Hepatic glutamine synthetase augmentation enhances ammonia detoxification

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Abstract
The urea cycle and glutamine synthetase (GS) are the two main pathways for waste nitrogen removal and their deficiency results in hyperammonemia. Here, we investigated the efficacy of liver-specific GS overexpression for therapy of hyperammonemia. To achieve hepatic GS overexpression, we generated a helper-dependent adenoviral (HDAd) vector expressing the murine GS under the control of a liver-specific expression cassette (HDAd-GS). Compared to mice injected with a control vector expressing an unrelated reporter gene (HDAd-alpha-fetoprotein), wild-type mice with increased hepatic GS showed reduced blood ammonia levels and a concomitant increase of blood glutamine after intraperitoneal injections of ammonium chloride, whereas blood urea was unaffected. Moreover, injection of HDAd-GS reduced blood ammonia levels at baseline and protected against acute hyperammonemia following ammonia challenge in a mouse model with conditional hepatic deficiency of carbamoyl phosphate synthetase 1 (Cps1), the initial and rate-limiting step of ureagenesis. In summary, we found that upregulation of hepatic GS reduced hyperammonemia in wild-type and Cps1-deficient mice, thus confirming a key role of GS in ammonia detoxification. These results suggest that hepatic GS augmentation therapy has potential for treatment of both primary and secondary forms of hyperammonemia.

KEYWORDS
carbamoyl phosphate synthetase 1 deficiency, glutamine synthetase, helper-dependent adenoviral vectors, hyperammonemia, urea cycle disorders

1 INTRODUCTION
The glutamate-ammonia ligase enzyme (GLUL), typically known as glutamine synthetase (GS) (E.C. 6.3.1.2), is a cytosolic enzyme that catalyzes adenosine triphosphate (ATP)-dependent production of glutamine from glutamate...
and ammonia. GS is highly conserved within living organisms, and its expression and activity have been detected in several tissues including liver and brain (mostly in astrocytes). As a serial pathway, together with the urea cycle enzymes expressed in the peri-portal region, peri-venous GS is a major ammonia detoxification system. Both systems are required for complete ammonia detoxification. In mice, liver-specific deletion of GS results in hyperammonemia leading to neuronal and behavioral abnormalities. Similar to inherited urea cycle disorders, patients with the ultrarare genetic GS deficiency have chronic hyperammonemia in addition to decreased levels of glutamine in body fluids. Moreover, GS expression is reduced in patients with liver cirrhosis and chronic hyperammonemia. Taken together, evidence both in mice and humans illustrates a key role of GS in ammonia homeostasis leading to the concept that the urea cycle and GS contributes equally to ammonia detoxification.

Based on these considerations, we reasoned that GS augmentation therapy can be an effective treatment against hyperammonemia, a life-threatening condition requiring the development of more effective therapeutic approaches.

2 | MATERIALS AND METHODS

Mouse procedures. Male wild-type (WT) 6-week-old C57BL/6 (Charles River Laboratories, Calco, Italy) and adult female transgenic Cps1<sup>tm1c/tm1c</sup> mice were randomly assigned to treatment groups. Investigators were not blinded to allocation during experiments and outcome assessment. All intravenous (i.v.) injections were performed by retro-orbital injections under isoflurane anesthesia. Vectors were prepared in sterile pharmaceutical-grade saline. WT and Cps1<sup>tm1c/tm1c</sup> mice were injected with a dose of 1 × 10<sup>13</sup> viral particles (vp)/kg of helper-dependent adenoviral (HDAd)-GS or HDAd-alpha-fetoprotein (AFP) (α-fetoprotein) as a control. The ammonium intraperitoneal (i.p.) challenge in WT mice was performed at 4 weeks postvector injection as described previously. Briefly, mice were overnight fasted before i.p. injections of 10 mmol/kg of ammonium chloride (Merck, Darmstadt, Germany) dissolved in water. Three days after the injections of HDAd expressing either AFP or GS, Cps1<sup>tm1c/tm1c</sup> mice were injected with 1.5 × 10<sup>10</sup> genome copies (gc)/mouse of serotype 8 adenoviral-associated viral (AAV8) vector expressing Cre recombinase under the control of the thymoxine binding globulin promoter (liver-specific) (University of Pennsylvania Vector Core, Philadelphia, Pennsylvania, USA). Cps1<sup>tm1c/tm1c</sup> mice were weighed daily and monitored for any signs of poor health. In Cps1<sup>tm1c/tm1c</sup> mice, the i.p. ammonia challenge was also performed 4 weeks after the injections of the HDAd vectors but with a lower dose of i.p. 5 mmol/kg ammonium chloride (Fisher-Scientific, Pittsburgh, Pennsylvania, USA) and without prior fasting. Blood samples were collected by retro-orbital bleedings at the indicated time points. Mice were sacrificed by cervical dislocation or isoflurane overdose.

HDAd vectors. Mouse GS cDNA was synthesized by Life Technologies (Carlsbad, California, USA). HDAd-GS and HDAd-AFP vectors both bear a liver-specific expression cassette driving the expression of either mouse GS or baboon AFP, respectively. HDAd vectors were produced as previously reported in 116 cells regularly tested and found negative for Mycoplasma by quantitative real-time polymerase chain reaction (PCR).

Analyses of serum and plasma samples. Serum or plasma ammonia levels were measured by an ammonia colorimetric assay kit (Cat# K370-100; BioVision Incorporated, Milpitas, California, USA) or (Cat# ab83360; Abcam, Cambridge, UK), respectively. Serum or plasma glutamine concentrations were measured by a colorimetric assay kit (Cat# K556-100; BioVision Incorporated, Milpitas, California, USA). Serum urea content was determined by an assay kit based on Jung’s method (Cat# K376-100; BioVision Incorporated, Milpitas, California, USA). For blood urea nitrogen (BUN), 10 μL of plasma per sample were pipetted into cups and placed into a Vet Excel Clinical Chemistry Analyzer (Alfa Wassermann Diagnostic Technologies, West Caldwell, New Jersey, USA). Calculation of urea nitrogen concentration was performed automatically by the clinical chemistry system. Hepatic ATP determination was performed by a fluorometric assay kit according to the manufacturer’s instructions (Cat# K354-100; BioVision Incorporated, Milpitas, California, USA). Liver lysates were made by homogenization in the corresponding hydrolysis buffer using a TissueLyser LT (Qiagen, Venlo, The Netherlands). Hepatic ATP levels were normalized for protein concentrations determined by Bradford Reagent (Bio-Rad, Hercules, California, USA).

Western blotting. Liver specimens were homogenized in radioimmunoprecipitation assay (RIPA) buffer in the presence of complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri, USA), incubated for 20 minutes at 4°C and centrifuged at 13 200g for 10 minutes. Pellets were discarded and cell lysates were used for western blots. Total protein concentration in cellular extracts was measured using the Bradford Reagent (Bio-Rad, Hercules, California, USA). Protein extracts were separated by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes. Blots were blocked with tris-buffered saline (TBS)-Tween-20 containing 5% nonfat milk for 1 hour at room temperature followed by incubation with primary antibody overnight at 4°C. Primary antibodies were: rabbit anti-GS (Cat#
ab16802; Abcam, Cambridge, UK), rabbit anti-ornithine aminotransferase (OAT) (Cat# ab137679; Abcam, Cambridge, UK), and rabbit anti-p115.13 Proteins of interest were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (GE Healthcare, Chicago, Illinois, USA). Peroxidase substrate was provided by enhanced chemiluminescence (ECL) Western Blotting Substrate kit (Pierce, Waltham, Massachusetts, USA). Densitometric analyses of the western blotting bands were performed using ImageJ Software (National Institutes of Health, Bethesda, Maryland, USA).

Immunofluorescence. Immunofluorescence was performed as previously described. Briefly, liver sections were fixed in 10% neutral buffered formalin, stored in 70% ethanol and embedded in paraffin blocks followed by cutting the sections at 4-μm thickness. Sections were then deparaffinized in xylene and rehydrated in serial ethanol washes. Antigen retrieval was performed in 10-mM sodium citrate buffer pH 6.0, after which the sections were permeabilized in 0.1% Triton X-100 and blocked with 10% normal goat serum. Slides were incubated with anti-carbamoyl phosphate synthetase 1 (CPS1) antibody (Cat# ab45956; Abcam, Cambridge, UK) and anti-GLUL antibody (Cat# ab64613; Abcam, Cambridge, UK), both at 1 μg/mL in the blocking buffer overnight at 4°C. Secondary antibody staining was performed at room temperature for 1 hour using Alexa Fluor 594 goat anti-rabbit (A-11012; Invitrogen, San Diego, California, USA) and Alexa Fluor 488 goat antimouse (A-11001; Invitrogen, San Diego, California, USA) antibodies. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) using VECTASHIELD Antifade Mounting Medium. Visualization of the stained sections was performed using Olympus IX71 Fluorescence Microscope.

RNA extraction and real-time PCR. Individual liver pieces were homogenized, and RNA was extracted by column purification (Cat# 11828665001; Roche Mannheim, Germany). Purified RNA was used to generate cDNA (Cat# 04379012001; Roche Mannheim, Germany) for qPCR with BioRad SYBR Green (Cat# 1725270; Bio-Rad, Hercules, California, USA), and IQ2 iCycler. β-actin gene was used as endogenous control. Fold changes were calculated using the delta-delta-Ct (DDCt) method. Cps1 primers were: forward CCAACATTCTCAGTGAAA; reverse TACTGCTTCTAAGCTTCTTT; Glut primers were: forward CCTACCTTGAAACAAAGGCA; reverse GTCTTCTCAGGTCACTACA; Actb primers were: CTAAGGCAAACCGTGAAAG; reverse ACCAGGGCATACAGGGACA.

Statistical analyses. Data are expressed as means ± SEM. A two-tailed unpaired Student's t test was performed when comparing two groups of mice. One-way ANOVA and Tukey's post-hoc tests were performed when comparing more than two groups relative to a single factor. Two-way ANOVA and Tukey's post-hoc tests or Sidak's multiple tests were performed when comparing two groups relative to two factors. No statistical methods were used to predetermine the sample size. A P-value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Upregulation of hepatic GS protects WT mice against acute hyperammonemia

HDAd vectors can efficiently deliver genes to hepatocytes to mediate long-term, high-level transgene expression without inducing chronic toxicity and have been successfully used in mouse models of a variety of inherited liver diseases. To investigate the potential of hepatic GS augmentation as a therapeutic approach for hyperammonemia, we constructed an HDAd vector expressing the murine GS under the control of a liver-specific expression cassette (HDAd-GS) (Figure 1A). Compared to C57BL/6 WT control mice injected with HDAd vector encoding the unrelated nontoxic, nonimmunogenic AFP reporter gene under the control of the same liver-specific expression cassette, administration of the vector expressing GS resulted in increased hepatic GS protein expression levels by western blot (band densitometric densitometric analysis showed a 39% reduction in serum ammonia at 0.5 hours after i.p. ammonium chloride challenge (P < 0.05; Figure 1C). The reduction of blood ammonia was accompanied by a concomitant increase in serum glutamine levels in mice with hepatic GS overexpression compared to HDAd-AFP-injected controls (Figure 1D), whereas serum urea was unaffected (Figure 1E). Hepatic content of ATP was similar between HDAd-GS-injected mice and AFP-treated controls (Figure S1), suggesting that no changes in energy levels were induced by GS overexpression. Taken together, these results suggest that liver-specific GS overexpression improved ammonia detoxification through an increase in hepatic glutamine synthesis in WT mice with acute hyperammonemia.

3.2 | Liver-specific GS overexpression improved ammonia detoxification in Cps1-deficient mice

To investigate whether increased hepatic GS expression is effective for therapy of hyperammonemia due to urea cycle disorders, we injected HDAd-GS or HDAd-AFP in mice with deficiency of CPS1, the initial and rate-limiting step in ureagenesis. Deletion of the Cps1 locus was achieved in
adult transgenic Cps1\(^{m1c/m1c}\) mice by i.v. injection of a low dose of AAV8 vector expressing the Cre recombinase inducing nonlethal increase in plasma ammonia. Cre-mediated reduction in hepatic Cps1 mRNA levels was similar in mice injected with HDAd-GS or HDAd-AFP vector. All values are shown as averages ± SEM (n ≥ 9/group). *P < 0.05; **P < 0.01 (two-way ANOVA). AFP, α-fetoprotein; GHpA, growth hormone polycadenylation signal; GS, glutamine synthetase; HDAd, helper-dependent adenoviral vector; ITR, inverted terminal repeat; LCR, locus control region; OAT, ornithine aminotransferase; ns, not significant; PEPCK, phosphoenolpyruvate carboxykinase; Pi, postinjection; WPRE, woodchuck hepatitis post-transcriptional regulatory element

FIGURE 1 Hepatic GS overexpression protects wild-type mice against acute hyperammonemia. (A) A helper-dependent adenoviral vector was produced to express murine GS under the control of the phosphoenolpyruvate carboxykinase (PEPCK) promoter (HDAd-GS). The vector also contains ITR, a packaging signal (Ψ), the ApoA1 intron (ApoA1in), the hepatic locus control region (LCR), and a growth hormone polyadenylation signal (GHpA). (B) Western blot of GS in livers of HDAd-GS-injected C57BL/6 wild-type mice compared to controls injected with a vector expressing an unrelated reporter gene (HDAd-AFP) at 12 weeks postvector injection. Ornithine aminotransferase (OAT) and p115 were used as loading controls. (C-E) Serum ammonia, glutamine, and urea at baseline and 0.5 hours after intraperitoneal injections of ammonium chloride (10 mmol/kg) in wild-type mice injected with HDAd-GS or HDAd-AFP vector. All values are shown as averages ± SEM (n ≥ 9/group). *P < 0.05; **P < 0.01 (two-way ANOVA). AFP, α-fetoprotein; GHpA, growth hormone polycadenylation signal; GS, glutamine synthetase; HDAd, helper-dependent adenoviral vector; ITR, inverted terminal repeat; LCR, locus control region; OAT, ornithine aminotransferase; ns, not significant; PEPCK, phosphoenolpyruvate carboxykinase; Pi, postinjection; WPRE, woodchuck hepatitis post-transcriptional regulatory element

This study supports the concept that liver GS plays a key role in ammonia homeostasis and provides a proof of concept that hepatic GS upregulation improves ammonia detoxification. Either chemical\(^{17,18}\) or genetic\(^ {4,7}\) inhibition of hepatic GS expression/activity resulted in increased blood ammonia, clearly showing an important contribution of GS in ammonia detoxification.

In the present study, we overexpressed GS in liver by the hepatotropic HDAd vectors and we observed improved ammonia detoxification in WT mice with acute
FIGURE 2 Expression of CPS1 in livers of HDAd-GS and HDAd-AFP-injected mice. (A) Cps1 mRNA expression in livers harvested at day 28 postvector administration in Cre-mediated Cps1-deficient (Cps1-def) mice injected with HDAd-GS or HDAd-AFP compared to control wild-type animals (n = 5/group). **P < 0.01 (One-way ANOVA).

(B) Representative images of immunofluorescence microscopy for CPS1 (red) in livers harvested at day 28 postvector administration in Cps1-def mice injected with HDAd-GS or HDAd-AFP compared to control wild-type animals.

(C) BUN in plasma harvested at day 28 postvector administration in Cre-mediated Cps1-deficient (Cps1-def) mice injected with HDAd-GS or HDAd-AFP compared to control wild-type animals. *P < 0.05 (one-way ANOVA). Data represent averages ± SEM. AFP, α-fetoprotein; BUN, blood urea nitrogen; CPS1, carbamoyl phosphate synthetase 1. GS, glutamine synthetase; HDAd, helper-dependent adenoviral vector; ns, not significant.

FIGURE 3 Expression of GS in livers of HDAd-GS- and HDAd-AFP-injected mice. (A) Glul real-time polymerase chain reaction on mRNA extracted from livers of Cps1-deficient (Cps1-def) mice injected with HDAd-GS or HDAd-AFP and harvested at day 28. **P < 0.01 (unpaired Student's t test) (n = 5/group). (B) Hepatic immunostaining for GS (green) in livers of Cps1-def mice injected with HDAd-AFP or HDAd-GS harvested at day 28. White arrow heads point to hepatocytes with GS expression outside the peri-venous area.

(C) Plasma glutamine/ammonia ratio at day 28 in Cps1-def mice injected with HDAd-GS or HDAd-AFP. *P < 0.05 (unpaired Student's t test) (n = 5/group). Data represent averages ± SEM. AFP, α-fetoprotein; GS, glutamine synthetase; HDAd, helper-dependent adenoviral vector.
hyperammonemia. The increased ammonia detoxification was associated with enhanced glutamine synthesis without changes in serum urea. Consistent with this data, increased activity of muscle GS induced by ornithine-phenylacetate treatment or muscle-directed gene transfer reduced blood ammonia levels in rat models of chronic liver failure and acute hyperammonemia. Nevertheless, we showed for the first time that increased hepatic GS is effective at reducing hyperammonemia. Despite expansion of GS protein localization in the peri-portal zone, hepatic energy stores were unaffected, suggesting that exogenous GS augmentation does not affect other functions of the liver. Whether a residual activity of the urea cycle is needed for GS augmentation therapy to be effective cannot be established because these studies were performed in mice with residual CPS1 activity. Nevertheless, this study supports that GS upregulation might be a therapeutic option for primary hyperammonemia in the deficiency of CPS1, the most severe urea cycle defect due to a defect in the initial incorporation of ammonia into intermediate compounds of the urea cycle. Available treatments for this disease, such as protein-restricted diet, supplementation with urea cycle intermediates, or ammonia scavenger drugs are often insufficient, and liver transplantation is the only available effective therapeutic option.

Secondary hyperammonemia is defined as an increase in blood ammonia driven by inhibition of ureagenesis as a consequence of accumulation of toxic metabolites or by substrate deficiencies. The most common examples of this type of hyperammonemia are organic acidemias in which urea synthesis is impaired by reduced production of N-acetylglutamate, the allosteric activator of ureagenesis and CPS1 inhibition. However, secondary hyperammonemia also occurs in fatty acid oxidation defects, carnitine-associated deficiency, lysinuric protein intolerance, and other disorders. It is likely that all these conditions can be ameliorated by enhancement of hepatic glutamine synthesis. In addition, hepatic GS upregulation might be effective in either acute or chronic acquired liver disease resulting in reduced urea cycle capacity. Moreover, treatments aiming at increasing GS activity can be combined with available treatments such as low protein diet, ammonia scavengers, or the recently described activation of hepatic autophagy to obtain more effective control of blood ammonia levels. Interestingly, improved ammonia control can be obtained by supplementation with glutamate (or its precursors) or phenylacetate to enhance ammonia removal by increased synthesis and clearance of glutamine, respectively.

Hepatic GS upregulation might also be achieved by systemic mRNA delivery, a therapeutic strategy that has been successfully used for correction on an inborn error of metabolism. Moreover, GS augmentation therapy might be achieved by uploading of GS onto lipid- or polymeric-based nanoparticles for systemic delivery or with membrane derived from natural cells such as erythrocytes or white blood cells. In addition, ammonia detoxification may be further improved by vesicles uploaded with recombinant GS enzyme through for liposome-supported peritoneal dialysis.

Small molecule drugs upregulating hepatic GS expression are another attractive approach. Interestingly, glucocorticoids are known to positively regulate expression of GS at the transcriptional level in multiple tissues. Dexamethasone was indeed found to be effective in mice at increasing ammonia detoxification. However, overexpression of GS in astrocytes is likely to be detrimental because it could lower the threshold of brain toxicity since cerebral edema depends on glutamine synthesis in astrocytes. Hence, treatments with low glucocorticoid doses or other drugs upregulating GS in livers but not in astrocytes are desirable.

Current guidelines for management of patients with suspected or confirmed diagnosis of urea cycle disorders recommend avoidance of steroids because they induce protein catabolism and can trigger hyperammonemia. This recommendation is based on anecdotal experience in patients with acute decompensation following steroid treatments. The results of our study suggest that GS upregulation is protective against hyperammonemia although the increased GS expression in astrocytes is likely to be detrimental.

In summary, our data show that liver-specific GS augmentation improved ammonia detoxification in WT and Cps1-deficient mice thus confirming a key role of GS in ammonia homeostasis. Hence, hepatic GS augmentation...
therapy has potential for treatment of both primary and secondary forms of hyperammonemia.

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COMPLIANCE WITH ETHICS GUIDELINES

CONFLICT OF INTEREST

L.R. Soria, M. Nitzahn, A. De Angelis, S. Khoja, S. Attanasio, P. Annunziata, D.J. Palmer, P. Ng, and N. Brunetti-Pierri have no conflicts of interest to declare. G.S. Lipshutz has served as a consultant to Audentes Therapeutics unrelated to the studies conducted herein.

INFORMED CONSENT

This article does not contain any studies with human subjects performed by any of the authors.

ANIMAL RIGHTS

Mouse procedures were performed in accordance with regulations and were authorized by both the Italian Ministry of Health and the Institutional Animal Care and Use Committee (IACUC) of David Geffen School of Medicine at UCLA, Los Angeles, United States.

AUTHOR CONTRIBUTIONS

L.R.S., G.S.L., and N.B.-P. designed the research; L.R.S., M.N., A.D.-A., and S.K., performed research; S.A., P.A., D.J.P., and P.N., made cloning and HDAd vectors; L.R.S., M.N., S.K., G.S.L., and N.B.-P. analyzed data; and L.R.S. and N.B.-P. wrote the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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