsored Urea Cycle Disorders Consortium (RDCRN UCDC) longitudinal study, OTC patients who were hospitalized owing to HA events during the course of the study were examined. Because each participant can experience more than one HA event, the generalized estimating equation (GEE) adjusted for age at the HA event was used. Observed frequency (%) for categorized variables and the mean (standard deviation) for continuous variables were reported. $P$-values less than 0.05 were used to indicate statistical significance.

**Acknowledgements**

The authors thank the members of the Urea Cycle Disorder Consortium for providing clinical data. Thanks to Dr Les Bieseker for his support and the support of the Physician Scientist Development Program at NHGRI. Thanks to Dr Pamela Schwartzberg and Dr Charles Venditti for their editorial insights during preparation of this manuscript. Thanks to Dr Maryna Eichelberger for producing the influenza virus and providing technical guidance.

**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

P.J.M., T.M. and G.A.D. conceived and designed the experiments. T.N.T., T.W., E.L., P.M.Z. and B.J.B. performed the experiments and acquired data. P.J.M. and H.S.L. analyzed the data. P.J.M. wrote the paper.

**Funding**

This work was supported by the intramural program at the National Institutes of Health (P.J.M.) and the Children’s Health Research Center (K12 HD052880) at Mount Sinai School of Medicine (P.J.M.).

**Supplementary material**

Supplementary material available online at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.013003/-/DC1

**References**


Supplemental Figure 1

A depiction of the urea cycle indicating the source of nitrogen groups for ureagenesis (Blue - ammonia; Red – aspartate).
Supplemental Figure 2

Protocol and methods used in PR8 infection. (A) Study design employed for defining infectious parameters.

Sac – sacrificed. (B) Inhalation apparatus used for PR8 virus delivery.
Supplemental Figure 3

Representative histologic evaluation of the liver on Day 5. (A) Representative H/E stain (N = 5). (B) Representative TUNEL stain (N = 5). (C) Oil Red O stain with baking (N = 5). For TUNEL stain, positive control is normal female rodent mammary gland included in the kit used. For Oil Red O stain, control liver tissue is from a mouse model of alcohol induced liver disease.
Supplemental Figure 4

Methods and feeding parameters for matched intake study design. (A) Study design for matched protein/caloric intake in infected and uninfected animals. Infected spf-ash decrease food intake over the 5 days of infection. To match food intake, the average amount eaten by spf-ash was determined previously, and intake for all other animals was subsequently matched on a g/kg/day basis. Sac – sacrificed. (B) Protein intake applied to individually housed animals. (C) Weight loss due to different conditions (N = 6-8 / group).