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Optimizing Therapy for Argininosuccinic Aciduria

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Abstract

Argininosuccinic aciduria (ASA) is a urea cycle disorder with a complex phenotype. In spite of a lower risk for recurrent hyperammonemic episodes as compared to the proximal disorders of ureagenesis, subjects with ASA are at risk for long-term complications including, poor neurocognitive outcome, hepatic disease and systemic hypertension. These complications can occur in spite of current standard therapy that includes dietary modifications and arginine supplementation suggesting that the presently available therapy is suboptimal. In this article, we discuss the natural history of ASA and the recent mechanistic insights from animal studies that have shown the requirement of argininosuccinate lyase, the enzyme deficient in ASA, for systemic nitric oxide production. These findings may have therapeutic implications and may help optimize therapy in ASA.

1. Introduction

Argininosuccinic aciduria, (ASA; MIM 207900), is the second most common urea cycle disorder (UCD) with an estimated incidence of 1 in 70,000 live births [1, 2]. ASA is caused by the deficiency of the urea cycle enzyme argininosuccinate lyase (ASL) that catalyzes conversion of argininosuccinic acid into arginine and fumarate. Subjects with ASA can present either in the neonatal period or later during childhood. Subjects with the severe neonatal-onset form of ASA typically present with hyperammonemia within the first week of life whereas those with the milder late-onset form can have either few or no episodes of hyperammonemia. Due to the ability to excrete significant amounts of argininosuccinate, a nitrogen rich compound, subjects with ASA have fewer hyperammonemic episodes as compared to those with more proximal enzymatic blocks of ureagenesis such as carbamoyl phosphate synthase 1 (CPS1) and ornithine transcarbamylase (OTC) deficiencies [3]. Intuitively it would thus be expected that the overall outcome in ASA would be better as compared to the more proximal UCDs. However, in contrast, it has been demonstrated that subjects with ASA have a greater risk for poor neurocognitive outcome, hepatic disease and systemic hypertension.

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2. ASA subjects have a complex phenotype

In a cross-sectional study, Tuchman and colleagues demonstrated that individuals with ASA had a statistically significant increases in neurological abnormalities, attention deficit hyperactivity disorder, developmental disability, and seizures as compared to those with other urea cycle disorders [1]. Seventy percent of ASA subjects in this study had developmental disability; fifty six percent had learning disability while one-third of the patients had seizures. However as this was a cross sectional study, the effect of factors such as number and severity of hyperammonemic episodes, or initiation and compliance with treatment on the clinical outcomes could not be ascertained. The institution of newborn screening programs has allowed for early diagnosis and treatment of ASA and has uncovered the natural history of the disease. Ficicioglu and colleagues discussed the outcome in 13 ASA subjects who were diagnosed by newborn screening in the United States [4]. All of the 13 subjects were asymptomatic at diagnosis, had never experienced significant hyperammonemic episodes, and a majority were treated with arginine supplementation. In spite of this, four of the 13 subjects had learning disability, three had mild developmental delay, three had seizures, and six patients had abnormal EEG. In a large cohort of ASA subjects from Austria, Mercimek-Mahmutoglu and colleagues reported on long-term follow up on 17 ASA subjects detected by newborn screening. In spite of normal plasma ammonia levels, twenty nine percent of subjects had Intelligent Quotient (IQ), in the low-normal ranges [2].

In addition to neurological deficits, subjects with ASA can develop hepatic complications. Anecdotal evidence for hepatomegaly, hepatitis, and even severe fibrosis have been previously published [5–7]. More recently, studies involving large cohorts of subjects with ASA have validated the concerns of hepatic involvement in this disorder. Tuchman and colleagues showed that ASA subjects had statistically significant elevations of plasma alanine aminotransferase as compared to those with OTC or argininosuccinate synthase (ASS1) deficiencies [1]. One long-term follow up study showed increased γ -glutamyl transferase and/or hepatic steatosis [2]. Interestingly, hepatic involvement has been noted even in individuals without significant hyperammonemia who were treated with protein restriction and arginine supplementation [2, 6]. However only a proportion of subjects with develop significant liver disease and many subjects have no evidence for hepatic involvement [4].

Systemic hypertension has been also recently recognized as a feature of ASA [8, 9]. Typically, secondary causes for hypertension are absent suggesting that this manifestation may be a direct consequence of ASL deficiency. Anecdotal reports suggest that the hypertension can be severe and difficult to control with conventional antihypertensive treatment [8].

These data imply that the complex phenotype observed in ASA involve mechanisms beyond the blockade in ureagenesis. The present therapeutic modalities used in the chronic treatment of ASA including dietary restriction and arginine supplementation are primarily geared towards prevention of hyperammonemia and have not been effective in preventing the neurocognitive deficits, hepatic disease, or hypertension [2, 4]. In this review, we summarize data from studies that have uncovered novel functions of ASL. Understanding the mechanistic basis of the complications in ASA may lead to newer treatment modalities for this complex disorder and prevent or alleviate the long-term complications.

3. Evolutionary aspects of ASL structure and function

ASL is the only mammalian enzyme that can generate endogenous arginine. In contrast to CPS1 and OTC expressions that are limited to certain tissues, most tissues express ASS1

and ASL [10, 11]. The widespread expression of the latter two enzymes is necessary for the cell-autonomous generation of arginine, a precursor for the synthesis of biologically important compounds such as nitric oxide (NO), polyamines, agmatine, glutamate, proline and creatine [12]. ASS and ASL are hence critical in two pathways in mammals: in the liver, they participate in ureagenesis with no net synthesis of arginine while in the rest of the tissues they are required for endogenous arginine synthesis (Figure 1). The importance of arginine and its metabolites for cell survival and proliferation is underlined by the conservation of ASL through evolution. The human ASL protein shares 56% of identity with its yeast homolog, ARG4 [13] and yeast mutants lacking ARG4 and thus auxotrophic for arginine can be rescued by the human ASL protein [14].

ASL belongs to a super-family of metabolic enzymes that includes fumarase, aspartase, δ crystallin, adenylosuccinate lyase, and 3-carboxy-cis, cis-muconate lactonizing enzyme [15, 16]. These enzymes function as tetramers and catalyze reactions in which fumarate is generated as a product. Among all the enzymes in this family, δ crystallin, a protein present in the avian and reptilian eye lens has a high degree of homology to ASL [17–19]. δ crystallin has structural functions that are necessary for maintaining transparency of the lens, that are distinct from its catalytic function necessary for the synthesis of arginine [20]. Given the similarities between the enzymes in this family, it is possible that similar to δ crystallin other members could also have moonlighting functions distinct from their requirement for catalysis. If this were to be the case, it raises the intriguing possibility of whether some of the long-term complications of ASA could be due to the lack of a ‘moonlighting’ function of ASL.

3.1 Deficiency of ASL leads to decrease in nitric oxide production

In order to understand the consequences of loss of ASL on arginine metabolism and gain insights into disease pathogenesis, we generated a novel hypomorphic mouse model of ASA [21]. ASA mice demonstrated a biochemical profile consistent with ASL deficiency with elevations of citrulline and argininosuccinic acid, and reduced arginine levels. These mice had evidence of multi-organ dysfunction involving the immune, hematopoietic, renal and cardiovascular systems that are not attributable to the blockade of ureagenesis. Consistent with the observations in human subjects, ASA mice also had hepatic involvement and systemic hypertension. Interestingly, the multi-organ dysfunction in ASA mice was associated with a significant decrease in systemic NO production as evidenced by measurement of tissue levels of nitrate and nitrosothiols (RSNO), surrogate markers of NO production and signaling [21]. Corroboratively, fibroblasts from humans with ASA demonstrate decreased RSNO levels, and plasma RSNO levels in ASA subjects were significantly lower when compared to controls [21]. These data show that the lack of ASL leads to a decrease in NO synthesis in both mice and humans.

3.2 Regulation of NO production is a function of ASL distinct from its role in ureagenesis

Distinguishing between the contributions of ASL deficiency in the liver vs. that in the other tissues to the phenotype of ASA is difficult. We hence corrected the ureagenesis defect in the ASA mice using liver-directed gene therapy [8]. While the gene therapy corrected the hepatic biochemical defect, normalized the growth and increased survival, the mice persisted to have NO deficiency as evidenced by significantly decreased RSNO levels in organs other than the liver. These data suggest that ASL is necessary outside of its role in ureagenesis and endogenous arginine synthesis, for NO production.

3.3 Role of NO deficiency in pathogenesis of ASA

The ASA mice had elevated systolic and diastolic blood pressures as compared to their wild-type littermates. *Ex vivo* experiments with aortic rings from these mice showed

endothelial dysfunction specifically due to the lack of NO production by vascular endothelium [8, 21]. Similarly, two human subjects with ASA showed impaired flow-mediated dilatation of brachial artery, a feature classically associated with deficient NO production from the vascular endothelium [21]. Interestingly, the ASA mice treated with liver-directed gene therapy continued to be hypertensive with evidence for decreased NO production from the vascular endothelium [8]. These data suggest that some manifestations of ASA like hypertension result directly from the tissue specific requirement of ASL for NO production and not due to the block in ureagenesis.

4. Arginine supplementation does not rescue the NO deficiency

If the deficiency of the substrate, arginine, is the predominant cause for the decrease in NO production, supplementation with exogenous arginine should correct this defect; however, this is not the case. *In vitro* experiments using human ASA fibroblasts and piglet endothelial cells knocked-down for ASL demonstrated decreased NO production even when supplemented with supra-physiologic concentrations of arginine [21]. Consistent with this, *in vivo* measurements of metabolite fluxes using stable isotope studies in human ASA subjects showed they had significantly reduced fractional transfer of guanidino nitrogen from $^{15}\text{N}_2$ -arginine to ^{15}N -citrulline, a dynamic marker of NO production. These data prove that in the absence of ASL, even exogenously administered arginine cannot be utilized by NOS for NO synthesis. This paradox was resolved by the finding that there is a 'structural requirement' of ASL to maintain a NO-synthesis-complex consisting of ASL, ASS1, NOS, heat shock protein 90, and cationic arginine transporter SLC7A1. In the absence of ASL, there is decrease in the assembly of this complex with loss of channeling of arginine to NOS for NO production. Similar to the evolution of the differential catalytic and structural functions for its homologous enzyme $\delta 2$ crystallin in the lens, ASL has a catalytic function necessary for urea and arginine synthesis and a structural function of channeling of arginine to NOS for NO production (Figure 2).

5. Genotype-phenotype correlations in ASA

There is generally a poor correlation between enzymatic activity and clinical outcome in ASA [22–24]. Patients with no detectable enzyme activity can have normal neurocognitive outcome whereas those with higher residual activity can have developmental delay [2]. One explanation for this poor correlation is the instability of the mutant ASL protein in the cell homogenate [25]. It is also difficult to predict the outcomes based on the genotype as the catalytic function of ASL depends on its tetrameric configuration and thus intragenic complementation among specific mutations can allow for residual activity [14, 23, 24]. By studying the growth of yeast-deletion mutants in arginine-free media, it was shown different allelic complementations could lead to residual enzymatic activity [14]. Using this model, it was demonstrated that in patients with late-onset form of ASA, at least one active site was formed thus resulting in significant residual activity. However, the recent findings of a structural function of ASL complicate the matter further. Mutations can have different effects on the catalytic vs. structural properties of ASL and it is likely that some patients would have minimal deficiencies in NO production while others may have significantly more NO deficiency depending on how much structurally intact ASL remains. Moreover, nutritional intake of NO sources may further modify the clinical manifestation of any potential NO deficiency. Hence, understanding genotype-phenotype-environment correlations could lead to a better personalization of therapy and potentially long-term outcome in ASA.

6. Increased free radical generation in ASA

The lack of arginine substrate availability can result in ‘uncoupling’ of NOS wherein free radicals are generated instead of NO [26–28]. In the active form, NOS is in the form of a dimer while in the inactive uncoupled state, it exists as a monomer. ASA mice indeed show uncoupling of NOS3 and elevation of free radicals in the aortae as well as in plasma and urine [8, 21], though the magnitude of these elevations support at best a secondary factor in the pathogenesis of this condition. Yet, increased free radical production could also contribute to tissue damage with the brain being especially sensitive to oxidative injury [29]. Free radicals may be particularly damaging in the context of ASA, as argininosuccinate could be converted to guanidinosuccinic acid, a known cellular and neuronal toxin [30–32]. Further studies are needed to determine whether free radicals contribute to the cognitive delay in ASA.

7. Implications of recent advances on therapy of ASA

The data from long-term studies suggest that arginine supplementation may not have sufficient impact on the long-term outcome in ASA patients, as previously believed [2]. In addition, mechanistic studies show that such supplementation does not replenish nitric oxide deficiency in ASA, and it is unclear whether arginine supplementation restores the other downstream metabolites. This raises two important issues 1) what is the appropriate dose of arginine? and 2) would NO donor therapy be beneficial in ASA?

7.1 Dose of arginine in ASA

Large amounts of exogenous arginine (400–700 mg/kg/day) will promote synthesis of argininosuccinate when arginine is used as the sole agent for nitrogen disposal in patients with ASA [22]. This dose of arginine facilitates adequate protein intake in infants and younger children while promoting efficient nitrogen excretion [22]. In fact, acute hyperammonemia is effectively treated with high dose intravenous arginine infusion which is often sufficient as monotherapy to control hyperammonemic episodes. Long-term oral therapy with a high-dose of arginine had been considered safe and efficacious and has been reported to lead to improved cognitive outcomes in a single observational study [33]. However the more recent long-term studies have not detected an improved outcome in ASA subjects supplemented with arginine as compared to those who were not [2, 4]. There has been a theoretical concern that while high-dose of arginine would lead to increased generation of argininosuccinate and improved nitrogen clearance, it may also serve as a hepatotoxic agent thereby contributing to the more severe liver disease seen in this condition.

In a recent double-blind, placebo-controlled, cross over trial, the effects of a high-dose of arginine vs. a low-dose of arginine on hepatic function tests were compared [34]. Twelve patients with ASA were randomized to receive either a low-dose of arginine therapy ($100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) combined with sodium phenylbutyrate ($500 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) (LDA arm) or a high-dose of arginine ($500 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) (HDA arm) for one week and liver function were assessed. Subjects had significantly increased levels of argininosuccinate, and aspartate aminotransferase levels while on the high-dose arginine arm. Interestingly, the elevations of plasma aminotransferases had a significant correlation with plasma argininosuccinate suggesting that argininosuccinate may have a role in hepatic injury. However, there were no differences in the synthetic liver function as assessed by prothrombin time, INR, and coagulation factor levels. These results suggest that a high-dose of arginine may not to be as innocuous as previously believed. Deciding on the optimal dose of arginine therapy for any individual is a difficult task. The primary goal in supplementing arginine to ASA subjects would be to prevent hyperammonemia. The dose required to maintain plasma arginine levels

in the normal ranges will differ between individuals. However, if high-dose of arginine is being used solely for the purposes of nitrogen excretion, consideration should be given to reducing the arginine dose and adding nitrogen scavengers.

7.2 NO supplementation therapy in ASA

As the deficiency of NOS-dependent NO generation is partly responsible for some of the manifestations of ASA, therapy with NOS-independent NO donor therapy should be beneficial. The anions nitrate (NO_3^-) and nitrite (NO_2^-) can be recycled in vivo to form NO. This nitrate-nitrite-NO pathway represents an important alternative source of NO to the classical L-arginine-NO-synthase pathway [35, 36]. ASA mice treated with sodium nitrite had comparable survival to traditional treatment that included arginine and sodium benzoate [21]. Addition of sodium nitrite to the traditional treatment regimen of arginine and nitrogen scavenger resulted in normalization of blood pressure [21]. The aortic rings in ASA mice showed normal relaxation with a NO donor. Corroborating these experiments, human ASA subjects with endothelial dysfunction and absence of flow-mediated dilatation of brachial artery showed normal dilatation with NO donors. These data imply that the NO donor therapy is beneficial in ASA especially in the treatment of hypertension.

One of the ASA subjects under our clinical care allowed us to translate our findings in a human therapeutic context [8]. This subject was diagnosed with idiopathic hypertension at the age of 5 years. In spite of continuous arginine supplementation, and therapy with four classes of first-line antihypertensive medications, his hypertension was poorly controlled for a period of over 10 years. At the age of 16 years, he presented with hypertensive urgency. Treatment with NOS-independent NO donor was initiated and resulted in sustained normalization of his blood pressures, permitting discontinuation of all other anti-hypertensive medications. Anecdotally, an improvement in several neuropsychological parameters pertaining to verbal memory and nonverbal problem solving was noted with the NO therapy. Whereas these human data are preliminary, they imply NO supplementation in ASA may be beneficial beyond the vascular complications and mandate that NO donor therapy be investigated in a systematic manner in the context of a randomized control trial (RCT). Irrespective, it may be prudent to recommend higher intake of nitrite rich foods as these foods are also low in protein and appropriate for UCD nutritional management.

8. Future directions

Accumulating evidences suggest that the complex phenotype in ASA is due to the roles of ASL outside of ureagenesis. Encouraging results with NO donor therapy in animal models and a human subject with ASA support the notion that understanding the pathogenesis of ASA would allow for optimization of treatment. A better understanding of the genotype-phenotype correlations would be needed to identify the subset of subjects who will benefit from NO therapy. A RCT would be necessary to further evaluate the effects of NO supplementation in ASA subjects. The availability of a relevant animal model for ASA would help explore the benefits of different treatment modalities. Hence, further studies exploring the roles of elevated levels of ASA, guanidinosuccinic acid, and free radicals in causation of specific phenotypes using the ASA mouse model would be of great value.

Currently, in addition to standard of care restricted protein intake and arginine supplementation in chronic management of ASA, vigilant monitoring for evidence of NO deficiency for example as evidenced by hypertension should be performed. Until RCTs studying the effects of nitrite supplementation is completed, recommendation of low protein, nitrite rich foods would be an appropriate recommendation given their low risk. Moreover, while acute hyperammonemia in ASA should continued to be treated with high dose intravenous arginine, in the context of chronic management, lower dose arginine in

conjunction with nitrogen-scavenging therapy especially in patients with elevated liver enzyme tests should be considered.

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Highlights

- We describe the complex phenotype in ASA
- We discuss the recent long-term studies delineating the natural history of ASA
- Highlight the mechanistic data that show requirement of ASL for NO production
- Discuss the limitations of current therapy and prospects for future novel therapies

Figure 1. The urea cycle and the citrulline-nitric oxide cycle

In the liver, ASS and ASS are involved in ureagenesis whereas in most other tissues, they are required for endogenous arginine synthesis. The production of nitric oxide is coupled within this Citrulline-NO cycle. NAGS: N-acetyl glutamate synthase; CPS1: Carbamoyl phosphate synthase 1; OTC: Ornithine transcarbamylase; ASS: Argininosuccinate synthase; ASL: Argininosuccinate lyase; ARG: Arginase 1; NOS: Nitric oxide synthase. Reproduced with permission from publishers John Wiley and Sons.

Figure 2. The catalytic and structural functions of ASL

ASL is not only required for endogenous arginine synthesis (a catalytic function), but is structurally required to maintain a complex that facilitates channelling of exogenous arginine to nitric oxide synthase (NOS) for Nitric oxide (NO production). The tetrameric ASL enzyme depicted in green represents that which is required for catalysis while the region in gray is needed for the NO-synthesis-complex that includes SCL7A1, the cationic arginine transporter.