

Review Article

Argininosuccinate synthase: at the center of arginine metabolism

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Abstract: The levels of L-arginine, a cationic, semi-essential amino acid, are often controlled within a cell at the level of local availability through biosynthesis. The importance of this temporal and spatial control of cellular L-arginine is highlighted by the tissue specific roles of argininosuccinate synthase (argininosuccinate synthetase) (EC 6.3.4.5), as the rate-limiting step in the conversion of L-citrulline to L-arginine. Since its discovery, the function of argininosuccinate synthase has been linked almost exclusively to hepatic urea production despite the fact that alternative pathways involving argininosuccinate synthase were defined, such as its role in providing arginine for creatine and for polyamine biosynthesis. However, it was the discovery of nitric oxide that meaningfully extended our understanding of the metabolic importance of non-hepatic argininosuccinate synthase. Indeed, our knowledge of the number of tissues that manage distinct pools of arginine under the control of argininosuccinate synthase has expanded significantly.

Keywords: Argininosuccinate synthase; arginine metabolism; nitric oxide; arginase; urea cycle, arginine recycling

Introduction

Arginine is required by all tissues in the human body for protein synthesis, and by some tissues for specialized needs. Any *de novo* biosynthetic pathway for arginine involves the conversion of citrulline to arginine catalyzed by argininosuccinate synthase and argininosuccinate lyase. Specifically, argininosuccinate synthase catalyzes the condensation of citrulline and aspartate to form argininosuccinate, the immediate precursor of arginine. Argininosuccinate lyase then splits argininosuccinate to release fumarate and arginine (**Figure 1**). First identified in liver, as a rate-limiting enzyme in urea synthesis, argininosuccinate synthase is now recognized as a ubiquitous enzyme in mammalian tissues, and not surprisingly, its expression, localization, and regulation differs significantly depending on the tissue specific needs for arginine.

Argininosuccinate synthase plays an important role as the rate-limiting step in providing arginine for an assortment of metabolic processes, both catabolic and anabolic. Thus, the metabolic pathways in which argininosuccinate syn-

these participates are linked to the varied uses of the amino acid arginine. There are five major pathways in which argininosuccinate synthase plays a key role. These are (a) urea synthesis, (b) nitric oxide synthesis, (c) polyamine synthesis, (d) creatine synthesis, and (e) the *de novo* synthesis of arginine to maintain serum levels.

In liver, where arginine is hydrolyzed to form urea and ornithine, argininosuccinate synthase is highly expressed, and hormone and nutrients constitute the major regulating factors [1]. In contrast, for nitric oxide-producing cells where arginine is the direct substrate for nitric oxide synthesis, argininosuccinate synthase is expressed at relatively low levels, and in most cases, its regulation has been tied to affecting the phenotypic patterns controlled by nitric oxide signaling [2].

The subcellular localization of argininosuccinate synthase also varies. For example, in endothelial cells argininosuccinate synthase co-localizes with eNOS at caveolae [3] and outside of the Golgi [4]. In kidney, subcellular localization is not well-defined, but seems to be cytosolic in

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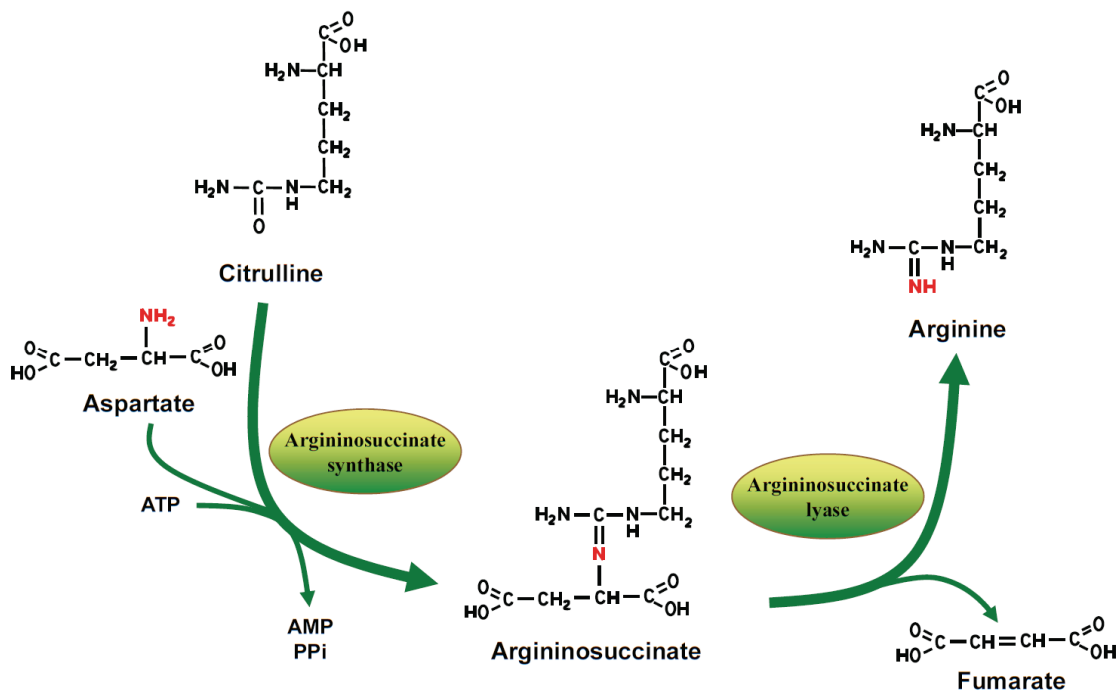


Figure 1. De Novo biosynthetic pathway for arginine.

relation to synthesis of arginine for guanidinoacetate production and for the *de novo* synthesis of arginine for export to maintain serum arginine levels. In liver, argininosuccinate synthase associates with the outside of the mitochondria to complete the urea cycle, which involves both the mitochondrial and cytosolic compartments [5].

The objective of this discussion is to highlight the varied metabolic processes that involve argininosuccinate synthase, its essential role in regulating unique cellular pools of available arginine, and the levels of control that regulate its expression.

Argininosuccinate synthase and the urea cycle

The urea cycle, also known as the Krebs urea cycle or ornithine cycle, was first discovered in 1932 by Hans Krebs [6]. The cycle is comprised of five enzymes and is essentially localized to the liver in mammals, although the kidney does share some capacity to produce urea. The first two enzymes of the urea cycle, carbamoyl phosphate synthetase I and ornithine transcarbamoylase are located within the mitochondrial matrix of periportal hepatocytes [7], while the

remaining three enzymes are located in the cytosol. Argininosuccinate synthase is located on the outer mitochondrial membrane [5], utilizing both citrulline and aspartate to produce argininosuccinate. Argininosuccinate lyase catalyzes the cleavage of argininosuccinate to produce arginine and fumarate. Subsequently, arginase splits the arginine to ornithine and urea, which ensures that the arginine generated by the actions of argininosuccinate synthase and argininosuccinate lyase is, for the most part, directed to urea production [8] (**Figure 2**). Urea is excreted and the ornithine is transported back into the mitochondria to complete the cycle.

Immunocytochemical studies by Cohen & Kudal [5] showed that argininosuccinate synthase and argininosuccinate lyase are located in the immediate vicinity of the mitochondria, predominantly next to the cytoplasmic surface of the outer membrane. This finding confirmed previous biochemical studies [9-11] suggesting that these enzymes are organized *in situ* next to the outer membrane of mitochondria. Perhaps our best understanding as to how argininosuccinate synthase associates with hepatic mitochondria comes from the findings related to adult-onset type II citrullinemia (CTLN2). Unlike classical

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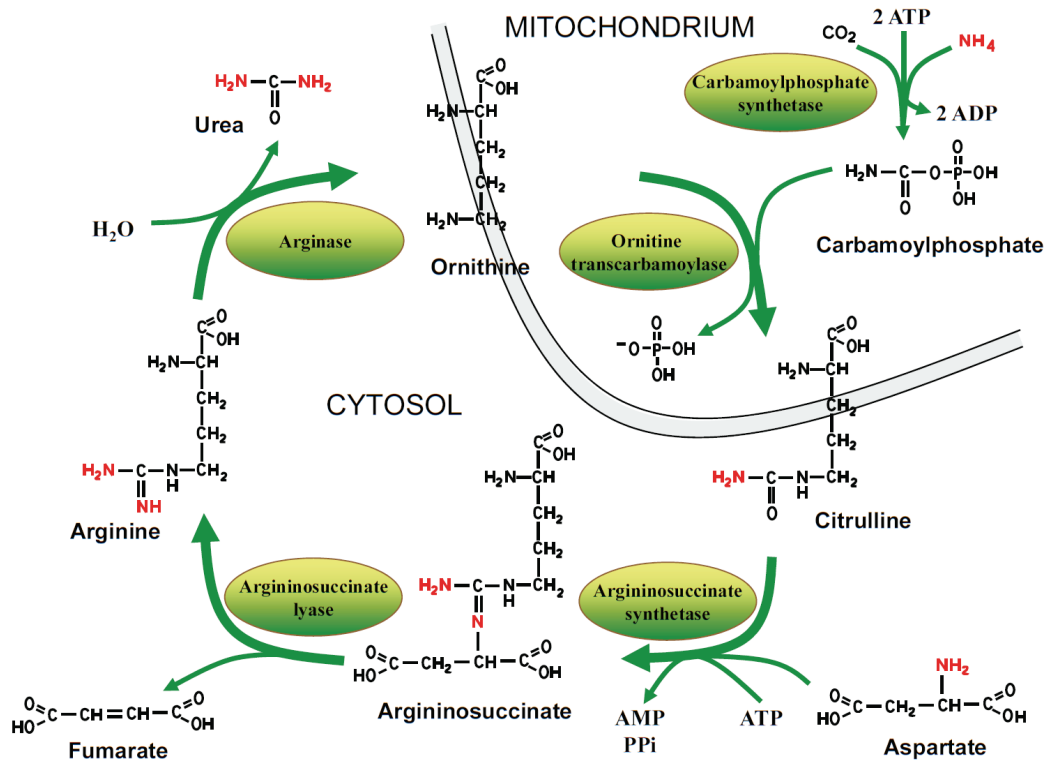


Figure 2. Urea cycle

type I citrullinemia (CTLN1) in children where the deficiency of argininosuccinate synthase results from a mutation in the gene [12], in CTLN2 there is no mutation in the argininosuccinate synthase gene, yet there is still a marked deficiency in liver argininosuccinate synthase protein [13]. To account for the liver specific deficiency of argininosuccinate synthase in CTLN2, Saheki *et al.* [13] showed association of CTLN2 with a defect in *SLC25A13* gene that encodes the mitochondrial Ca^{2+} -dependent aspartate/glutamate transporter known as citrin [14]. Saheki's findings suggested that citrin forms a complex with the three "soluble" cytoplasmic enzymes of the urea cycle, including argininosuccinate synthase. This interaction with citrin may be direct or mediated through some other protein or proteins. Nevertheless, the loss of spatial control attributable to the mutated citrin strongly correlated with reduction of liver argininosuccinate synthase protein in CTLN2, possibly through destabilization and/or degradation [13].

Urea cycle enzymes appear to be closely associ-

ated with one another such that substrates and products can be shuttled from one enzyme to another by channeling [11]. Support for channeling comes from studies demonstrating the channeling of the extramitochondrial ornithine to the mitochondrial ornithine transcarbamoylase via its transporter [15], from studies showing the co-localization of carbamoyl phosphate synthetase I and ornithine transcarbamoylase with the inner mitochondrial membrane [16], from labeling studies demonstrating channeling of citrulline from ornithine transcarbamoylase in the mitochondria to argininosuccinate synthase in the cytoplasm, and from studies demonstrating channeling of arginine between argininosuccinate lyase and arginase [10].

Thus the spatial relationship of argininosuccinate synthase relative to ornithine transcarbamoylase in the mitochondria, and argininosuccinate lyase in the cytosol, together with the relatively high expression levels for each of these enzymes, ensures that both substrate and product, and in particular arginine, do not readily exchange with other cellular pools, or

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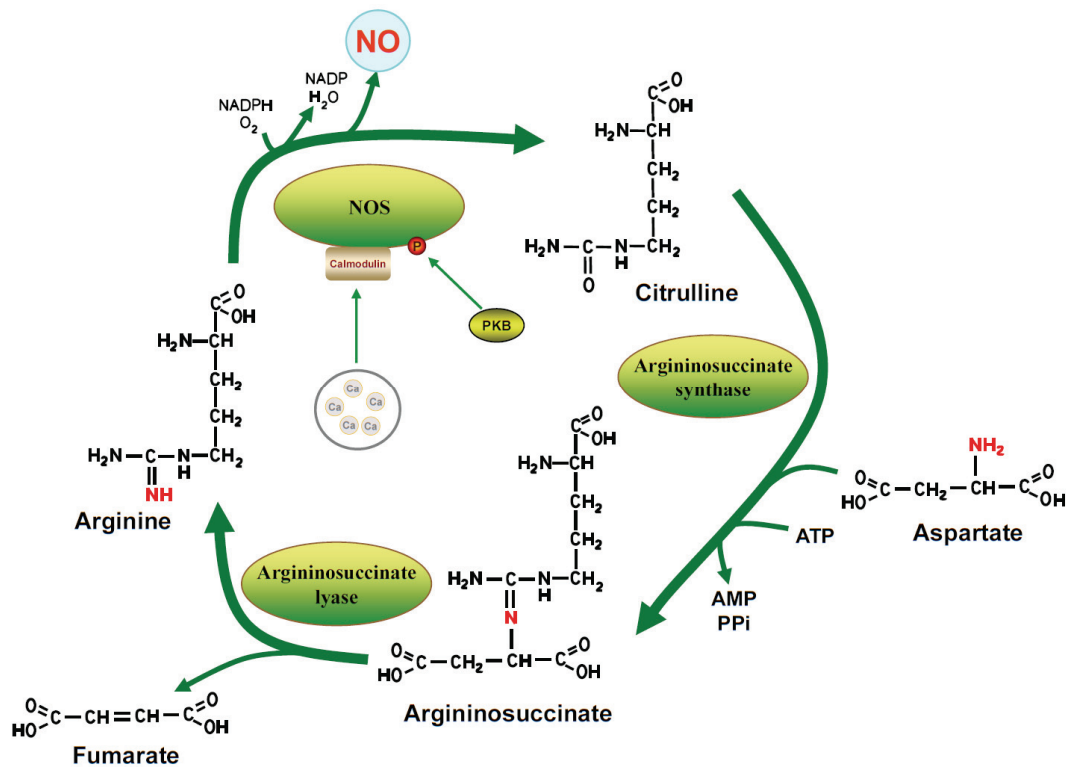


Figure 3. Citrulline-nitric oxide cycle

even with extracellular sources. Moreover, hepatocytes do not easily exchange arginine with serum, evidenced by their limited capacity to take up arginine [17]. As a result, any other intracellular pool of arginine, as well as serum arginine, is protected [18, 19].

The significance of this arrangement can be illustrated by the grave outcomes observed during liver failure, liver transplants, and other liver diseases, that result in disruption of liver tissue and the release of hepatic arginase. Under these conditions, serum arginine levels can be severely diminished, limiting the available arginine for other tissues [20-23].

Under normal conditions, and in the short term, the urea cycle has sufficient capacity to respond to immediate increases in waste nitrogen load [1, 24, 25], and the rate of urea production essentially depends on the activity of carbamoyl-phosphate synthase I [1]. On the other hand, under conditions where the urea cycle is functioning maximally, argininosuccinate synthase becomes the rate-limiting step [24]. Therefore,

it is not surprising that expression of argininosuccinate synthase, relative to urea cycle function, is highly regulated and governed by changes in dietary protein intake, and by hormones such as glucagon, insulin, and glucocorticoids [1]. There are also studies in cultured hepatocytes demonstrating nutrient control over argininosuccinate synthase expression where the addition of arginine was suppressive and the addition of citrulline induced argininosuccinate synthase expression [23, 26, 27].

Both the response to dietary changes and the response to hormones seem to be largely coordinated through transcriptional regulation of the argininosuccinate synthase gene (*ASS1*). Argininosuccinate synthase mRNA levels are induced by glucocorticoids, cAMP, and glucagon; whereas, insulin and growth hormone seem to act on the developmental regulation of argininosuccinate synthase by modulating the glucocorticoid effect [28, 29]. Interestingly, it has only recently been shown that regulation of argininosuccinate synthase transcription by cAMP maps to a highly conserved cAMP response element

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10 kb upstream of the transcription start site in the argininosuccinate synthase promoter [28].

Argininosuccinate synthase involvement in nitric oxide metabolism

Endothelium derived relaxing factor, first discovered by Furchgott [30] in 1980, was later determined to be a free radical compound called nitric oxide [31]. Nitric oxide is a highly reactive molecule that readily diffuses through cell membranes. As a consequence, nitric oxide cannot be stored inside producing cells like other endogenous messengers; rather, its signaling capacity must be controlled at the levels of synthesis and local availability. The importance of this temporal and spatial control of nitric oxide production is highlighted by vascular endothelial cells where virtually all phenotypic properties are related to nitric oxide bioactivity. Endothelial nitric oxide synthase (eNOS) utilizes L-arginine as the principal substrate, converting it to L-citrulline and nitric oxide. L-citrulline is recycled to L-arginine by two enzymes, argininosuccinate synthase and argininosuccinate lyase, providing the essential arginine for nitric oxide production in endothelial cells [32]. Together, the three enzymes, eNOS, argininosuccinate synthase and argininosuccinate lyase, make up the citrulline-nitric oxide cycle [29] (**Figure 3**).

Although expression of the complete urea cycle is only described in liver, argininosuccinate synthase and argininosuccinate lyase are expressed in an entire range of cell types, often related to their involvement in providing the essential arginine for nitric oxide production. In fact, the capacity to recycle citrulline to arginine appears to be a prerequisite for all nitric oxide-producing cells [33].

For example, cells that constitutively express isoforms of NOS, such as vascular endothelial cells and neurons, all have the capacity to regenerate arginine from citrulline [34-36]. Citrulline formation by pulmonary endothelial cells was shown to increase when nitric oxide production was stimulated, and to diminish when subjected to chronic hypoxia [37]. In neural tissue, histochemical staining for the localization of nitric oxide synthase demonstrated that argininosuccinate synthase coexists with nitric oxide synthase [38]. Furthermore, argininosuccinate synthase and argininosuccinate lyase co-localize in discrete populations of myenteric and

submucosal neurons in the canine proximal colon [36].

Likewise, the induction of iNOS, that leads to increased production of nitric oxide in non-hepatic cell types, is always accompanied by the enhancement of argininosuccinate synthase expression [33]. Argininosuccinate synthase and argininosuccinate lyase also co-localize with nNOS throughout the canine gastrointestinal tract [39]. In the guinea-pig trachea, human bronchus and murine proximal colon, the inhibition of electrically induced nitrergic responses by competitive NOS inhibitors was shown to be overcome by both arginine and citrulline treatment. The ability of citrulline treatment to overcome NOS inhibition was attributed to its conversion to arginine by argininosuccinate synthase and argininosuccinate lyase [40, 41].

More recently, Van Geldre *et al* [42] demonstrated that citrulline recycling was active in rat gastric fundus, and that the recycling of citrulline maintained sufficient arginine during the long-term relaxations of the gastric fundus after food intake. While Swany *et al* [43] suggested that in the rat cerebellum the citrulline-nitric oxide cycle maintains the essential supply of arginine to support the increased production of nitric oxide.

When Xie *et al.* [44] transfected argininosuccinate synthase into vascular smooth muscle cells, the resulting overexpression of argininosuccinate synthase dramatically enhanced the capacity of transfected cells to produce nitric oxide over that of untransfected cells despite non-limiting levels of extracellular arginine. As a result, Xie *et al.* [44, 45] concluded that the capacity to recycle citrulline to arginine is "rate-limiting" to nitric oxide production. Su *et al.* [37] arrived at a similar conclusion showing that endotoxin inhibited induction of argininosuccinate synthase, and as a consequence, production of nitric oxide in hypoxic pulmonary artery endothelial cells was impaired.

Compelling evidence for the essential role of argininosuccinate synthase in supporting nitric oxide production came from a series of experiments using RNA interference to specifically silence argininosuccinate synthase expression in vascular endothelial cells [32]. Diminished levels of argininosuccinate synthase expression that resulted from siRNA treatment were shown

to correlate with impaired nitric oxide production in the presence and absence of effectors. Unanticipated, however, was the significant loss of viable endothelial cells observed after siRNA treatment. The fact that the loss of viability followed reduced expression of Bcl-2, and an increase in caspase activity, strongly suggested a relationship between impaired nitric oxide production and apoptosis. This relationship was supported by the observation that exposure to a nitric oxide donor rescued cell viability despite siRNA treatment [32]. More importantly, these results demonstrated that argininosuccinate synthase was even required for the maintenance of basal production levels of nitric oxide in order to support cell viability.

In endothelial cells, argininosuccinate synthase and argininosuccinate lyase were shown to colocalize with endothelial nitric oxide synthase (eNOS) and specialized structures of the plasma membrane called caveolae [3, 46]. Caveolar domains assemble at the Golgi and traffic to the plasma membrane as stable transport platforms [47]. This may explain the subcellular association of eNOS with Golgi and perinuclear membrane [4, 48, 49]. Evidence also suggests that interaction with other proteins may play a role in eNOS targeting. For example, Schilling *et al* [50] described two novel eNOS-interacting proteins named NOSIP (for eNOS-interacting protein) and NOSTRIN (for eNOS-trafficking inducer), both of which influence the subcellular localization of eNOS. For NOSIP, targeting of eNOS to the cytoskeleton seems to be one of the protein's major functions [51]. On the other hand, when NOSTRIN is overexpressed, this leads to the translocation of eNOS from the plasma membrane to intracellular vesicular structures [52], possibly involving an endocytic process [53]. In comparison, much less is known about argininosuccinate synthase targeting and how the co-localization of argininosuccinate synthase and eNOS is maintained to regulate the local availability of substrate for nitric oxide production.

Argininosuccinate synthase in the maintenance of serum arginine levels

In adult human intestines, glutamine/glutamate and proline are converted to citrulline. This citrulline is subsequently released by enterocytes to be taken up mainly by the kidneys for conversion to arginine [4]. Because a significant

fraction (~40%) of ingested arginine undergoes catabolism [33], the majority of circulating arginine used for other tissues is provided by the kidneys [54] and from protein turnover [33]. Thus, arginine is often considered to be "semi-essential" since in healthy adults endogenous production, essentially by the kidneys, can be sufficient to meet normal metabolic needs [33].

It has been speculated that in the mammalian neonate, enteric arginine synthesis is necessary because mother's milk is relatively deficient in arginine; whereas, the precursors of arginine, proline and glutamine, are quite plentiful [55]. This may also be the reason why the fetal intestine expresses argininosuccinate synthase, providing the metabolic capacity to produce arginine before birth. Nevertheless, at about two to three years of age, the intestine loses the ability to synthesize arginine as a consequence of the loss of argininosuccinate synthase expression. Subsequently, endogenous arginine biosynthesis becomes an inter-organ process, where the net production of citrulline occurs almost exclusively in the enterocytes of the small intestine [56] and absorption of citrulline from circulation takes place essentially in the cortex of the kidney [57] where it is converted to arginine.

In the adult human, elevated expression and colocalization of carbamoyl phosphate synthase I (CPS1) and ornithine transcarbamoylase (OTC) in enterocyte mitochondria favors intestinal synthesis of citrulline from ammonia, HCO_3^- and ornithine, while the relatively low levels of cytosolic argininosuccinate synthase and argininosuccinate lyase minimizes further conversion of citrulline to arginine. Based on these differences, the intestinal urea cycle enzymes maximize the net synthesis of citrulline from glutamine, and the specific release of citrulline [9].

Similar to the poor uptake of arginine by the liver, citrulline does not undergo significant hepatic uptake and therefore is available for renal arginine synthesis [56]. In the kidney, conversion of citrulline to arginine is catalyzed by argininosuccinate synthase and argininosuccinate lyase. Unlike other pathways involving argininosuccinate synthase, the rate-limiting step in the synthesis of arginine by the kidney seems to be based on citrulline availability [29, 56, 57]. Support for this observation comes from the finding that citrulline supplementation stimulates arginine synthesis in the kidneys, leading to in-

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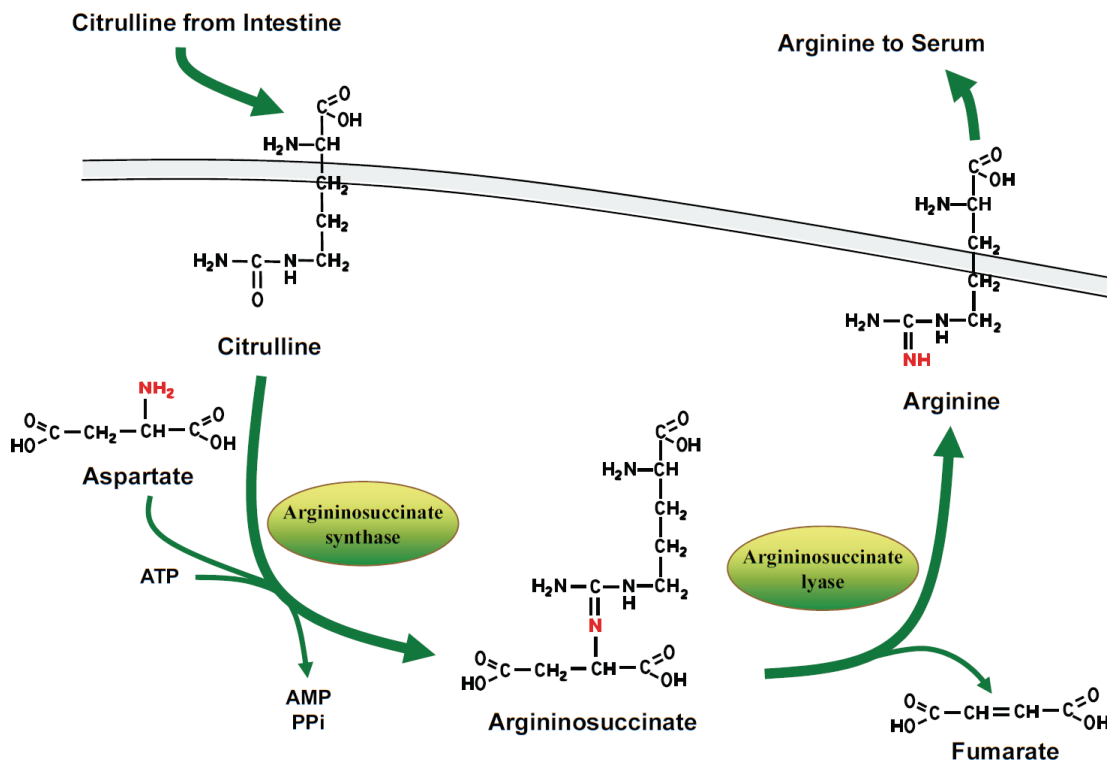


Figure 4. Synthesis of arginine by kidney.

creases in plasma arginine [58].

Thus in adults, the majority of endogenous arginine synthesis involves the intestinal/renal axis. Citrulline is released by the intestines followed by uptake in the kidney. In the kidneys, expression of argininosuccinate synthase and argininosuccinate lyase then promotes conversion of citrulline to arginine and its subsequent release into plasma (Figure 4).

Argininosuccinate synthase provides the essential arginine for creatine biosynthesis

Creatine is distributed throughout the body, with approximately 95% found in skeletal muscle [59] and most of the remaining 5% in brain, liver, kidney and testis. The daily requirement for creatine is achieved either through intestinal absorption of dietary creatine or by *de novo* creatine biosynthesis [60]. Two enzymes are involved in creatine biosynthesis. AGAT (L-Arginine-glycine amidinotransferase) converts arginine and glycine into ornithine and guanidinoacetate, and GAMT (Guanidinoacetate me-

thyltransferase) catalyzes S-adenosyl-L-methionine-dependent methylation of guanidinoacetate to yield creatine and S-adenosyl-L-homocysteine. The formation of guanidinoacetate is normally the rate-limiting step of creatine biosynthesis, and in this respect, the feedback repression by creatine, the end product of the pathway, acts to conserve the utilization of the essential amino acid, methionine, and semi-essential amino acid, arginine.

Again, different body tissues seem to play a role in creatine synthesis. In humans, synthesis of guanidinoacetate occurs mainly in the kidney followed by transport to the liver where guanidinoacetate is subsequently methylated to produce creatine [60]. Thus the kidney-liver axis for creatine synthesis provides creatine to peripheral tissues, and in particular to muscle.

In the human kidney, guanidinoacetate is synthesized by the transamidation of the guanidine group from arginine to glycine. The synthesis of guanidinoacetate is dependent on the endogenous synthesis of arginine from citrulline cata-

Argininosuccinate synthase for arginine metabolism

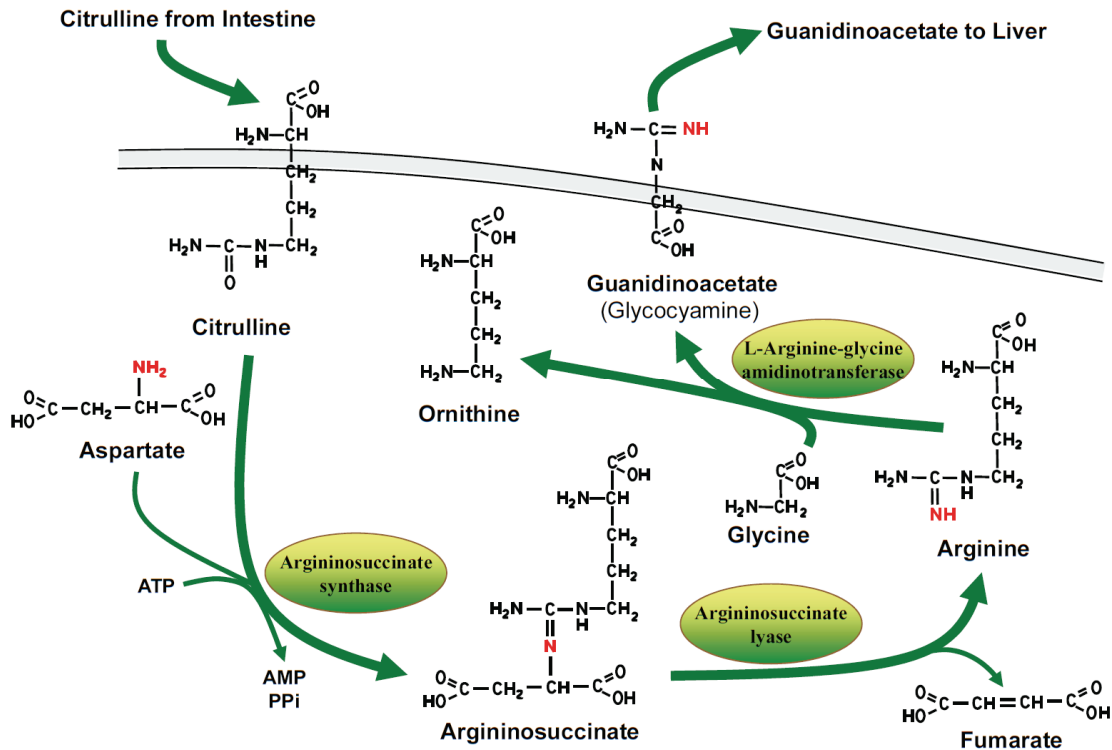


Figure 5. Synthesis of guanidinoacetate by the kidney.

lyzed by argininosuccinate synthase and argininosuccinate lyase. However, regulation of argininosuccinate synthase in kidney tissue, as it relates to the synthesis of guanidinoacetate, is not well-defined. Evidence, however, suggests that in this case again, the rate-limiting step may be the availability of citrulline that is provided by the intestines, and the negative feedback regulation of creatine at AGAT [60] (Figure 5).

Argininosuccinate synthase defines the pool of arginine for arginase and subsequent polyamine synthesis

The arginine derived from argininosuccinate synthase and argininosuccinate lyase may also be diverted by arginase to the production of ornithine for polyamine biosynthesis [9]. Ornithine is a precursor of putrescine, the base molecule of polyamines. In this case, arginine production by argininosuccinate synthase regulation would presumably be tied to cell cycle regulatory events, and perhaps to the induction and regulation of ornithine decarboxylase in response to specific hormones, growth factors,

or cell cycle regulatory signals. Importantly, argininosuccinate synthase appears to provide a distinct pool of intracellular arginine for arginase and the subsequent synthesis of polyamines, much like that observed for nitric oxide production [61]. This observation is supported by results demonstrating that polyamine synthesis is dependent on the intracellular localization and synthesis of arginine from citrulline [62, 63].

Argininosuccinate synthase expression in tumor tissue

Some tumor types downregulate argininosuccinate synthase expression producing an intrinsic dependence on extracellular arginine due to the inability to synthesize endogenous arginine for growth [64]. This dependence on extracellular arginine is known as "arginine auxotrophy", and tumors of this type include hepatocellular carcinoma, malignant melanoma, malignant pleural mesothelioma, osteosarcoma, prostate and renal cancer [64]. Although arginine deprivation therapy, as an anticancer strategy, has been investigated for several decades [65-69], it is

only recently that it has shown encouraging activity in patients with specific tumor types [70].

Arginine deiminase (ADI), an enzyme isolated from *Mycoplasma* [71, 72], hydrolyzes arginine to citrulline and ammonia. In its native form, it is strongly antigenic with a half-life of 5 hours [73]. However, conjugation to 20,000 mw polyethylene glycol (ADI-PEG20) decreases antigenicity and dramatically increases serum half-life, allowing weekly administration that reduces plasma arginine to undetectable levels [65]. For example, ADI-PEG 20, with its prolonged half-life and ability to suppress detectable levels of circulating arginine, has been assessed in patients with malignant melanoma and hepatocellular carcinoma. Both are arginine auxotrophic tumors [65]. Izzo *et al.* [74] reported a 47% (complete and partial) response rate to this drug in a phase I/II study of patients with advanced hepatocellular carcinoma. Whereas, Ascierto *et al.* [75] documented an overall 25% (complete and partial) response rate for this treatment in patients with advanced metastatic melanoma. Importantly, several patients in both studies were observed to have a sustained response to treatment. Based on reports like these, ADI-PEG 20 has received an orphan drug designation in both the United States and, more recently, in Europe for the treatment of hepatocellular carcinoma.

Just recently, Kobayashi *et al* [76] suggested that reduced expression of argininosuccinate synthase in patients with osteosarcoma may provide a novel predictive biomarker related to their risk of metastasis. Moreover, the authors also suggested that it may be possible to exploit the arginine auxotrophic phenotype of this osteosarcoma by depleting extracellular arginine [70].

In contrast, there are also tumor types that demonstrate a dependency on elevated argininosuccinate synthase expression. For example, Szolsarek *et al* [77] demonstrated that epithelial ovarian tumor tissue was highly dependent on elevated argininosuccinate synthase expression. Based on this observation, the authors suggested that the dependency of this ovarian tumor on elevated argininosuccinate synthase may be exploited, providing an important diagnostic marker as well as potential therapeutic target.

Regulation of argininosuccinate synthase expression

Since the early publications on argininosuccinate synthase in the 1970s, our understanding of the properties and biological role of this enzyme has changed greatly. Initially, the non-hepatic role of this enzyme was assumed to be a vestige; and as such, the role and regulation of argininosuccinate synthase, outside of that needed for urea production, developed slowly. However, interest in argininosuccinate synthase, and particularly its regulation, has grown due in great part because of its involvement in nitric oxide production [29].

In humans, there are 14 copies of the argininosuccinate synthase gene, including 13 pseudogenes scattered across the genome. The *ASS1* gene, located on chromosome 9, appears to be the only functional gene involved in argininosuccinate synthase expression. This is supported by the findings that mutations in the chromosome 9 copy of *ASS1* cause citrullinemia [12, 13, 78].

About 800 bp of the promoter region for the human *ASS1* gene have been characterized [79] showing a TATA box, six potential Sp1 binding sites (GC boxes) and one potential AP-2 site [80, 81]. The three GC boxes in the immediate promoter were shown to act synergistically and to be required in order to achieve full promoter activation [81]. Surprisingly, no CAAT sequence (C/EBP binding site), nor GRE (glucocorticoid responsive), or CRE (cAMP responsive-) elements were found in the early characterization of the argininosuccinate synthase promoter.

Recently, however, Guei *et al* [28], through functional analysis of DNase I hypersensitive sites, located a cAMP response element for the human *ASS1* gene at ~10 kb upstream of the transcription start site. This discovery helped reconcile earlier findings showing liver-specific enhancement of *ASS1* gene expression by *TSE1* (tissue-specific extinguisher locus 1) regulation [82, 83], and the fact that transcription of the *ASS1* gene in rat liver was stimulated by cAMP in nuclear run-on assays [84]. *TSE1* encodes the regulatory subunit RI alpha of protein kinase A (PKA) [85].

According to Tsai *et al* [86], the proximal region of the *ASS1* promoter also contains an E-box

that is recognized by c-Myc and HIF-1 α , as well as a GC-box that is recognized by Sp4 in melanoma cells. Under non-induced conditions, the E-box element bound HIF-1 α , and under induced conditions (arginine depletion) HIF-1 α was replaced by c-Myc, at least in two of the melanoma cell lines, but not in another. In all cases, however, Sp4 was shown to constitutively bind the GC-box regardless of arginine availability. Taken together, their results suggested that the transcriptional induction of *ASS1* expression, relative to arginine depletion, involved the interplay between the positive transcriptional regulators c-Myc and Sp4, and the negative regulator HIF-1 α . Moreover, this level of regulation in melanoma cells was taken to confer resistance to arginine deiminase based arginine depletion studies.

Since most all phenotypic properties of endothelial cells relate to nitric oxide signaling, eNOS has been highly studied in response to physiological cues such as shear stress and cytokines [87-90]. On the other hand, relatively little is known about the regulatory mechanisms that control argininosuccinate synthase expression in this context. Nevertheless, what has been elucidated, thus far, has provided a picture that supports the central role of argininosuccinate synthase and its coordinate regulation with eNOS in nitric oxide production.

For example, Mun *et al* [91] examined the differential expression of genes in human umbilical vein endothelial cells (HUVECs) under static and laminar shear stress conditions. Among the 20 genes whose expression was increased by laminar shear stress conditions, and simultaneously decreased by cellular senescence, was *ASS1*. Based on their findings, the authors suggested that argininosuccinate synthase expression was critical both for basal and laminar shear stress-induced nitric oxide production. This observation was consistent with Goodwin *et al* [32] who demonstrated through siRNA knockdown studies, that argininosuccinate synthase expression was required to support both basal and induced nitric oxide production in vascular endothelial cells.

Goodwin *et al* [92] also showed that both argininosuccinate synthase and eNOS expression were significantly reduced by treatment with physiological levels of TNF- α in vascular endothelial cells. Not surprisingly, the loss ar-

gininosuccinate synthase and eNOS expression was accompanied by a dramatic decrease in the capacity of the TNF- α -treated cells to produce nitric oxide. Moreover, the down regulation of argininosuccinate synthase expression by TNF- α was shown to be mediated through a similar transcriptional mechanism by which TNF- α down-regulates eNOS, involving Sp1/Sp3 elements in the proximal promoter [87]. Inhibitor studies suggested that repression of argininosuccinate synthase expression by TNF- α was mediated, at least in part, via the NF- κ B signaling pathway [93]. Critically, these results revealed the extent to which TNF- α impairs vascular endothelial function via the coordinate down-regulation of both eNOS and argininosuccinate synthase expression [92].

On the other hand, the peroxisome proliferator-activated receptor gamma (PPAR- γ) agonist, troglitazone, was shown to support nitric oxide production in vascular endothelial cells through the up-regulation of argininosuccinate synthase expression [94]. Because the induction of argininosuccinate synthase was inhibited by treatment with the transcriptional inhibitor, 1-D-ribofuranosylbenzimidazole (DRB), this demonstrated that troglitazone mediated its response essentially at the transcriptional level. In keeping with this finding, a distal PPAR- γ response element (PPRE) at -2471 to -2458 was identified that mediated the PPAR- γ agonist response. These results extended the repertoire of mechanisms by which PPAR- γ agonists promote vascular health [95, 96]. In the case of troglitazone, one of its vascular protective properties directly relates to the maintenance of an adequate supply of arginine for nitric oxide production through the up-regulation of argininosuccinate synthase [94].

Less is known at a more global level involving co-regulators and co-repressors that interact with specific transcription factors on the *ASS1* gene promoter to alter rates of expression. Thus, critical information related to the interplay of promoter elements that allow tissue specific expression of argininosuccinate synthase is quite limited.

Argininosuccinate synthase has been shown to associate with raft-like membrane microdomains, caveolae, at the plasma membrane [3]. The major constituent of caveolae is caveolin-1 [97], which is expressed in many cell types, in-

cluding endothelial and epithelial cells. Importantly, a major phenotype of caveolin-1 knock-out mice relates to nitric oxide signaling. Nitric oxide release from caveolin-1-deficient cells is higher compared to wild-type cells, and secondary effects of nitric oxide are increased, demonstrating activation of eNOS through de-inhibition in the absence of caveolin-1 [98]. Conversely, direct binding of eNOS to the scaffolding domain (positions 82-101) of caveolin-1 has been shown to decrease nitric oxide production *in vitro* and in cell culture [46, 99, 100]. However, little is known as to whether caveolin may play a similar role in affecting either the activity or the subcellular localization of argininosuccinate synthase. Indeed, the only known factor shown to affect localization of argininosuccinate synthase is citrin where mutations in the *SLC25A13* gene that encodes citrin were shown to cause adult-onset type II citrullinemia [13]. In this case, the mutated citrin does not allow argininosuccinate synthase mitochondrial association. As a consequence, argininosuccinate synthase appears to be rapidly degraded accounting for the low levels found in hepatocytes of affected individuals [13].

Open reading frames in the 5'-leaders of messenger RNAs are emerging as important mediators of transcript-specific translational controls. In most instances, upstream open-reading frames (uORFs) regulate the expression of a downstream ORF on the same mRNA (*cis*-regulation). In this case, those mRNAs with uORFs are typically expressed as the only species of that mRNA in a particular cell type [101]. In contrast, the transcription of argininosuccinate synthase mRNA, at least in vascular endothelial cells, is initiated from multiple start sites, generating mRNAs with different lengths and overlapping 5'-untranslated regions (UTRs) [102]. In fact, nearly 10% of argininosuccinate transcripts are longer variants that contain an out-of-frame, upstream open reading frame (uORF) encoding a ~4.4 kDa protein, referred to as Argininosuccinate synthase Regulatory Protein (ARP). ARP downregulates argininosuccinate synthase expression *in trans* at the translational level [103]. Consistent with the rate-limiting role of argininosuccinate synthase in the citrulline/nitric oxide cycle, the increase in argininosuccinate synthase protein that resulted from the knockdown of ARP was accompanied by an increase in nitric oxide production. Likewise, when ARP was over-expressed in en-

dothelial cells, argininosuccinate synthase expression was suppressed, resulting in reduced nitric oxide production [103].

Since argininosuccinate synthase is responsible for maintaining the available arginine for varied metabolic processes, it seems logical that acute or immediate changes in activity may also be necessary to accommodate the multiple roles that the arginine product plays in cell function. However, such immediate responses require regulation that goes beyond changes in transcription. Nevertheless, there are very few documented reports that discuss posttranslational events that may regulate the function of argininosuccinate synthase. In fact the bulk of published literature suggests that argininosuccinate synthase regulation occurs primarily at the transcriptional level [29].

There are, however, some recent findings that have changed this view. For example, it has been reported that the NADPH sensor protein, HSCARG, interacts with argininosuccinate synthase to downregulate its activity in epithelial cells [104]. In fact, it has been suggested that the protein-protein interaction of HSCARG with argininosuccinate synthase may provide a mechanism by which the epithelial cell maintains an intracellular balance between its redox state and nitric oxide levels.

On the other hand, Hao *et al* [105] showed that human argininosuccinate synthase can be inactivated by reversible nitrosylation at Cys-132 in response to lipopolysaccharide-treatment in both cultured smooth muscle cells and in mice. Mutagenesis studies confirmed that the nitrosylation of Cys-132 was both necessary and sufficient to inhibit argininosuccinate synthase. Based on their results, the authors suggested that post-translational modification of argininosuccinate synthase by nitrosylation might provide important feedback mechanism whereby nitric oxide can limit arginine availability in nitric oxide producing cells [105].

Possibly the first report demonstrating that argininosuccinate was a phosphoprotein came in 2008 when Imani *et al* [106], using a phosphoproteomic analysis, reported that argininosuccinate synthase in HeLa cells was a phosphoprotein. However, their study did not consider the biological relevance of argininosuccinate synthase phosphorylation. Subsequently, Corbin *et*

al [107] showed that argininosuccinate synthase was indeed phosphorylated in vascular endothelial cells in response to VEGF treatment by protein kinase A (PKA). Interestingly, PKA was already known to enhance nitric oxide production via phosphorylation and activation of eNOS [48].

Although reports on the post-transcriptional regulation of argininosuccinate synthase are presently meager in number, it is becoming clear that there exists an assortment of new mechanisms that affect argininosuccinate synthase expression and activity. As such, argininosuccinate synthase offers a distinctive paradigm for regulation that has expanded beyond transcriptional regulation to now include localization, translation and post-translational controls. This array of regulatory strategies which a cell uses to define and control arginine levels further endorses the physiological significance of argininosuccinate synthase in mammalian arginine metabolism.

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