A mouse model of argininosuccinic aciduria: biochemical characterization

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Abstract
Argininosuccinate lyase (AL) has several roles in intermediary metabolism. It is an essential component of the urea cycle, providing a pathway for the disposal of excess nitrogen in mammals. AL links the urea cycle to the tricarboxylic acid (TCA) cycle by generating fumarate. Finally, AL is required for the endogenous production of arginine. In this latter role it may function outside ureagenic organs to provide arginine as a substrate for nitric oxide synthases (NOS). Increasing evidence suggests that argininosuccinate synthetase (AS) and AL are more globally expressed, and the coordinate regulation of AS and AL gene expression with that of the inducible form of NOS (iNOS) provides evidence that this may facilitate the regulation of NOS activity. Deficiency of AL leads to the human urea cycle disorder argininosuccinic aciduria. We produced an AL deficient mouse by gene targeting in order to investigate the role of AL in endogenous arginine production. This mouse also provides a model of the human disorder to explore the pathogenesis of the disorder and possible new treatments. Metabolic studies of these mice demonstrated that they have the same biochemical phenotype as humans, with hyperammonemia, elevated plasma argininosuccinic acid and low plasma arginine. Plasma nitrites, derived from NO, were not reduced in AL deficient mice and there was no significant difference in the level of cyclic GMP, the second messenger induced by NO.

Keywords: Argininosuccinate lyase; Nitric oxide; Mouse; Urea cycle

Introduction
Argininosuccinate lyase (AL; EC 4.3.2.1) is responsible for cleaving argininosuccinate into fumarate and arginine. AL is a 52-kDa cytosolic enzyme that functions as a tetramer and participates in two important metabolic pathways: the urea cycle, where it participates in the disposal of waste nitrogen, and the arginine–citrulline cycle, in which arginine is metabolized by nitric oxide synthases (NOS) to generate nitric oxide and citrulline. Because of its role in the urea cycle, AL is highly expressed in the liver and kidney, where nitrogen in the form of ammonium is processed into urea [1]. The urea cycle enzymes preferentially use substrates produced within the cell rather than from extracellular sources.

This channeling of substrates in the urea cycle has been demonstrated by in situ studies of permeabilized rat hepatocytes [2,3]. AL may also play a critical role in the production of nitric oxide (NO). AL participates in the arginine–citrulline cycle to generate arginine from which NOS generates NO and regenerates citrulline. AL and argininosuccinate synthetase (AS) both participate in this cycle and are expressed in all tissues that have been examined [4,5]. Evidence to suggest a link between de novo arginine synthesis and NO production is provided by co-induction studies. Rats injected with lipopolysaccharide (LPS), a known inducer of NO synthesis, have been used for kinetic studies of NO production. In these studies, inducible NOS (iNOS), AS, and AL were all co-induced, inferring that citrulline–arginine recycling appears to be important in NO synthesis [6]. This suggests that AL provides iNOS with a preferred cellular source of arginine, reminiscent of the channeling of substrates in the urea cycle [2]. Further evidence in hu-
mans comes from stable isotope studies in adult males fed a regular diet followed by an arginine-free diet. Restriction of dietary arginine leads to a decrease in serum arginine but does not alter the rate of whole body NO synthesis [7]. This implies that endogenous arginine synthesized by AL is the preferred source (over dietary arginine) for NOS.

Deficiency of AL in humans was first described by Coryell et al. [8], and is commonly referred to as argininosuccinic aciduria. Individuals with this autosomal recessive disease typically come to medical attention early in life with symptoms of hyperammonemia such as seizures, lethargy, or coma. The disease is characterized biochemically by hyperammonemia, detectable argininosuccinic acid in serum, urine and CSF, low serum arginine concentrations and elevated serum citrulline levels. Treatment currently consists of a low-protein diet, decreasing nitrogen flux through the urea cycle, and arginine supplementation [1]. Despite this treatment, patients often exhibit intellectual impairment and delayed motor skills. These sequelae are thought to result from chronic hyperammonemia, although it has also been suggested that a deficit in TCA cycle intermediates may contribute to the phenotype [9]. Patients with AL deficiency may also suffer from progressive hepatic disease [10], and may require orthotopic liver transplantation due to cirrhosis. Because individuals with AL deficiency have fewer and less severe episodes of hyperammonemia than patients with other urea cycle disorders, it has been suggested that the liver pathology is due to the toxicity of argininosuccinic acid or its anhydride metabolites. Alternatively, liver disease in argininosuccinic aciduria may result at least in part from impaired NO synthesis as a consequence of the inability to synthesize arginine. It is possible that reduced NO production may lead to impaired blood pressure regulation in the liver. Perturbed hepatic blood flow may result in hepatic fibrosis. Alternatively, accumulation of argininosuccinic acid, which is normally undetectable in the plasma, may be fibrogenic and lead to cirrhosis.

Since NO has been shown to regulate basal blood flow and is produced from arginine [11–15], investigating the effect of reduced endogenous arginine synthesis on NO production may provide insights into the role of arginine synthesis on the regulation and pathophysiologic effects of NO. NO achieves its effect by acting on intracellular guanylyl cyclase, resulting in conversion of GTP to cGMP. cGMP then acts on phosphodiesterases, cGMP-gated ion channels, and cGMP-dependent protein kinases which produce the cardiovascular, neuronal, pancreatic and other effects associated with NO [21,22]. cGMP is a potentially more stable indirect measure of NO production. Here we describe the generation and initial biochemical characterization of a mouse strain deficient in AL activity.

![Image](https://example.com/image1.png)

**A**

![Image](https://example.com/image2.png)

**B**

Fig. 1. The targeting strategy for the mouse AL gene. (A) Restriction sites used for generating the targeting plasmid are indicated (B, *BamH*I; Ev, *EcoR*V; Xb, *Xho*I; E, *EcoR*I). (B) The probe depicted as a black bar detects a recombinant band of 3.9 kbp following homologous recombination and replacement of exons 8 and 9 with the neomycin gene. A representative Southern blot is shown with the 5 kbp wild type band and the 3.9 kbp recombinant band.
Materials and methods

Gene targeting of the AL gene

A previously isolated cDNA fragment [16] was used to screen a 129SvEv mouse genomic library by plaque hybridization to isolate a 12 kbp portion of the mouse AL gene. Using standard molecular biology cloning strategies, exons 8 and 9 were replaced with a 1.4 kbp neomycin cassette which serves to delete 2 of the 13 exons and generate an out of frame mRNA beginning at exon 10 (Fig. 1). This AL gene construct was electroporated into the embryonic stem cell line AB2.2, the cells subject to positive and negative selection with G418 and gancyclovir, and numerous ES cell clones were identified by Southern blotting using the mini-Southern procedure [17]. We generated mice heterozygous for the mutant allele and bred them to produce homozygous deficient mice. Genotyping was carried out using the PCR primers: (5′ AAG ATG GGT TCC GCA GAT TAG GAT C 3′; 5′ TGT AGG CAT CTG AGA GTT GCA CAA A 3′; 5′ TCT TGT CGA TCA GGA TGA TCT GGA C 3′). PCR was used to identify a wild type 600 bp sized fragment containing exons 8 and 9 and a smaller 400 bp fragment primed from the neomycin cassette. Animal care conformed to institutional standards following an approved animal protocol.

Biochemical assays

Argininosuccinate lyase activity in homogenized liver samples was measured by colorimetric assay of urea production from argininosuccinate, and the activities of ornithine transcarbamylase, carbamyl phosphate synthetase, argininosuccinate synthetase, and arginase were measured as previously described [18]. Serum amino-acid analysis was performed by high pressure liquid chromatography utilizing the ninhydrin reaction and detection at 570 nm (440 nm for proline). Plasma ammonia levels were assayed based on reductiveamination of 2-oxoglutarate, using glutamate dehydrogenase and reduced NADPH. The decrease in absorbance at 340 nm due to the oxidation of NADPH is proportional to the plasma ammonia concentration. Guanidinoacetate and creatine concentrations were measured in aqueous extracts of homogenized heart, skeletal muscle, and liver using tandem mass spectrometry [19].

Measurement of nitric oxide degradation products and related metabolites

Measurement of the degradation products of NO was performed on urine samples using the Greiss reaction, a colorimetric detection of nitrite [20]. cGMP levels in urine and homogenized liver were measured using the commercially available Biotrak cGMP enzyme immunoassay (EIA) (Amersham-Pharmacia Biotech) that combines the use of a peroxidase-labeled cGMP conjugate, a specific antiserum which can be immobilized to pre-coated microtiter plates and a one-pot stabilized substrate solution. Measurement of cGMP in concentrations of femtomoles/well was carried out by spectrophotometric measurement at 450 nm.

Results

Generation of AL deficient mice

Following microinjection of ES cells into blastocysts, heterozygous mice were generated from chimeric founder mice (Fig. 1). Twenty-two litters (179 pups) from heterozygous intercrosses were genotyped by PCR shortly after birth. Genotypes of offspring were consistent with the expected Mendelian ratio (1:2:1), indicating that there is no increased in utero lethality in homozygous null (AL\(^{-/-}\)) or heterozygous mice. AL\(^{-/-}\) pups are indistinguishable in weight (AL\(^{-/-}\) average 1.37 g vs. wild type average 1.45 g; \(\chi^2 = 0.002\)) or appearance at birth from wild type littermates and all initially feed well. Within 48 h AL\(^{-/-}\) mice became less active, stopped feeding and rapidly expired.

Characterization of urea cycle enzymes and biochemical parameters

In AL\(^{-/-}\) mice there was a complete deficiency of AL activity (Fig. 2). Enzyme activity in AL\(^{-/-}\) liver ranged from 14–77 \(\mu\)mol/h/g tissue, while wild type liver had AL activity of 673–1540 \(\mu\)mol/h/g tissue, consistent with a null mutation generated by deleting a large portion of the carboxy terminal end of the protein. To determine whether the absence of AL leads to a down-

![Fig. 2: Selected serum amino-acid levels. Values are indicated with the standard error for each group (n = 4). The animals deficient for argininosuccinate lyase have elevated levels of glutamine (gln), citrulline (cit), and argininosuccinic acid (ASA) and low levels of arginine (arg) relative to wild type littermates. ASA is undetectable in wild type mice. All differences are highly significant (P > 0.001). These biochemical abnormalities recapitulate the human disease argininosuccinic aciduria.](image-url)
regulation of the overall pathway, other urea cycle enzymes were assayed in liver tissue. AL\(^{-/-}\) liver has reduced levels of arginase activity relative to control liver (Table 1).

Serum amino-acid analysis corroborates the enzyme findings. AL\(^{-/-}\) mice have statistically significant elevations of serum argininosuccinic acid, citrulline, and glutamine and low serum arginine levels when compared to wild type littermates (Fig. 2; \(P < 0.001\)). Plasma ammonia levels were assayed in samples from AL\(^{-/-}\) and wild type mice. AL\(^{-/-}\) mice had a statistically significant increase in plasma ammonia levels compared to wild type mice (Fig. 3; \(P < 0.05\)). These results are all consistent with the loss of AL activity in this mutant mouse strain, resulting in hyperammonemia.

Since arginine is a precursor in the synthesis of creatine via guanidinoacetate, we measured the concentration of each compound in pooled homogenized skeletal muscle, heart, and liver samples. There was no difference detected between AL\(^{-/-}\) and wild type mice in the pooled tissues (wild type 0.73 ± 0.135 \(\mu\)M (\(N = 6\)) vs AL\(^{-/-}\) 0.658 ± 0.094 \(\mu\)M (\(N = 5\)).

Because NO has a short half-life (\(t_{1/2} = 10\ s\)), direct measurement is difficult. Therefore, measurement of the NO degradation product nitrite was measured by the Greiss reaction. There was no observable difference of plasma nitrite concentration among between wild type (mean = 34.1 ± 10.2) and AL\(^{-/-}\) animals (mean = 26.7 ± 13.7).

To examine the signal transduction pathway activated by NO, additional experiments were performed to determine guanosine 3',5'-cyclic monophosphate (cGMP) levels in the urine of the affected and control animals. Pooled urine from eight litters was assayed. The mean level in AL\(^{-/-}\) mice was 11,080 (SE = 1081) femtomoles/well while mean urine cGMP in wild type animals was 11,410 (SE = 589) femtomoles/well. There was no significant difference in the concentration of cGMP in the urine of AL\(^{-/-}\) mice when compared to wild type mice.

### Discussion

The urea cycle of mammals serves two functions; the de novo biosynthesis of arginine and the disposal of excess nitrogen beyond that needed for net protein biosynthesis. Arginine is also used as a substrate in several other pathways including polyamine, creatine and NO biosynthesis. Argininosuccinic aciduria is a rare, autosomal recessive disorder of nitrogen metabolism that results from a deficiency of the enzyme argininosuccinate lyase. Individuals with argininosuccinic aciduria typically come to medical attention in the newborn period or during infancy as a result of vomiting, lethargy, developmental delay and hyperammonemia. The biochemical hallmarks of this disease are elevations of the amino acids argininosuccinic acid and citrulline and low levels of plasma arginine. Despite treatment to normalize plasma arginine levels and decrease episodes of hyperammonemia, individuals often have impaired motor and intellectual development, although this is not a universal finding [9]. In addition, many patients develop progressive liver dysfunction and fibrosis. The pathophysiology of the liver disease is unclear. It seems unlikely to be related to the hyperammonemia, since individuals with other urea cycle disorders typically have more episodes of hyperammonemia and do not develop cirrhosis with the frequency seen in individuals with argininosuccinic aciduria. This has led to the hypothesis that argininosuccinic acid is toxic to hepatocytes and results in cirrhosis, although a

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**Table 1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>AL(^{-/-}) mice</th>
<th>Wild type littermates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Means (± SE) in (\mu)mol/h/g liver</td>
<td>Means (± SE) in (\mu)mol/h/g liver</td>
</tr>
<tr>
<td>Carbamyl phosphate synthetase</td>
<td>17.2 (±2.5)</td>
<td>12.2 (±1.5)</td>
</tr>
<tr>
<td>Ornithine transcarbamylase</td>
<td>597 (±86)</td>
<td>713 (±164)</td>
</tr>
<tr>
<td>Arginase*</td>
<td>12.8 (±1.6)</td>
<td>21.3 (±2.7)</td>
</tr>
<tr>
<td>Argininosuccinate lyase*</td>
<td>40 (±19)</td>
<td>968 (±286)</td>
</tr>
</tbody>
</table>

*Arginase activity is lower in AL\(^{-/-}\) mice relative to control mice. \(P < 0.05\).

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**Fig. 3.** Plasma ammonia levels from mutant mice and control littermates. Plasma ammonia levels in AL\(^{-/-}\) (\(n = 5\)) average four-times the levels seen in wild type littermates (\(n = 3; \ P < 0.05\)). The standard errors are shown for the two groups.
correlation of plasma argininosuccinate levels with liver disease has not been established. Two alternate mechanisms of liver and brain injury related to arginine deficiency seem plausible. Low tissue arginine levels may reduce NO production and impair liver blood flow regulation, and may lead to fibrosis [21,22]. Support for this theory comes from two observations in humans. A study of normal individuals infused with the endothelial NO inhibitor L-NMMA into the brachial artery demonstrated a 50% fall in basal blood flow and an attenuated response to the vasodilator acetylcholine [23]. Similarly, blood pressure perturbations have been reported in a hypoargininemic newborn with argininosuccinic aciduria [24]. The infant exhibited hypertension at the time of presentation, which resolved with intravenous arginine infusion. In the same report a second infant administered an intravenous “arginine challenge” to evaluate pituitary function exhibited reduced blood pressure with each infusion of arginine. In addition, rats administered the arginine analog/NO inhibitor Nω-nitro-L-arginine have sustained hypertension in comparison to control animals. This effect can be attenuated by pretreatment with L-arginine [25].

NO is an important signaling molecule used in a vast number of biologic settings including the regulation of vascular tone, neurotransmitter release and learning, immune function and skeletal muscle and cardiac energy regulation. NO functions by activating guanylyl cyclase to produce cGMP from GTP, which leads to a series of aforementioned effects [26,27]. Recent studies indicate that endogenously synthesized (as opposed to dietary) arginine as a substrate for NOS is important for NO production. That NOS preferentially uses endogenously produced arginine is supported by evidence of channeling of substrate via microcompartmentalization within the cell [28]. Thus, low tissue arginine levels would be expected to impede NO production by decreasing the availability of the substrate arginine and through impaired induction of NOS. To test the hypothesis that low arginine levels restrict NO production, we measured degradation products (nitrites) and the second messenger (cGMP). There was no significant difference in either plasma nitrite levels of mutant animals or urine cGMP levels. A better method to test this hypothesis would be to directly measure NO production. Unfortunately, the small size and short life span of AL deficient mice combined with the short half-life of NO did not permit direct measurement. Measurement of NO formation in cultured astrocytes following induction with cytokines is in progress.

Finally, low arginine levels may reduce creatine production and thus reduce the energy capacity of the brain, and/or the synthesis of NO in the brain may be impaired [29,30]. AL is known to be expressed in several cell types within the brain and its expression is induced by elevated ammonia in a cell culture system [31,32]. No difference in the tissue concentrations of creatine or its precursor guanidinoacetate was detected in the mutant mice, however, this may reflect the young age of the mutant mice and residual levels from maternal production. Future experiments using a regulatable genetic rescue of the AL neonatal lethality should allow a more definitive determination of the role of endogenous arginine synthesis in NO formation and the mechanism of liver fibrosis in argininosuccinic aciduria.

Acknowledgments

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References


