Published in final edited form as:
Mol Genet Metab. 2008 January ; 93(1): 7–14. doi:10.1016/j.ymgme.2007.08.123.

Contrasting Features of Urea Cycle Disorders in Human Patients and Knockout Mouse Models

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Abstract

The urea cycle exists for the removal of excess nitrogen from the body. Six separate enzymes comprise the urea cycle, and a deficiency in any one of them causes a urea cycle disorder (UCD) in humans. Arginase is the only urea cycle enzyme with an alternate isoform, though no known human disorder currently exists due to a deficiency in the second isoform. While all of the UCDs usually present with hyperammonemia in the first few days to months of life, most disorders are distinguished by a characteristic profile of plasma amino acid alterations that can be utilized for diagnosis. While enzyme assay is possible, an analysis of the underlying mutation is preferable for an accurate diagnosis. Mouse models for each of the urea cycle disorders exist (with the exception of NAGS deficiency), and for almost all of them, their clinical and biochemical phenotypes rather closely resemble the phenotypes seen in human patients. Consequently, all of the current mouse models are highly useful for future research into novel pharmacological and dietary treatments and gene therapy protocols for the management of urea cycle disorders.

Keywords

urea; hyperammonemia; knockout; N-acetylglutamate synthase; carbamyl phosphate synthetase I; ornithine transcarbamylase; argininosuccinate synthetase; argininosuccinate lyase; arginase

Introduction

The Urea Cycle

The urea cycle is the only metabolic pathway capable of removing excess nitrogen from the body [1]. Occurring in the liver, ammonium nitrogen from dietary protein sources and from the breakdown of endogenous protein is converted into urea, which unlike ammonia is

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nontoxic, water-soluble, and is easily excreted from the body through the kidneys as a component of urine. The urea cycle is originally thought to have evolved in amphibians through adaptation to an air-breathing terrestrial lifestyle, necessitating a novel method whereby ammonia could be efficiently expelled [2]. While fish are able to passively excrete ammonia across their gills, terrestrial animals are not able to excrete enough water to dilute ammonia to a nontoxic level without becoming dehydrated [3]. Six separate enzymes comprise the urea cycle, namely N-acetylglutamate synthase (NAGS), carbamyl phosphate synthetase I (CPS-I), ornithine transcarbamylase (OTC), argininosuccinate synthetase (AS), argininosuccinate lyase (AL), and arginase (ARG), and a deficiency in any one of the enzymes causes a urea cycle disorder (UCD) in humans [4]. NAGS, CPS-I, and OTC function in the mitochondria, while AS, AL, and ARG exist in the cytosol (Figure 1). Except for OTC deficiency, which is X-linked, all of the UCDs are inherited in an autosomal recessive pattern, and their overall frequency is approximately 1 in 30,000 live births [5] (Table 1).

Diagnosis and Treatment of Urea Cycle Disorders

Hyperammonemia, or high plasma ammonia, is the primary diagnostic finding associated with all of the urea cycle disorders, and patients with UCDs usually present as neonates. They are born normally but often reveal initial symptoms within the first few days to weeks of life. Generally, the most severe course of disease is associated with the most proximal blockage in the cycle. For example, patients with complete CPS-I or OTC deficiency frequently present during the first few days of life, patients with AS or AL deficiency tend to present during the first month, and patients with ARG deficiency usually present later in childhood [6]. Urea cycle disorders are generally severe, and if left untreated for too long, the resulting hyperammonemia can cause mental retardation, seizures, and early morbidity [1]. Therefore, the initial focus for patients with UCDs is centered on lowering ammonia levels in the plasma.

Since hyperammonemia originally results from protein breakdown, the first step in the treatment of UCDs is to stop all protein intake and substitute an oral or intravenous high energy source such as glucose [7]. If the plasma ammonia is extremely high and stopping protein intake does not sufficiently normalize ammonia levels, hemodialysis or hemofiltration are used. Other oral and intravenous compounds also exist that can assist in the removal of excess nitrogen without the need to produce urea. Sodium benzoate was the first compound introduced; benzoate conjugates with glycine to form hippurate which can be easily excreted in the urine, and for every mole of benzoate administered, one mole of nitrogen is removed [8]. Sodium phenylacetate was introduced next; however, sodium phenylbutyrate is more commonly used today for the treatment of UCDs due to the unpleasant odor of phenylacetate. Phenylbutyrate is converted to phenylacetate which conjugates with glutamine, and the resulting compound is easily excreted in the urine [1,5]. In contrast to benzoate, two moles of nitrogen are eliminated for every mole of phenylbutyrate administered.

Once hyperammonemia has been detected and managed, determining which of the urea cycle disorders (or other metabolic defect) exists requires further measurement of plasma and urinary amino acids and urinary organic acids [9]. Abnormal urinary organic acid levels are suggestive of congenital lactic acidoses, organic acidemias, and fatty acid oxidation defects as being causal for the hyperammonemia, whereas in urea cycle disorders urinary organic acid levels are often unremarkable. Furthermore, the determination of plasma citrulline helps differentiate proximal from distal defects; plasma citrulline is either absent or only present in trace amounts in NAGS deficiency, CPS-I deficiency, and OTC deficiency; it is normal in ARG deficiency; and it is markedly elevated in AS and AL deficiencies. Finding argininosuccinic acid in plasma and urine permits a diagnosis of AL deficiency, whereas levels are normal in AS deficiency. Elevated urinary orotic acid levels combined with trace amounts of plasma citrulline can differentiate OTC deficiency from CPSI and NAGS deficiencies, where urinary orotic acid

levels are normal. Finally, plasma arginine levels are reduced in all urea cycle disorders except for ARG deficiency, so elevated plasma arginine can be used to diagnose ARG deficiency as being causal for the observed hyperammonemia. These biochemical abnormalities are discussed in more detail below.

Due to the severe outcome if UCDs are not effectively managed, patients are considered candidates for orthotopic liver transplantation and gene-replacement therapy options [9-13]. However, organ transplantation is associated with significant morbidity and likelihood of future mortality, and though gene-replacement therapy has had success in transiently rescuing animal models of urea cycle disorders, it is not yet safely available to human patients [14-18].

Human Disorders and Animal Models

N-Acetylglutamate Synthase

Human NAGS Deficiency—NAGS deficiency (OMIM 237310) is due to a defect in N-acetylglutamate synthase (NAGS), a very low abundance enzyme which catalyzes the formation of N-acetylglutamate (NAG) from glutamate and acetyl-CoA [19]. A deficiency caused by null alleles results in hyperammonemia early in the newborn period due to the secondary deficiency of CPS-I; without NAG, CPS-I is catalytically inactive [20]. However, late onset hypomorphic alleles also exist, and those patients can often go undiagnosed until adulthood [21,22]. Symptoms of NAGS deficiency are similar to those of CPS-I deficiency and include elevated plasma glutamine, reduced citrulline, and normal urinary orotic acid. The largest hurdle for the reliable detection of NAGS deficiency has been the difficulty in cloning the human gene on chromosome 17, which only occurred recently due to a low degree of sequence conservation throughout evolution [23]. Due to its low abundance, a large amount of liver tissue is required in order to perform enzyme activity assays, and this has likely caused many patients to go undiagnosed in the past [24]. However, with the recent cloning and sequencing of the human NAGS gene, a molecular diagnosis of the disease is now possible [20,25].

NAGS Knockout Mouse Model—Since the mouse homolog on chromosome 11 has been identified and cloned only recently, there are as of yet no animal models of NAGS deficiency [26].

Carbamyl Phosphate Synthetase I

Human CPS-I Deficiency—CPS-I deficiency (OMIM 237300) results from a defect in carbamyl phosphate synthetase I, a highly abundant protein in the liver which is responsible for converting ammonia to carbamyl phosphate [4]. CPS-I deficiency is very rare and patients with this condition exhibit lethally severe hyperammonemia most often in the neonatal period, though a less severe, late-onset form also exists [27-29]. The overall incidence of the disease is estimated at one in 800,000 to one in 1,000,000 [30]. Its proximity with NAGS in the intramitochondrial pathway results in similar biochemical abnormalities in CPS-I deficiency, with alterations in particular amino acids as described above; however, these findings alone cannot discriminate between CPS-I and NAGS deficiencies. Enzyme activity and mutation analysis are necessary for a correct diagnosis [31]. However, due to the large size of the gene located on chromosome 2, and the heterogeneity of the mutant alleles, functional characterization of mutations may be necessary to determine causality [32-34].

The CPS-I Knockout Mouse Model—A mouse model for CPS-I deficiency has been constructed by Schofield et al. [35] through disruption of exon 17 of the gene, and all knockout animals die within 36 hours of birth with highly elevated plasma ammonia levels present within

6 hours of birth. Exon 17 was chosen because it includes sequences encoding the most 5′ nucleotide binding domain in the gene. Enzyme activity in liver is completely abolished in homozygotes, and no obvious liver pathological abnormalities are present. The murine gene for CPS-I is located on chromosome 1 and has more than 30 exons, similar to the human gene [36,37]. Unfortunately, it is our understanding that the original mouse colony containing the null mutation no longer exists.

Ornithine Transcarbamylase

Human OTC Deficiency—Ornithine transcarbamylase catalyzes the formation of citrulline from carbamyl phosphate and ornithine, and a disruption of this enzyme is known as OTC deficiency (OMIM 311250). OTC deficiency is the most common of the urea cycle disorders with an estimated frequency of 1 in 14,000 [38,39]. In contrast to the other urea cycle disorders, OTC deficiency is X-linked, and thus hemizygous males exhibit the most severe symptoms with hyperammonemia within the first few weeks of life; on the other hand, female heterozygotes can have quite variable features [1,40-43], mostly due to the mosaic pattern of X inactivation in their hepatocytes [44]. The mutant alleles present in symptomatic female heterozygotes are likely mutations which severely effect OTC activity [40]. Similar to NAGS and CPS-I deficiencies, elevated plasma glutamine and alanine and decreased citrulline are also seen in OTC deficiency. Urinary orotic acid may also be elevated in this disorder (unless the patient is under good control).

The OTC Deficient Mouse Models—Two mouse models currently exist for OTC deficiency. The best characterized model is the sparse fur (spf) mouse, first reported by DeMars et al. [45], while the other model is the spf^{ash} (abnormal skin and hair) mouse, first described by Doolittle et al. [46]. In the spf mouse, a C->A missense mutation in exon 4 changes a histidine to asparagine (H117N) and results in only 10% of normal liver enzyme activity, whereas in the spf^{ash} mouse model, a different G->A missense mutation in exon 4 changes an arginine to histidine (R129H) and results in abnormal splicing and only 5% of normal liver enzyme activity [46-49]. In both models, affected animals display several characteristic biochemical hallmarks of OTC deficiency, including elevated plasma ammonia and glutamine, decreased plasma arginine and citrulline, and increased urinary orotic acid, and the mean lifespan of hemizygote males is 42 days, with 93% mortality by 88 days [50]. While the correction of the underlying defect using gene therapy has had great success in the mouse models [51-54], it has not been as successful in human OTC-deficient patients [55]. In adult OTC-deficient mice, first generation adenoviral vectors showed only transient rescue, while helper-dependent adenoviral vectors have allowed for metabolic correction lasting greater than six months [14,56,57]. However, in a phase 1/2 clinical trial using first generation adenoviral vectors in an OTC-deficient patient, fatal acute toxicity resulted [55].

Argininosuccinate Synthetase

Human AS Deficiency—Citrullinemia type I (OMIM 215700) is caused by a defect in argininosuccinate synthetase (AS), which normally functions to convert citrulline into argininosuccinate in the cytosol of hepatocytes [58]. The human gene is located on chromosome 9, and the incidence rate is approximately one in 100,000 [59,60]. Most patients present during the early neonatal period with acute hyperammonemia, and highly elevated plasma citrulline is the hallmark of this disorder. A late onset form resulting in a milder course of disease also exists [61]. Enzyme assay is possible in fibroblasts, though an invasive procedure is required in order to obtain liver tissue; therefore, a genetic analysis is usually more desirable. Of the mutations described so far, four lead to deletions of an entire exon, one leads to a deletion of the first seven bases of exon 16, one leads to an insertion between exons 15 and 16, one leads to an insertion in exon 14, and fourteen are missense mutations in the coding sequence [62-65]. Citrullinemia type II (OMIM 603471) also exists; however, this disorder is

due to defects in a mitochondrial aspartate-glutamate transporter and not due to mutations in the human AS gene, and plasma citrulline levels are not nearly as high as those found in classic AS deficiency [66,67].

The AS Knockout Mouse Model—A knockout mouse model for AS deficiency has been generated by O'Brien's group [68], and homozygous animals expire within a few days after birth, exhibiting markedly elevated plasma citrulline and decreased arginine. A naturally occurring bovine model for AS deficiency also exists but will not be discussed in this review; for more information please refer to [16,69-71]. With the start codon located in exon 3, exon 4 was interrupted in order to create a null mutant [72]. The murine AS gene is located on chromosome 2 [73]. Clinically and biochemically, the mouse model resembles the human disorder, and these animals have been successfully used in the development of adenoviral gene therapy protocols for the treatment of urea cycle disorders [16,74]. Originally, a first generation adenoviral vector with the RNA polymerase II promoter controlling expression of the human AS cDNA was used in a homozygous AS-deficient animal along with arginine and sodium benzoate therapy, and this resulted in an increase in lifespan from 4 to 11 days [16]; however, treatment of these mice using adenoviral vectors with the CMV promoter controlling expression of the human AS cDNA allowed their lifespan to be extended to 16 days [74]. A second viral infusion at two weeks of age increased their lifespan further to 36 days, while daily arginine and sodium benzoate injections allowed the mice to live up to 40 days [74].

Argininosuccinate Lyase

Human AL Deficiency—Argininosuccinate lyase (AL) participates in the cleavage of argininosuccinate into fumarate and arginine, and a deficiency of AL results in argininosuccinic aciduria (OMIM 207900) [75]. It is the second most common urea cycle disorder after OTC deficiency, with a frequency of approximately 1 in 70,000 live births in the United States, and the gene is located on chromosome 7 [31,76,77]. Patients typically present early in life with hyperammonemia, elevated plasma citrulline, and measurable argininosuccinic acid in plasma and urine, and the hyperammonemic episodes are less frequent and less severe than in many of the other urea cycle disorders [1]. AL enzyme activity can be determined in cultured fibroblasts or erythrocytes; however, elevated plasma ammonia and amino acids including argininosuccinic acid are usually sufficient for proper diagnosis of this disorder [1]. Of the mutations known to exist in AL deficient patients, most are missense mutations in conserved regions of the gene, though other types of mutations (nonsense, splice-site, silent, deletion, insertion) do exist at a low frequency [78]. Exons 4, 5, and 7 appear to be mutational hotspots [78], while a deletion hotspot has recently been identified in exon 13 [79].

The AL Knockout Mouse Model—Mice deficient in argininosuccinate lyase were constructed by Reid Sutton et al. [80] through replacement of exons 8 and 9, creating a frameshift in the mRNA beginning with exon 10, and all homozygotes expire within 48 hours after birth and have elevated plasma ammonia, argininosuccinic acid, glutamine, and citrulline and low plasma arginine. The mouse argininosuccinate lyase gene is located on chromosome 5 [81]. AL knockout animals show a deficiency in AL enzyme activity in liver as well as a deficiency in liver arginase activity, likely due to the lowered plasma arginine levels. Furthermore, no alterations in plasma nitrite, tissue creatine, or urine cGMP are found [80]. No report currently exists in the literature regarding the successful adenoviral rescue of this knockout mouse model.

Arginase

Human ARG Deficiency—Arginase deficiency (ARG deficiency, OMIM 207800) is among the least frequent of the urea cycle disorders with an incidence of 1 in 2,000,000 live births and results from a defect in arginase (AI), the final enzyme of the cycle, located on

chromosome 6 [82]. It is characterized by symptoms of progressive neurological and intellectual impairment, spasticity, persistent growth retardation, and episodic hyperammonemia [83]. Arginase converts arginine to ornithine and urea by cleaving the guanidino group from arginine, and since the ability to synthesize arginine from ornithine exists in almost all organisms, the evolution of arginase is thought to have enabled the completion of a cycle which allowed for the excretion of urea in terrestrial organisms [2,84].

The primary diagnostic finding in arginase deficiency is hyperargininemia, or a buildup of arginine in plasma. All other urea cycle disorders are characterized by low plasma arginine levels, and therefore, dietary supplementation with all essential amino acids other than arginine is a necessary therapy for arginase deficiency. Hyperammonemic crises are far less frequent and the peak ammonia levels are not as great as seen in other UCDs; furthermore, the spasticity seen in hyperargininemic patients does not exist in patients with other UCDs, and its cause is not very well understood. Missense mutations have been found in arginase deficient patients, and many occur in highly conserved regions of the gene [85,86].

Early studies whereby an increased protein intake in patients invariably increased levels of urea without a subsequent increase in arginine led to the conclusion that a second isoform of arginase likely existed and was encoded by a separate gene. It was demonstrated in 1983 [87] and proven in 1996 that this was indeed the case [88-90]. The gene for arginase II (AII) is located on chromosome 14.

The Arginase I Knockout Mouse Model—In order to further understand the consequences of arginase I deficiency, our group constructed and characterized an AI knockout mouse. The mouse is born normally and thrives for 10-12 days before succumbing to hyperammonemia, with only moderate hyperargininemia, and exhibits symptoms such as decerebrate posture, encephalopathy, and tremors in the extremities [91]. Liver pathology reveals enlarged hepatocytes with a variety of intracytoplasmic inclusions. Homozygotes display significantly increased plasma ammonia and arginine with plasma ornithine greatly reduced [91]. In contrast to human patients where kidney arginase II activity can often be induced up to 40-fold [6], only a 2-fold upregulation in AII activity is seen in the mouse model based on immunoprecipitation of kidney extract with an anti-AII antibody [91]; this low level of activity may not be able to compensate for a loss of liver arginase I activity in mice, resulting in a more severe outcome than that seen in the human patients.

The Arginase II Knockout Mouse Model—No known human disorder currently exists resulting from a deficiency of arginase II, which suggests either an embryonic lethal or an asymptomatic condition. When the arginase II knockout mouse was constructed by O'Brien's group [92], it proved to be completely viable and fertile with no obvious pathological abnormalities. Homozygotes exhibit 2-fold increases in plasma arginine at 8-10 weeks of age, with no significant alterations in any other amino acid. Tissue polyamines are also normal [92].

The Arginase I/II Double Knockout Mouse Model—Last year, our group bred the arginase single knockout animals together and created an arginase double knockout mouse [93]. The double knockout has the same phenotype as the arginase I knockout animal, with death occurring at two weeks of age following severe hyperammonemia. Plasma and liver arginine were greatly increased while plasma and liver ornithine were greatly reduced [93].

Conclusions

Thus far, mouse models for all of the urea cycle disorders (except for NAGS deficiency) have been constructed, and all except the ARG I deficient mouse seem to accurately replicate the

clinical and biochemical features seen in the human disorders. The models for OTC and AS deficiencies have enabled the testing of novel gene therapy protocols that are not possible to test directly in human patients at this time, and further investigation into the treatment and understanding of each disorder is currently ongoing. Though total correction of the underlying defects through gene therapy is the ultimate goal, further research into additional pharmacological and dietary treatments should be continued. The mouse models described here are highly useful substrates for both approaches.

Acknowledgments

This work was supported at UCLA by USPHS Grant HD 06576, by the DOD Grant PC020651, and by the Mental Retardation Research Center at UCLA.

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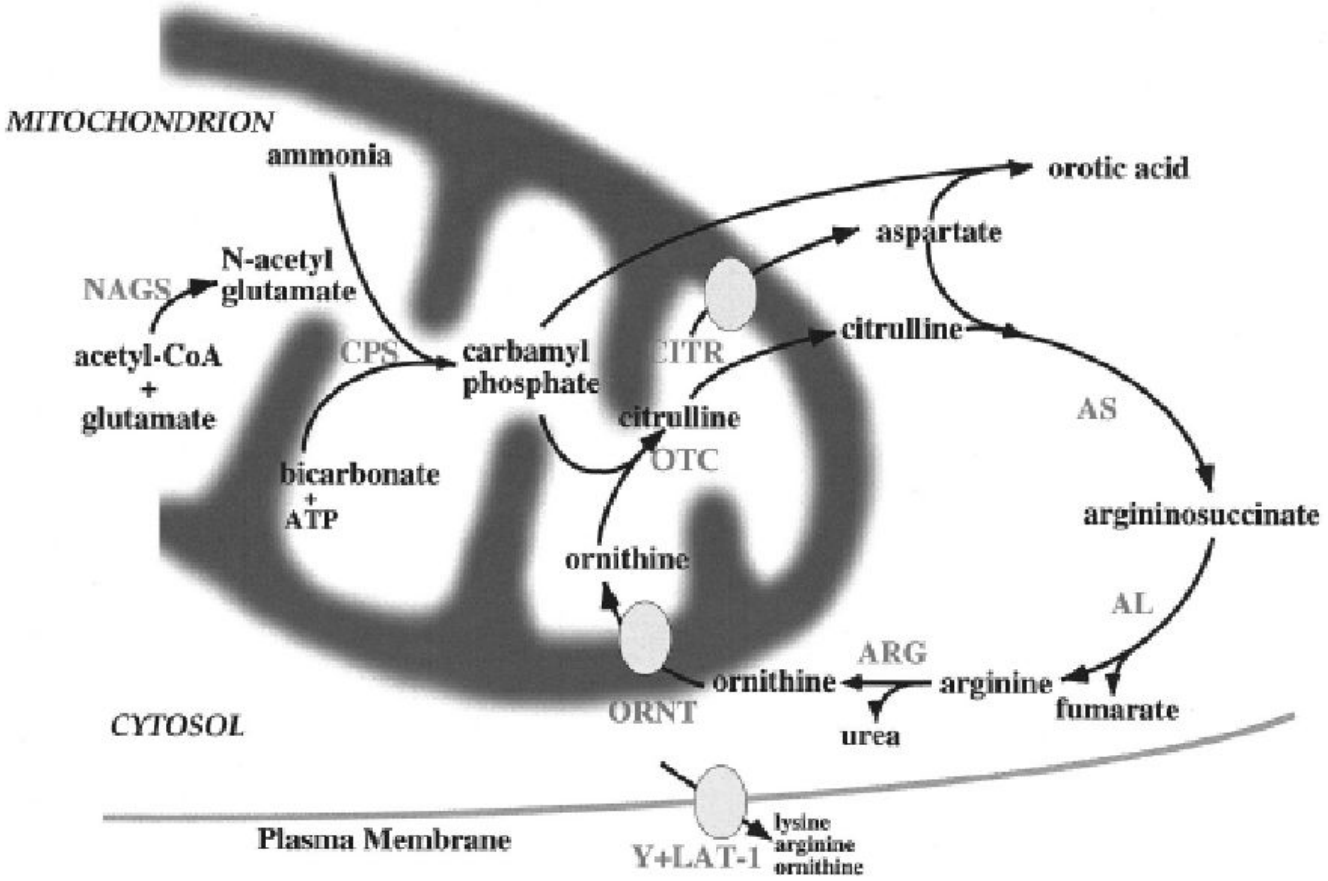

Figure 1.
Enzymes of the urea cycle, reproduced with permission from [94]. Ammonia in the mitochondria is converted to urea in the cytosol, and ornithine is transported back into the mitochondria to continue the cycle.

Table 1

Human and mouse homologs of urea cycle enzymes

Enzyme	Disorder	Human gene	Mouse gene	Mouse Model?
N-acetylglutamate synthase	NAGS deficiency	Chr. 17	Chr. 11	No
Carbamyl phosphate synthetase I	CPS-I deficiency	Chr. 2	Chr. 1	(Yes)
Ornithine transcarbamylase	OTC deficiency	Chr. X	Chr. X	Yes
Argininosuccinate synthetase	Citrullinemia type I	Chr. 9	Chr. 2	Yes
Argininosuccinate lyase	Argininosuccinic aciduria	Chr. 7	Chr. 5	Yes
Arginase	Hyperargininemia	Chr. 6	Chr. 10	Yes

Note: The CPS-I knockout mouse model is no longer available.