Implications of arginine deficiency for growth and organ maturation. Studies on hair, muscle, brain and lymphoid organ maturation

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Chapter I

Arginine biosynthesis and metabolism

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Chapter I

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Abbreviations and enzymes with enzyme codes used:

A-I    [EC 3.5.3.1] : hepatic arginase
A-II   [EC 3.5.3.1] : kidney-type arginase
AGAT   [EC 2.1.4.1] : arginine:glycine amidinotransferase
AAT    [EC 2.6.1.1.] : aspartate aminotransferase
ASL    [EC 4.3.2.1] : argininosuccinate lyase
ASS    [EC 6.3.4.5] : argininosuccinate synthetase
CPS    [EC 6.3.4.16] : carbamoylphosphate synthetase
GMT    [EC 2.1.1.2] : s-adenosylmethionine:guanidinoacetate methyltransferase
OAT    [EC 2.6.1.13] : ornithine aminotransferase
ODC    [EC 4.1.1.17] : ornithine decarboxylase
OTC    [EC 2.1.3.3] : ornithine transcarbamoylase
P5C    [EC n.a.] : pyrroline-5-carboxylate synthetase
PDG    [EC 3.5.1.2.] : phosphate-dependent glutaminase
PO     [EC n.a.] : proline oxidase
SI     [EC 3.2.1.48-10] : sucrase-isomaltase

EC = enzyme code, n.a. = not assigned
1-Biosynthesis of arginine

1.1-Intestinal citrulline biosynthesis

The organ perfusion studies by Windmueller and Spaeth\(^1,2\) established that de novo synthesis of citrulline, as well as ornithine and proline, takes place in the small intestine (Fig. 1). Citrulline synthesis requires NH\(_3\), CO\(_2\), and ornithine. The NH\(_3\) is derived from glutamine via the action of glutaminase. Together with bicarbonate, NH\(_3\) can form carbamoylphosphate by the action of carbamoylphosphate synthetase (CPS-1; EC 6.3.4.16), an enzyme that requires N-acetylglutamate for activity. The carbamoyl group is transferred to ornithine to form citrulline in a reaction catalyzed by ornithine transcarbamoylase (OTC; EC 2.1.3.3). In addition to glutamate, proline can function as a precursor for the intestinal ornithine synthesis (Fig. 1)\(^3\), via the formation of P5C by proline oxidase, and the subsequent non-enzymatic conversion into L-glutamyl-γ-semialdehyde\(^4\).

The enzymes involved in citrulline synthesis, phosphate-dependent glutaminase, OAT, and aspartate aminotransferase are found in a variety of tissues\(^5,6\), but CPS I, OTC, and N-acetyl glutamate synthetase expression are restricted to the liver and intestinal mucosa\(^7\). P5C synthetase, which catalyzes the multistep conversion of glutamate via glutamyl-γ-semialdehyde into P5C\(^4\), is present almost exclusively in the small intestinal mucosa, with only trace amounts in other tissues\(^4\). P5C is, in turn, converted into ornithine by OAT. For this reason, the intestine is the only organ that has the capacity of net production of ornithine. In (most) other organs, including liver, OAT converts ornithine into glutamate. Human patients suffering from a deficiency in P5C synthetase develop hyperammonemia and hypoornithinemia\(^8\). In addition, these patients have decreased proline levels, implying that P5C-S may also have a P5C-R like activity. The essential role of the intestine to provide citrulline for arginine biosynthesis elsewhere, is also demonstrated by the arginine deficiency that results from inhibition of intestinal
citrulline synthesis, as a result of inhibition or deficiency of OTC, OAT, or by massive resection of the small bowel.

In intestinal tissue, arterial and luminal glutamine is the main metabolic contributor of intestinal citrulline biosynthesis. The magnitude of the role of proline in citrulline synthesis is disputed. In pigs, there is no arterial uptake of proline by the gut, suggesting that only dietary proline is metabolized into citrulline. Indeed, a prominent role for proline is suggested by the observation that, at least in pigs, proline is among the most abundant amino acids in the sow’s milk.

Fig. 1: Scheme of the relation between glutamine and the ornithine cycle. Enzymes that catalyze the reactions are: 1- phosphate-dependent glutaminase [EC 3.5.1.2], 2- pyrroline-5-carboxylate synthetase [EC n.a.], 3- ornithine aminotransferase [EC 2.6.1.13], 4- carbamoyl-phosphate synthetase [EC 6.3.4.16], 5- ornithine transcarbamoylase [EC 2.1.3.3], 6- argininosuccinate synthetase [EC 6.3.4.5], 7- argininosuccinate lyase [EC 4.3.2.1], 8- hepatic arginase [EC 3.5.3.1] / kidney-type arginase [EC 3.5.3.1], 9- proline oxidase [EC n.a.], 10- aspartate aminotransferase [EC 2.6.1.1]. Enzymes 6, 7 and 8 are cytosolic, 1-5 and 9 are mitochondrial, and enzyme 10 is both cytosolic and mitochondrial.

Though proline oxidase (PO) is reported to be present mainly in liver and kidney, other groups have detected a relatively high level of PO activity in the intestinal
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mucosa of pig and rat\textsuperscript{16,17}. This difference has been attributed to instability of the enzyme during homogenization of, specifically, mucosal tissue\textsuperscript{6}.

1.2- Arginine biosynthesis

1.2.1-Renal arginine biosynthesis.

In the adult kidney, arginine is made endogenously by converting citrulline into arginine, mainly in the renal proximal tubules\textsuperscript{1,18}, via the cytosolic enzymes ASS and ASL. The kidney accounts for 60\% of arginine synthesis from circulating citrulline\textsuperscript{19} (see section 2.2.2). Although the kidney expresses CPS-I, (unpublished observation), as well as glutaminase and glutamate dehydrogenase (GDH)\textsuperscript{20,21} in the preweanling period, the adult kidney lacks the capacity for carbamoylphosphate synthesis.

The citrulline that is metabolized into arginine by the kidneys derives primarily from the intestine\textsuperscript{1,22}. The amount of citrulline taken up for arginine synthesis has been shown to be proportional to the amount released by the intestine\textsuperscript{1,23}. Furthermore, a positive correlation between renal citrulline uptake and arginine production has been shown in humans\textsuperscript{24}, sheep\textsuperscript{25} and rats\textsuperscript{26}. The capacity of the kidney to synthesize arginine exceeds the capacity of the intestine to synthesize citrulline several-fold, indicating that renal arginine synthesis depends on intestinal citrulline synthesis\textsuperscript{27}. In addition, the renal arginine flux is highly regulated: renal ASS and ASL mRNA as well as activity are upregulated during a high protein diet\textsuperscript{28}, upon starvation\textsuperscript{29}, or upon an acute decrease in circulating arginine levels under pathological conditions\textsuperscript{30}.

Next to the cytosolic arginine-synthesizing enzymes, the kidney expresses A-II (EC 3.5.3.1)\textsuperscript{31}, but the expression of arginase, ASS and ASL is segregated in different parts of the nephron. arginine synthesis is restricted to the upstream portions of proximal convoluted tubules, whereas arginase activity is present mainly in the cortical and outer medullary portions of straight proximal tubules. Hence, there is little or no co-expression of these enzymes in the same cell\textsuperscript{32}, allowing for net renal arginine biosynthesis from the citrulline reabsorbed from glomerular
filtrate in proximal convoluted tubuli cells, while in straight tubuli, arginase-derived urea passively diffuses into the luminal fluid entering Henle's loops 32.

1.2.2-Extra-renal arginine biosynthesis

ASS mRNA is expressed in the neurons of the myenteric plexus of the adult small intestine 33, suggesting the existence of a neuronal arginine-citrulline cycle for regeneration of arginine for nitric oxide synthesis. Such a cycle has been described in several other nitric oxide producing cell-types, like endothelial cells 34,35, macrophages 36, and neurons 37-39. The picture therefore emerged that citrulline synthesis is confined to the small intestinal enterocytes, whereas arginine biosynthesis occurs in many tissues, the kidney being the most prominent. Presumably, the co-localization of ASS and ASL with NOS serves to assure that cellular NO synthesis becomes independent of circulating arginine. Thus, ASS, ASL, and nNOS immune reactivity (or NADPH-diaphorase activity) have been shown to colocalize in the central nervous system of rat 37,38 and pig 39. Whether ASS and ASL indeed colocalize with nNOS in the intestinal neurons and whether this renders these neurons independent of extracellular arginine through intraneuronal recycling of L-citrulline to L-arginine, is not clear. In vitro, inhibitory neuromuscular transmission in murine proximal colon was stimulated by arginine, but not by citrulline alone (0.1-2 mM), which in principle indicates that enteric nerves were not able to convert citrulline into arginine 40. In contrast, when nitrergic transmission was blocked by the NOS antagonist L-NAME, sustained neuronal exposure to citrulline (0.2 mM) reversed the effects of L-NAME 40,41, revealing the capacity of enteric nerves to recycle L-citrulline to L-arginine to sustain NO synthesis. Most likely, enteric neurons thus do not depend on extracellular arginine for generation of NO. Measurement of the inhibitory component of the intestinal enteric neuron system, evoked by NANC neurons, in organ bath experiments using intestinal tissue from our arginine-depleted transgenic F/A mice 42, may provide evidence for the (in)dependence of enteric neurons on extracellular arginine availability.
Similar to neurons, macrophages, activated by LPS or IFNγ, have been shown to express ASS, ASL, and A-I and A-II. Despite the upregulation of ornithine cycle enzymes required for regeneration of arginine from citrulline, macrophages remain dependent on extracellular arginine, as is shown by the upregulation of their arginine transport capacity (via the enhanced expression of CAT-2) in conjunction with iNOS induction. The upregulation of either A-I (A-II is not upregulated) or iNOS may be subject to the nature of the inflammatory response: the upregulation of A-I may be directed by Th2-derived anti-inflammatory cytokines, whereas Th1-associated pro-inflammatory cytokines induce iNOS expression. In section 4, the potential role of macrophage A-I induction in counteracting the macrophage NO output is further discussed.

1.3-Hepatic arginine metabolism

The liver does not participate in whole-body arginine synthesis, despite very high levels of ASS and ASL. This is because the very high arginase content of hepatocytes prevents the net production of arginine, which emphasizes the importance of the liver for urea production (ammonia detoxification) rather than for arginine biosynthesis. Moreover, the liver lacks expression of the high affinity cationic amino acid transporter (CAT-1), which is required for efficient hepatic import or export of arginine from the circulation. In concert with the argument that the liver does not contribute arginine to the circulation, patients with inherited ornithine cycle deficiencies, who need supplementation of arginine, continue to do so after transplantation of the liver.

1.4-ontogeny of arginine metabolism

In rapidly growing suckling rats, endogenous arginine biosynthesis is crucial to compensate for the insufficient supply of arginine via the milk. During the suckling period, the kidney has a limited capability for the synthesis of arginine. Evidence is accumulating that, in mammals, the intestine rather than the kidney plays a major role in arginine biosynthesis in this period. Thus, the enterocytes of

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the small intestine express CPS, AGA synthetase, OTC, ASS and ASL \cite{11,27,55,56}, i.e. the enzymes required for arginine production from glutamine \cite{3}, but do not express arginase \cite{33}. Similarly, enzymes that convert glutamine and proline to ornithine, glutaminase, P5CS and OAT, are all present in concentrations well above adult levels in the period before weaning \cite{57,58,59}, whereas the activity of pyrroline-5-carboxylate reductase (P5CR), the enzyme that can divert pyrroline-5-carboxylate towards proline, shows a reciprocal pattern \cite{56}. The intestine appears to play a similar role in human neonates as well, as destruction of the enterocytes in necrotizing enterocolitis also results in a selective decrease in circulating arginine \cite{61}. In mammals, ASS and ASL, disappear from the enterocytes in the perinatal period, concurrent with the appearance of endogenous arginase \cite{33}. After weaning, the role of the gut therefore becomes confined to citrulline biosynthesis.

The ontogeny of arginine metabolism in intestinal epithelium is associated with its maturation, as expression of ornithine cycle enzymes changes during migration along the crypt-villus axis. From the formation of villi onwards, glutaminase \cite{62}, OAT, CPS and OTC mRNA are concentrated near the base of the villus, whereas ASS and ASL mRNA are only present near the top \cite{33,42}. We have suggested that this baso-apical difference in gene-expression relates to the time after stem-cell division, and migration of the enterocytes along the crypt-villus axis \cite{33}. The enterocytes near the top of the villus are "older" than those at the base, implying that the presence of OAT, CPS and OTC protein in all villus enterocytes, including those at the top of the villus, is due to the half-life of these proteins that were synthesised when the enterocytes were still localized at the base of the villus. Because their mRNAs are only present in the apical enterocytes, ASS and ASL apparently become expressed only several days after enterocyte cell division and/or migration along the villus axis. The transit time of the enterocytes between the crypt and the top of the villus, which decreases from 11-14 days in the first postnatal week, to 2-3 days after weaning \cite{63,64}, may thus explain the difference between the pre- and post weaning expression pattern \cite{33}. In contrast with this hypothesis, however, a decrease in enterocyte migration rate, through the transgenic
overexpression of E-cadherin, is not accompanied by a change in distribution of the terminal differentiation marker liver-type FABP along the crypt-villus axis, suggesting that differentiation, at least for liver-type FABP, is largely cell nonautonomous \(^{65}\).

Together, the observed expression pattern of ornithine cycle enzymes suggests the existence of a metabolic compartimentation of citrulline synthesis in the enterocytes at the base of the villus and arginine synthesis in the top of villus enterocytes. Such a two-compartment system along the villus axis may enable the gut to simultaneously meet the needs for the synthesis of citrulline and arginine during the suckling period. Further examination of the expression of arginine transporters, or measurement of arginine and citrulline concentration in the two compartments should confirm this hypothesis.
2-Dietary demand of arginine

2.1-Dispensable and indispensable amino acids

Protein requirements have long been assessed by the sum of requirements of the essential amino acids and the nitrogen from non-essential amino acids. By preparing diets containing mixtures of amino acids as the only sources containing dietary nitrogen, the quantitative requirement for each amino acid was determined. In rats, it was found that of the 20 amino acids, that are incorporated into protein, 10 could not be excluded from the diet: valine, leucine, isoleucine, methionine, threonine, lysine, phenylalanine, tryptophan, histidine, and arginine. Excluding any one of these essential amino acids, except arginine, resulted in weight loss, and eventually death. However, although exclusion of arginine led to decreased weight gain, but allowed the animals to survive. The endogenous synthesis of arginine was apparently adequate for survival, but not for optimal growth. The classification of arginine as being a dispensable or indispensable amino acid is therefore a matter of definition, as arginine can be synthesized endogenously, but in limited quantities only. Irrespective of definitions, the limited endogenous biosynthetic capacity is most obvious under conditions of increased demand for- or catabolism of arginine. Indeed, arginine supplementation has been shown to ameliorate recovery and reduce hospital state under catabolic conditions, such as sepsis or trauma, also reviewed in.

2.2-The homeostasis of creatine reflects a delicate arginine balance

The highest level of creatine is found in muscle, Sertoli cells, and brain, where it plays an essential role in the energy metabolism. The biosynthesis of creatine first comprises the formation of guanidino acetic acid (GAA) from L-arginine and glycine by L-arginine-glycine amidinotransferase (AGAT EC 2.1.4.1., Fig. 2), followed by the transfer of a methyl group by S-adenosylmethionine:guanidinoacetate methyltransferase (GMT). The delicate balance of the dietary supply and endogenous biosynthesis of arginine with its daily
expenditure is nicely illustrated by the direct relation between the dietary intake of arginine and the body pool size of its product creatine \(^{76,77}\). A young adult male excretes on average of 23 mg (0.2 mmol) of creatinine per kg body weight per day. Thus, 0.2 mmol arginine per kg body weight is required for creatine synthesis \(^{66}\). The recommended daily allowance for protein is 0.8 gram protein per kg of body weight \(^{66}\), supplying 0.25 mmol of arginine per kg of body weight. Creatine synthesis alone, therefore, requires 80% of the daily intake of arginine. This tight balance may well explain why the creatine pool size is influenced significantly by changes in the quantities of arginine, glycine or creatine in the diet \(^{76,78}\).

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**Figure 1: Metabolic routes of creatine synthesis from arginine.** Enzymes that catalyze the indicated reactions are given in italics. AGAT transamidinates the guanidino group of arginine to glycine, but also to the other substrates indicated. Proposed transamidination substrates: glycine (Gly), ß-aminopropionic acid (GPA), γ-aminobutyric acid (GABA), δ-aminovaleric acid (δ-AVA), and products: ß-guanidinopropionic acid (GPA), ß-guanidinobutyric acid (GBA), and δ-guanidinovaleric acid (GVA). Products formed by reactive oxygen (see text): guanidinosuccinic acid (GSA), methylguanidine (MG) and guanidine (G). GSA and MG are most probably formed from argininosuccinate (AS) and CTN, respectively, via a reaction with a reactive oxygen species (see also section 6).

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### 2.3-Arginine demands in growing mammals
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Davis et al compared the supply of amino acids via the milk and their accumulation in body proteins of rat to estimate the degree to which each amino acid in the milk is utilized for protein accretion. The essential amino acids were all utilized for protein synthesis with efficiencies of around 75%, indicating that only 25% of amino acids are degraded in the suckling period. This finding corroborates an earlier finding that excretion of urea in this period is very small, see also chapter IV. Similar findings were reported for other species like pig, calf, sheep, chicken and human. However, 170 and 270% of ingested arginine and glycine, respectively, i.e. the precursors of creatine, was found in the lean body mass, indicating that they were actively synthesized.

2.4-Species differences in arginine requirement and biosynthesis

As described above, it has been shown that dietary arginine is required for optimal growth of species like rodents and pigs, but for obligate carnivorous species like cats and mink, it is an essential amino acid, due to the virtual absence of P5C synthetase and OAT in the intestine of these carnivores (see also 3.1). The resulting deficiency in ornithine biosynthetic capacity renders their ornithine availability directly dependent on dietary arginine. The result of this can be devastating: cats develop hyperammonemia and show clinical symptoms of ammonia toxicity within 2 hours after consumption of an amino acids containing meal without arginine, whereas non-carnivorous species show only minimal signs of intoxication when fed arginine-free diets. Since ornithine and citrulline occur only in very low concentrations in ordinary dietary proteins, feline species depend on dietary arginine to spike the ornithine cycle and maintain high capacity for ammonia detoxification. Thus, in carnivorous species, dietary arginine supply can be regarded critical for maintaining the urea cycle capacity for detoxifying ammonia.
3-inborn errors of metabolism leading to congenital arginine deficiency

3.1 Ornithine aminotransferase

The small intestinal mucosa is the only site of synthesis of pyrroline-5-carboxylate (P5C) from glutamate or proline\(^4,28\) (Fig. 1). P5C is a precursor for the non-enzymatic conversion to glutamate-\(\gamma\)-semialdehyde, which, in turn, is the substrate of OAT for ornithine formation. Carnivorous species, like the cat, have only 18% (per gram of mucosa), and 5% (per kilogram body weight) of intestinal P5C activity compared with rat. This limitation in the de novo synthesis of ornithine forms the metabolic basis for the severe hyperammonemia found in cats fed an arginine-deficient diet\(^90\).

Patients that lack OAT\(^91,92\) develop hyperornithemia and gyrate atrophy (GA), which is characterized by night blindness and diminished peripheral vision in the first and second decades of life, followed by progression to total blindness. In GA patients, hyperornithemia is associated with a reduction in the formation of creatine\(^93\), via inhibition of AGAT activity by the excess ornithine\(^94\). This creatine deficiency has been proposed to account for pathophysiology of the disease\(^95\), as the mass of skeletal muscle tissue in GA patients is only 24% of that of controls. Unexpectedly, arginine was found to be an essential amino acid in young growing patients with this inborn error\(^93\). Mice, in which the OAT gene was genetically targeted, can serve as an animal model for GA\(^12\). In young mice with this deficiency, hypo-ornithemia (56 \(\mu\)M, 50% of normal) and –argininemia (26 \(\mu\)M, less than 10% of normal), and an impaired growth rate are seen, demonstrating the requirement for ornithine biosynthesis from pyrroline-5-carboxylate at this age\(^12\). Interestingly, OAT-deficient mice develop hyperornithemia (>1mM) as adults, elegantly demonstrating that the OAT enzyme is directed towards ornithine synthesis in the neonatal small intestine and liver, but is reversed towards P5C.
synthesis in many adult tissues. A similar phenomenon seems to occur in human patients as they grow older.\textsuperscript{91}

3.2 Ornithine transcarbamoylase

The human deficiency of OTC, the gene being located on the X-chromosome, was first described in 1962.\textsuperscript{96} It is regarded as the most common ornithine cycle deficiency in man.\textsuperscript{10} Affected male infants generally die in the first few weeks after birth, due to acute hyperammonemic coma, though some chronic cases are known.\textsuperscript{97} Well studied murine models of OTC deficiency are the \textit{sparse-fur} (spf) and \textit{sparse-fur with abnormal skin and hair} (spf-ash) mice. These mutant mice suffer from congenital hyperammonemia and hypoargininemia and can serve as a useful model to study the neurochemical consequences of congenital urea cycle disorders,\textsuperscript{97} including hyperammonemic encephalopathy. Spf-ash mice are characterized by retarded body- and fur growth, and an excitable hyper-reactive temperament.\textsuperscript{10,98} Biochemically, these mice are characterized by a significantly elevated orotic acid excretion, elevated serum ammonia, glutamine and tryptophan levels,\textsuperscript{10,99} and a decrease in all other amino acid levels.\textsuperscript{99} Especially the ornithine cycle intermediates arginine, ornithine and citrulline are decreased to 20-50\% of normal.\textsuperscript{99}

In children, encephalopathy as a result of chronic hyperammonemia often seen in OTC deficiency is associated with changes in cerebral neurotransmitter systems.\textsuperscript{100} Similarly, Spf mice display behavioral abnormalities and brain damage,\textsuperscript{101} which can be attributed to alterations in a number of neurotransmitter systems, (NO, ATP, and glutamate), ammonia, or the tryptophan-derived excitotoxin quinolinic acid.\textsuperscript{104} Moreover, Spf mice have an elevation in the levels of neurotoxic guanidino compounds, such as GSA.\textsuperscript{105} Recently, we described behavioral aberrations, similar to those in Spf mutants, in our mouse model of arginine deficiency (F/A mice).\textsuperscript{42} We showed that this model is more selective in its amino acid deficiency than Spf mutants, as F/A mice have normal levels of ammonia, urea, and all amino acids except arginine. Although, the formation of GSA
is increased in both F/A and Spf mice, this compound is not increased in the brain of F/A mice, whereas it is in Spf-ash mice $^{106}$. Hence, a comparison of the two models suggests that the deficiency of arginine, or its direct metabolites (NO, agmatine or creatine), is the determining factor in the development of neuromotor deficits in both models. This conclusion would also justify the arginine treatment of OTC-deficient hyperammonemic patients $^{52}$.

3.3 Argininosuccinate synthetase

Citrullinemia is an autosomal recessive disorder, caused by a deficiency of argininosuccinate synthase (ASS). It is characterized by elevated levels of blood citrulline, ammonia, and orotic aciduria $^{107}$. Patients suffer from a disturbance of consciousness and coma, and most die with cerebral edema within a few years of onset $^{108}$. In man, ASS deficiency can manifest as a neonatal (CTLN1), or an adult-onset form (CTLN2) and as an asymptomatic form $^{52,109}$. CTLN1 is associated with mutations in the ASS gene, leading to due to ASS enzyme defects, whereas CTLN2 is a form of the disease that is defined by a decrease in ASS protein, but with normal kinetic properties $^{110}$. The protein being effected in CTLN2 may be a calcium-dependent mitochondrial transporter with a role in urea cycle function $^{108}$. The phenotype of ASS-deficient mutant mice closely resembles that of humans who lack the ASS enzyme, $^{107}$. Homozygous mutant animals develop high levels of blood citrulline, become hyperammonemic, and die within one or two days after birth.
4-Metabolism of arginine

4.1-Intestinal utilization of glutamine and arginine

The small intestine of the adult rat extracts 25-33% of arterial glutamine in a single pass, which accounts for 30% of whole body glutamine utilization\(^\text{17,111}\). Until the pioneering work of Windmueller on glutamine utilization by rat jejunum\(^2\), it was tacitly assumed that all dietary amino acids absorbed by the small intestinal mucosa entered the portal circulation intact and became available for extra-intestinal tissues\(^\text{17}\). However, intraluminally delivered glutamine is utilized extensively by the small intestinal mucosa: 66% of intraluminal glutamine is metabolized in a single pass by rat jejunum\(^\text{112,113}\). Thus, most of the glutamine is metabolized in the small intestinal epithelium and does not reach the portal circulation\(^\text{17}\). Subsequently, it was shown that glutamine, and not glucose, is the main metabolic fuel of enterocytes. Extraction of glutamine from the circulation is directly related to blood glutamine level, and in conditions associated with a decreased circulatory glutamine level, such as starvation, glutamine utilization in the intestine declines, whereas, on the other hand, the hepatic glutamine uptake increases\(^\text{111}\).

In adult rats, 40% of the luminal arginine, absorbed by the intestinal mucosa, is catabolized in a single pass, and the remaining 60% enters the intestinal venous blood intact\(^\text{17,113}\). A similar percentage was found for humans\(^\text{114,115}\). These numbers indicate that substantial amounts of dietary arginine are not available for extra-intestinal tissues. For pig enterocytes, it was shown that 93, 4, and 1% of arginine was metabolized to ornithine, citrulline and CO\(_2\) respectively\(^\text{55}\). In adult pig enterocytes, the majority of the arginine that is metabolized to ornithine (56%), is subsequently metabolized to proline. However, in contrast to post-weaning pigs, proline is an essential amino acid in neonatal pigs\(^3\). This can be explained by the lack of arginase in the neonatal pig intestine.
4.2-Arginase I and II

4.2.1-Tissue distribution

Arginase is the most widespread of urea cycle enzyme in terms of tissue distribution. In mammals, the presence of at least two different arginase genes has been established. In man, hepatic arginase I (A-I) is primarily found in liver, but also in extra-hepatic tissues such as erythrocytes and enterocytes. Studies in man and rats have demonstrated that liver and kidney-type arginase (A-II) differ from one another in both immunogenic and electrophoretic properties. Arginase II is more widespread and found primarily in kidney, adult intestine, stomach, brain, mammary gland, pancreas, epididymis, lung and skin. A-I and A-II may be coexpressed in the rodent kidney, small intestinal enterocytes, rat aortic endothelial cells and murine macrophages. The expression of A-II is highly species-dependent: the A-II content of small intestine and mammary gland of the rat is a factor 20 higher than that in man or dog.

4.2.2-Enzyme properties

Ureotelic mammals excrete the ammonia that is formed during the catabolism of amino acids as urea. Arginine regulates urea synthesis by allosterically activating the synthesis of N-acetyl glutamate, the essential co-factor of CPS-1. The arginase reaction is highly exergonic with a change in free energy of -23 kcal/mol, making the reaction irreversible. Arginase has by far the highest specific activity of the ornithine cycle enzymes (a V_max of more than 4000 μmol/min/mg), but the high K_m of 1-15 mM causes it to work at much lower velocity in vivo. Its activity strongly depends on the presence of its cofactor Mn^{2+}. Reflecting arginase’s high capacity, tissue arginase activities and tissue arginine content are inversely related: liver, with the highest arginase content, has the lowest arginine content, while kidney, muscle and spleen have only 1 % of liver arginase activity, but have a 5 to 10 fold higher arginine content (Fig. 3). In addition to L-arginine, other substrates for arginase are: L-canavanine, L-
homoarginine, L-argininic acid, and agmatine, but with 7-10 fold higher $K_m$. D-arginine and GABA are not substrates $^{129}$, indicating that the suitability of alternate substrates for liver arginase is determined by the presence of a guanidino group, the length of the amino acid carbon skeleton and the substituents around C-α. Lysine inhibits arginase $^{132}$, and functions as an arginine antagonist in mammals $^{133}$. As a result, the metabolic effects of feeding an arginine-deficient diet resemble the effect of feeding a diet containing a high lysine relative to arginine content.

4.2.3-Subcellular localization
A-I and A-II have a different subcellular localization: A-I is found in the cytosol $^{134}$, though a substantial fraction of A-I (10% of total activity) is associated with microsomes and mitochondria $^{135}$. A-II is strictly associated with in the mitochondria $^{136}$. It has been suggested that the mitochondrial localization of A-II facilitates the synthesis of glutamate and proline from ornithine $^{116}$, given the intramitochondrial localization of ornithine aminotransferase.

4.2.4-Regulation of arginase expression
Activated murine macrophages metabolize arginine by two alternative pathways involving either the inducible enzyme NO synthase (iNOS) or arginase. Macrophages contain both A-I and A-II, with the A-II form predominating. Activation of macrophages, by LPS or γIFN, induces a concurrent induction of iNOS and A-I $^{137}$ $^{138}$. In addition, arginine synthesizing enzymes ASS and ASL have been shown to be co-induced with iNOS $^{139}$, implying that activated macrophages can regenerate arginine from citrulline and are independent of extracellular arginine for NO synthesis. However, cellular arginine import is elevated in macrophages when NOS expression is activated $^{47}$, via enhanced expression of CAT-2 $^{139}$, indicating that activated macrophages require extracellular arginine for sustained substrate supply for the generation of NO.
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Fig. 3: relationship between arginase activity and arginine extractable content in several tissues. Adapted from Gopalakrishna, 1979. Open boxes: arginase activity; closed boxes: arginine content.

4.3-Agmatine.

Agmatine is a metabolite of arginine, formed via arginine decarboxylase (ADC). Agmatine and ADC activity have been described to be widespread in brain, liver, kidney, intestine, adrenal glands and macrophages, but the function of agmatine is not well understood. Agmatine can be hydrolyzed by agmatine urohydrolase (agmatinase; EC 3.5.3.11) to putrescine, and hence agmatine is a metabolic precursor for the biosynthesis of higher polyamines. On the other hand, agmatine may suppress cell proliferation by inhibiting ODC activity and subsequent polyamine biosynthesis. In addition, agmatine may play a role in cell signaling by controlling NO production by inhibiting NO synthases. Recently, agmatine has been identified as an neurotransmitter in the brain, that binds the N-methyl-D-aspartate (NMDA) subclass of glutamate receptors in hippocampal neurons. Modulation of the receptor was shown to be mediated by the interaction between the guanidino group and the neuronal channel pore. Though
the authors claimed this treat to be selective for agmatine\textsuperscript{145}, other (neurotoxic) guanidino compounds (see Chapter 6), like guanidinosuccinic acid (GSA) and methylguanidine (MG) have been shown to share similar properties\textsuperscript{146,147}.

4.4-\textit{Proline}.

Ornithine can be converted by the enzyme ornithine aminotransferase (OAT) and proline oxidase via proline-5-carboxylate into proline. All these reactions occur in the mitochondrion\textsuperscript{6}. Proline may stimulate collagen synthesis in hydroxylated form\textsuperscript{148}. This may offer an explanation for arginine's ameliorating effect on the wound healing process and cell proliferation\textsuperscript{149}.

4.5-\textit{Polyamines}.

Ornithine also serves as precursor in polyamine synthesis and is decarboxylated by the rate-limiting enzyme ornithine decarboxylase (ODC; EC 4.1.1.17) to putrescine, the first polyamine in the biosynthetic pathway that can further be converted into spermine and spermidine. Polyamine synthesis has been correlated with increased arginase and ODC activity in the kidney\textsuperscript{150} and the small intestine\textsuperscript{151}. Polyamines are known to exert regulatory properties in cell proliferation\textsuperscript{143,152} and, in that way, are involved in tissue growth and wound repair.

4.6-\textit{Nitric oxide}.

Another, quantitatively minor, pathway in the urea cycle is the NO synthase (NOS) pathway in which the free-radical NO and citrulline are stoichiometrically formed by the action of NOS (EC 1.14.13.39)\textsuperscript{153}. Three distinct isoforms of NOS are recognized, representing three different gene products\textsuperscript{154}. Two of these isoenzymes, defined as cNOS, are expressed constitutively and are calcium-dependent, whereas one isoform, termed inducible NOS (iNOS or NOS II), is only synthesized \textit{de novo} after stimulation and is non-calcium dependent\textsuperscript{155,156}. One cNOS subtype, named endothelial NOS (eNOS or NOS III), is mostly membrane-bound and is present in several cells, mostly endothelial cells\textsuperscript{157,158}. The other
cNOS subtype is cytosolic and mostly present in neuronal tissue and therefore defined as neuronal NOS (nNOS or NOS I). The iNOS isoform is cytosolic can be expressed in a great variety of cells including macrophages and epithelia. The cNOS enzyme is permanently present in cells and is (transiently) activated by temporary rises in free Ca\(^{2+}\). The iNOS isoform is not present at basal levels but can be induced in response to inflammatory cytokines and endotoxin, including LPS.
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5- Regulation of metabolism by arginine

5.1 Effect of arginine on protein synthesis and degradation

During disease and injury the organism responds by changing the rates in the synthesis and/or degradation, i.e. turnover of proteins. Supplementation of intravenous or enteral arginine is associated with alterations in protein metabolism. These effects may, at least in part, be mediated by the arginine-mediated hormone release such as insulin and growth hormone (GH), which are known to produce anabolic effects by inhibiting the loss of body protein and stimulating amino acid transport into the cells (see 5.2). The rate of translation of specific mRNAs determines the rate of protein synthesis, whereas post-translational modification of proteins affects protein function. Arginyl-tRNA is not only an immediate precursor for protein biosynthesis, but it is also involved in the post-translational conjugation of arginine with N-termini of proteins bearing N-terminal aspartate or glutamate, thereby targeting these proteins for degradation via the ubiquitin-dependent proteolytic pathway. Arginine may thus be involved in modulation of protein breakdown by post-translational arginylation.

5.2 Endocrine activity arginine

Arginine exerts a secretagogue effect by stimulating the secretion of the pituitary, pancreatic, and adrenal hormones. The pituitary GH has been shown to increase in response to intravenous administration of arginine in humans, but also after oral administration. Arginine was also found to exert the release of prolactin, both after intravenous and oral administration. Furthermore, arginine administration stimulates the secretion of several pancreatic hormones like insulin, glucagon, pancreatic polypeptide, and the GH-inhibiting hormone somatostatin. This secretagogue effect of arginine has been shown to act via mechanisms dependent, as well as independent of the production of nitric oxide. Several recent reports show that the effects of arginine constituting the induction of vasodilatation, inhibition of platelet...
aggregation and blood viscosity are mediated, in part, by arginine-induced endogenous released insulin $^{175,176}$. Besides these pancreatic hormonal responses, arginine was also found to stimulate the secretion of catecholamines $^{175,177}$ by the adrenal gland.
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6-Guanidino compounds

6.1- the ornithine-guanidine bi-cycle

So far, approximately 120 naturally occurring guanidino compounds have been described in plants and tissues 178 (for structural formulas: see Chapter VI, appendix II). Previously, Natelson and Sherwin proposed the existence of a guanidino cycle 179, which, next to the ornithine cycle, would be important in nitrogen metabolism. Though the intermediates have been detected in bacteria, the existence of this cycle has never been proven in mammals. This guanidine cycle was purported to be an alternative pathway to ensure the synthesis of creatine phosphate, needed for muscular contraction. In mammals, which do not possess urease, urea can be oxidized by the cytochrome P-450 system to give hydroxyurea 179. Hydroxyurea can either enter the ornithine cycle via the formation of carbamoylphosphate, or can be hydrolyzed to give hydroxylamine and enter the guanidine cycle. Guanidinoacetic acid (GAA), the precursor for creatine (CT), can be formed from glycine and arginine (via the ornithine cycle), or from glycine and canavanine (via the guanidine cycle) 180. According to Natelson, the two cycles would be operative in parallel, and were therefore specified as the 'ornithine-guanidine bi-cycle' 181 (Fig. 4). The existence of such a bi-cycle however, has never been shown in mammals.

6.2- guanidino compounds formed after transamidination.

A major route of formation of guanidino compounds from arginine is the transfer of the amidino group from arginine to glycine, yielding ornithine and GAA, the immediate precursor for creatine (CT) and creatinine (CTN). A proposed scheme of reactions is given in Fig 2. The reaction, catalyzed by the enzyme AGAT (EC 2.1.4.1), is reversible 182, and catalyzes the rate-determining step in creatine synthesis 180. AGAT accepts a wide variety of substrates as amidine acceptors: in addition to the physiological substrate α-amino acetic acid (glycine), α-aminoproprionic acid (- α-alanine), α-aminobutyric acid or α-aminovaleric acid can
function as substrate, yielding guanidinopropionic acid (GPA), guanidinobutyric acid (GBA), and guanidinovaleric acid (GVA), respectively.\textsuperscript{178,180,182} (Fig. 2).

![Diagram of the Ornithine Cycle and the Guanidine Cycle via the "ornithine-guanidine bi-cycle" of Natelson et al.](image)

Fig. 4: Proposed interaction of the ornithine cycle and the guanidine cycle via the "ornithine-guanidine bi-cycle" of Natelson et al. Excess urea can be re-utilized via this cycle. Details are provided in the text. Guanidine cycle substrates and products: hydroxyamine (OH-amine), homoserine (Hser), ureido homoserine (ureido-Hser), canavanino succinate (CavSA), canavanine (Cav), canalinine (Ca). Intermediates in creatine synthesis: arginine (Arg), guanidinocarboxylate (GAA), creatine (CT), glycine (Gly).

In murine models of arginine deficiency, either as a result of OTC-deficiency (spf and spf-ash mice)\textsuperscript{99}, or of A-I overexpression\textsuperscript{42}, GPA, GBA, and GAA are decreased, demonstrating that availability of arginine as amidin donor determines the formation of these GCs.

6.3-formation of GSA by reactive oxygen species

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The formation of guanidino compounds under several pathological conditions is demonstrated, and some GCs, such as the neurotoxic guanidinosuccinic acid (GSA), guanidine (G), and methylguanidine (MG), have been reported to add to the (neuro)pathology of several metabolic diseases. Elevated levels of the guanidine derivatives MG as well as GSA, have been detected in the urine and plasma of patients with uremia, and both compounds were shown to be toxic \(^{146,147,183}\). The exact mechanism of GSA synthesis remains to be established. Several alternative biochemical routes of GSA formation have been previously postulated. According to this proposed guanidine cycle pathway, GSA can be formed from canavaninosuccinate. As already stated, the existence of a guanidine cycle in mammals has never been demonstrated. Perhaps a more plausible route of GSA synthesis was proposed by Cohen \textit{et al.}\(^{183}\): GSA may be produced due to a shift in activity of AGAT to use aspartate rather than glycine as amidine donor. Another proposed enzymatic pathway of GSA synthesis is based on the cleavage of argininosuccinate by ASL to carbamoyl-aspartate and ornithine, instead of arginine and fumarate, and subsequent formation of GSA. This reaction may be stimulated by urea, which is known to inhibit ASL activity \(^{185}\), thus favoring AS accumulation. Indeed, addition of urea to the medium dose-dependently increases GSA synthesis in isolated rat hepatocytes. In addition, urea levels correlate positively with GSA and MG concentration in patients with renal failure \(^{186}\) and after partial nephrectomy in rodents \(^{187,188}\). On the other hand, the decreased GSA synthesis in human patients with hyperargininemia as a result of arginase deficiency \(^{189}\) cannot easily be explained by this hypothesis.

However, evidence is accumulating that the conversion of AS in GSA and betaine may be non-enzymatic, via the reaction of argininosuccinic acid with the action of an oxygen radical species. Such a conversion has already been shown to occur \textit{in vitro}, in isolated rat hepatocytes \(^{183}\). In a similar series of experiments, OTC inhibition by DL-norvaline inhibited GSA synthesis in isolated hepatocytes, suggesting that the urea cycle enzymes catalyze intermediate reactions in the GSA synthetic pathway \(^{190}\). Following urea injection, hepatic GSA levels also increased

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in vivo, but there was little change in hepatic arginine \(^{190}\), indicating that GSA synthesis is stimulated by urea, and is directly dependent on arginine availability. The latter is corroborated by the increased GSA synthesis in arginine-deficient transgenic mice \(^{42}\).

Similarly, MG has been shown to be generated after the reaction of an oxygen radical with CTN \(^{191,192}\). In uremia MG may arise from the degradation of CTN by the gut flora \(^{193}\). The concentration of MG varies with the GSA concentration in patients with renal insufficiency. Together, the toxic GCs MG and GSA most probably are generated by the cleavage of CTN or AS respectively, by reactive oxygen. Such a mechanism would provide the rational for the use of active oxygen scavengers for treatment of uremia.

6.4-Pathological effects

The clinical syndrome of uremia is due to the failure of not only the excretory but also the metabolic, regulatory and endocrine functions of the kidney. In addition to elevations in CTN and urea, acute and chronic renal failure is recognized by a state of hypercitrullinemia \(^{194}\). Citrulline proved to be a more sensitive indicator than urea or creatine, as citrulline levels correlated with the degree of nephrectomy, and hypercitrullinemia developed in the range of 10% to 33% nephrectomy without any changes in urea and creatine \(^{194}\). Thus, hypercitrullinemia is a specific marker of normal function of the proximal tubule, whereas an impaired creatine and urea clearance apparently can be compensated. A number of guanidino derivatives are also considered to be important indicators of renal failure \(^{193}\). GSA positively correlates with plasma urea \(^{186}\) and indicates the severity of renal failure \(^{195,196}\). MG also appears in plasma after renal failure \(^{197}\). GSA and MG are recognized as candidate markers, which reflect the pathological stage of nephritis \(^{147,198,199}\). The enhanced production of certain GCs, in particular GSA and MG, has been associated with the etiology of the uremic syndrome. Their increased synthesis has been reported in renal insufficiency \(^{184,196,200,201}\), in partially nephrectomized rats and mice \(^{187}\), during endotoxemia-induced sepsis \(^{202}\).
and in patients with hypothyroidism\textsuperscript{203}. Furthermore, an increased GSA concentration is found during under hyperammonemic conditions due to consumption of an arginine-free diet in carnivorous species\textsuperscript{204}, or under normal ammonia concentrations in mice models of arginine deficiency\textsuperscript{42}.

Uremia has been shown to be associated with neurological disorders\textsuperscript{205-207}, and a proportion of patients with renal failure suffers from central and peripheral nervous system impairments\textsuperscript{206}. Mildly uremic mice display acute behavioral deficits, which may relate to analogous symptoms in uremic patients suffering from uremic encephalopathy\textsuperscript{208}. The role of GSA in uremic neurotoxicity and coma is still controversial and needs further investigation, but GSA, G, and MG have been shown to be (neuro)toxic \textit{in vitro}\textsuperscript{209}. Moreover, GSA has been shown to cause convulsions after intracerebroventricular injection in mice\textsuperscript{147}. The elevated levels of GSA and MG may well play a role in the neurological consequences of uremia via activation of NMDA receptors\textsuperscript{147,210}. Mice, suffering from low levels of arginine and elevated level of circulating GSA and MG displayed behavioral deficits, such as hyperactivity as well as several more specific deficits in neuromotor abilities and coordinated movements\textsuperscript{42,98}.
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7-Arginine transporters

7.1-Amino acid transporters

Five systems are known to mediate transport of cationic amino acids over the cell membrane: system $y^+$, $y^+$L, $b^+$, $b^{0}$, and $B^{0}$. These transport systems can be distinguished by their affinity for cationic amino acids, their dependence on sodium, and their capacity to share transport with zwitterionic amino acids \[211\]. Systems $b^+$, $b^{0}$, and $B^{0}$, first discovered in rat blastocysts \[212\], are found in brush border membranes, and have a broad specificity for zwitterionic and dibasic amino acids. Systems $b^+$ (cationic amino acids-preferring) and $b^{0}$ (cationic and neutral amino acids-preferring) are sodium-independent, and $B^{0}$ (cationic and neutral amino acids-preferring) is sodium-dependent. Transport of arginine, ornithine, and lysine across plasma membranes is mostly performed via the sodium-independent amino acid-transport system, formerly designated $y^+$ \[213,214\].

7.2-System $y^+$

The major mammalian cationic amino acid-transport system is the nearly ubiquitous system $y^+$, which facilitates Na$^+$-independent arginine, lysine, and ornithine transport driven by membrane potential \[215\]. Expression in oocytes revealed the ecotropic murine leukemia virus receptor, now named CAT-1 \[216\], as a CAT \[217\]. Since, four homologous human and rodent genes make up the family of cationic amino acid transporters (CATs) that encode system $y^+$, CAT-1, 2A, and a splice variant CAT-2B, CAT-3 and CAT-4 \[214\].

CAT-1 expression is nearly ubiquitous and produces a single protein. Under basal conditions, CAT-1 is absent from hepatocytes \[215\], probably to prevent unnecessary transport and metabolism of arginine by the hepatic arginase in the hepatocytes. However, CAT1 expression is present in regenerating liver, and in the cases when hepatic cationic amino acid transport is needed, such as following feeding, cellular growth and illness.
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A full length cDNA clone from a previously identified murine T-lymphoma cell line (Tea, T early activation gene) showed significant homology (61%, 218) with CAT-1 and amino acid-transport capacity in oocytes 219. Two splice variants of this gene were identified and termed CAT-2A and -B. mCAT-2 expression is highly tissue-specific and is expressed using at least two widely separated promoters 51. CAT-2A is expressed in the liver and is regarded as the low affinity variant, whereas CAT-2B is the high affinity variant, expressed in T-cells. CAT2A is highly expressed in liver, skeletal muscle, especially after trauma, and skin, while CAT2B expression is high in brain, lung, testis, uterus, and activated, but not resting T-cells (expression was found in T-cell thymoma cells, but was low in thymocytes 215). CAT 2A and 2B proteins are 97% identical, as they differ only one 41 amino acid domain 215,219.

Recently, two new members of the CAT family have been cloned; a brain-specific isoform, which again has strong similarity with CAT-1, termed CAT-3 220, and CAT4 (previously termed HCAT3). The latter gene is highly expressed in skeletal muscle, intestine, kidney, and placenta and in eye and in retinal pigmented epithelium. CAT4 is homologous to the amino acid permease CD98 light chain 221. A defect in CAT4 expression at the basolateral membrane of epithelial cells in kidney and intestine has been associated with the development of lysinuric protein intolerance, a rare autosomal recessive disease, characterized by poor feeding, vomiting, diarrhea, and episodes of hyperammonemic coma 222,223.

7.3-Regulation of arginine transporters

CAT1 and CAT2 proteins expressed in Xenopus oocytes exhibit high-affinity-low capacity cationic amino acid transport activities 224. There is a steric constraint for arginine binding to the CAT-1 transporter as α-methylation completely abrogates arginine binding 217. Similarly, the affinity for D-arginine is 20 times less than for the L-arginine 225. On the contrary, L-homoarginine is specifically transported with high affinity, making it a useful probe to study CAT-1 activity, as physiological concentrations of L-homoarginine are low. The kinetic
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constants $V_{\text{max}}$ (maximal transport rate when excess substrate is present) and $K_m$ (substrate concentration at which the transport rate is half maximal) for arginine uptake depend on the CAT isoform. In hepatocytes in the basal state, the $y^+$ system (CAT2A) is barely detectable having a $K_m$ value of 2-5 mM and a $V_{\text{max}}$ of 3.3 nmol $^{-1} \cdot \text{mg of protein}^{-1} \cdot \text{min}^{-1}$ 225 i.e. a 10-70 fold higher apparent $K_m$ and $V_{\text{max}}$ than the CAT1 and 2B (100-150 µM 214). The high $K_m$ value of CAT2A for arginine (way above the normal plasma concentration) underscores the minimal rate of arginine uptake by hepatocytes under normal physiological conditions 225. The precise cellular localization of arginine transporters may account for the "arginine paradox", the dependence of endothelial NO synthesis on extracellular arginine availability, while the intracellular arginine concentrations (0.1-1.0 mM) greatly exceed the $K_m$ of eNOS for arginine (2-10 µM). The apparent $K_m$ for arginine of NO synthesis by intact endothelial cells is approximately 75-150 µM 6, which is in the range of normal physiological plasma concentrations, and of the $K_m$ values of the arginine transport systems.

Glucocorticoids and insulin induce expression of the CAT1 gene in liver cells through induction of transcription and stabilization of its mRNA 50. The CAT1 gene is subject to adaptive regulation by arginine availability in vitro, as arginine depletion increases CAT1 mRNA stability, causing an increase in CAT1 protein, and transport capacity 226. CAT2A is, in contrast to CAT1 and 2B, hardly trans-stimulated 227. Therefore, the 41 amino acid domain which lacks in the protein sequence of CAT2A is thought to be responsible for substrate recognition and possibly the mechanism of substrate translocation 215,219.

Quiescent lymphocytes from spleen, lymph nodes, and Peyer’s patches constitutively express CAT2B, although these cells exhibit little transport of arginine via the systems $y^+$ or $y^+L$ 214,215. Upon mitogenic activation, however, transcripts of CAT2B rapidly accumulate 215, underscoring a role for arginine in this T-cell activation process 228. In addition, system $y^+$ can be induced by inflammatory cytokines in hepatocytes 229 and macrophages 46.
Other cationic amino acids and positively charged analogues are effective inhibitors of arginine uptake by system $y^+$. For example, arginine uptake can be competitively inhibited by lysine, ornithine, canavanine, and certain NOS inhibitors, such as N-monomethyl-L-arginine and N-iminoethyl-L-ornithine. N-nitro-L-arginine and N-nitro-L-arginine methyl ester, or aminoguanidine, on the other hand, have no effect on arginine uptake.
8-Arginine as immunonutrient.

8.1 Arginine and immune function

Only limited information is presently available as to how nutrients can influence immune function or the development of lymphoid organs. An intriguing feature of the nutritional benefit of arginine is, next to its function as a building block for protein synthesis, its immunosupportive effect, especially under catabolic conditions. Arginine was already identified as an immunonutrient more than 20 years ago, in studies on wound healing. Since, arginine has often been indicated to influence immune system responses, either via the increased production of polyamines, effects on tumor growth, by ameliorating survival during sepsis, or directly on T-cell gene expression. Based on these studies, arginine has widely been added to postoperative supplemental formulas, like Impact® (Novartis), at doses as high as 100 gr per kg formula, but the actual molecular basis of the beneficial effect of arginine on lymphocyte biology has remained unclear through the years.

8.2 Potential mechanism of action

ADP-ribosylation of arginine residues

In the transgenic arginine-deficient mice described in Chapter V, the development of the lymphoid system is affected. This finding may hint towards an important aspect of arginine’s immunosupportive effect. The influence of the arginine deficiency seems to be restricted mainly to the early development of B-cells in the bone marrow, as we found no defective T-cell maturation in the thymus, or reduction of T-cell number in peripheral lymphoid organs. Despite their hampered maturation, transgenic B-cells residing in the spleen were able to proliferate normally upon B-cell specific in vitro stimulation. We are currently testing the proliferative ability of splenic T cells, but preliminary data already
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indicate an impaired production of IFNγ, and possibly IL4 and 6, upon CD3/CD28 *in vitro* stimulation, corroborating other reports\(^ {228}\).

In addition to a temporary hampered B cell maturation, the F/A transgenic mice suffer from a temporary inhibition of muscle development. An attractive hypothesis on how arginine may influence both lymphocyte responses and muscle growth is a possible enhancement of ADP-ribosylation of arginine residues in lymphocyte membrane proteins. Mono-ADP-ribosylation is a posttranslational modification of proteins in which the ADP-ribose moiety of NAD\(^+\) is transferred to the guanidino group of arginine on a target molecule\(^ {241}\). This reaction is catalyzed by mono-ADP ribosyltransferases (ART), which are exo-enzymes imbedded in the cell membrane. ART is abundantly expressed in myocytes, as well as immune cells. A membrane protein that was identified as a substrate for ART was α7-integrin\(^ {242}\). Mice, lacking this integrin suffer from muscular dystrophy, which starts five days after birth\(^ {243}\), a phenotype similar to the arginine-deficient F/A-mice.

The involvement of ART in immune response regulation is already indicated by the observation that strains of rats, which lack one of the ART enzymes (ART-1), develop autoimmunity\(^ {244}\). ART-1 and 2, isoforms of the murine enzyme, which are active on CD4\(^+\) and CD8\(^+\) T-lymphocytes, and may be functional in downregulating their activation state\(^ {245}\). ART-1 and 2 have been shown to specifically ribosylate members of the integrin family of adhesion molecules, like lymphocyte-function associated protein-1 (LFA-1)\(^ {246}\), or T-cell membrane proteins important in eliciting T-cell mediated immune responses, like CD27, CD28, CD43, CD44 and CD45R\(^ {242}\). ADP-ribosylation of T-lymphocytes is known to attenuate T-cell proliferation, cytotoxicity, and cytokine excretion\(^ {247}\), probably because T-cell receptors fail to associate in a contiguous and functional receptor cluster after stimulation\(^ {242}\). B-cell responses are not affected by ADP-ribosylation\(^ {245}\). Hence, the observed inactivity of T-lymphocytes (preliminary data), but intact B-cell response upon stimulation in our F/A-2 transgenic mice perfectly matches the functional consequences of an over-ribosylation of T-cells. Such an over-ribosylation during hypo-argininemia could be due to the fact that ARTs not only
ADP-ribosylate arginines in the backbone of proteins, but also free arginine. Thus, arginine and agmatine are the naturally competitive inhibitors of ADP-ribosylation of cell-surface molecules, and a decrease in circulating arginine, as seen in our F/A transgenics, then results in an enhanced ribosylation of cell surface proteins. However, the involvement of ADP-ribosylation in the arginine deficiency phenotype remains to be demonstrated.
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9-Scope of the thesis

The amino acid arginine is more than a building block for the synthesis of protein; it serves important physiological functions, such as the detoxification of ammonia, and it is a precursor for the synthesis of nitric oxide (Science’s molecule of the year in 1990), agmatine, creatine and polyamines. Furthermore, it serves as a secretagogue for glucagon and growth hormone, and evidence is accumulating that it has intriguing capacities in promoting lymphocyte function. Lactating mammals have been shown to accumulate large amounts of arginine, compared to mother’s milk, implying endogenous arginine biosynthetic capacity. This thesis aims to establish the specific functions of arginine for neonatal development.

In Chapter II, we identified the suckling small intestine as an organ with arginine biosynthetic capacity, after studying the spatial distribution of ornithine cycle enzymes along the rat small intestinal villus axis. The enterocytes of neonatal and suckling rats have the capacity to synthesize arginine, but this capacity is shown to be lost after weaning. Furthermore, we found a compartmentation of the citrulline and arginine synthesis over the villus axis: the basal enterocytes seem to synthesize citrulline, and the apical enterocytes arginine. A possible role of the changes in turnover rate of enterocytes during development in the establishment of this metabolic compartmentation is discussed.

We investigated the significance of this intestinal arginine metabolism for neonatal development further in a murine model of chronic arginine deficiency. To this end, two lines of transgenic mice which express different levels of arginase I in their enterocytes were generated, in order to establish lines of mice with a graded severity of arginine deficiency. In Chapter III, both lines were analyzed, and indeed this approach led to a, surprisingly selective, reduction in circulating arginine concentration, further underscoring the importance of enterocytes in maintaining arginine homeostasis. We describe the development of a pronounced phenotype in the arginine-deficient mice, which corresponds with the degree of arginine
deficiency and which includes the retardation of hair- and muscle growth, and an impaired development of the lymphoid tissue, in particular Peyer’s patches.

As mentioned earlier, arginine serves as a precursor for the synthesis of creatine and other guanidino compounds. In Chapter IV, the metabolic effects of a deficiency of arginine, in particular of arginine’s guanidino group, are considered. Decreases in the concentration of arginine lead to corresponding decreases in its transaminidation products and, hence, creatine synthesis. However, hypoargininemic conditions also induce the accumulation of certain neurotoxic guanidino compounds, associated with the development of psychomotor deficits, a phenomenon which parallels the enigmatic guanidino compound disturbances seen in patients with renal failure or hyperammonia. These data hint to a role for an enhanced oxidative stress under these conditions.

Another feature of arginine is its purported immunosupportive effect, which has never been explained, despite more than 20 years of research on this matter. As described in Chapter III, hypoargininemia affects the development of lymphoid organs, reflected in the virtual absence of Peyer’s patches. In Chapter V, this feature is further explored. Arginine deficiency leads to reduced B-cell numbers in the peripheral lymphoid organs, but T cells numbers are unaffected. Further research into the cause of this reduction led to the bone marrow, where it was unexpectedly revealed that arginine deficiency impaired early B-cell maturation at the transition from the pro- to pre- B cell stage.
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