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Arginine revisited: Minireview article

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Summary. Arginine is a precursor of proteins and employed in urea synthesis. It is also the precursor of many other compounds, such as creatine, nitric oxide, polyamines, agmatine, proline. In this review, its transport and that of other basic amino acids are examined, along with its transformation into nitric oxide, agmatine and proline, and the mutual regulation of the individual pathways.

Keywords: Arginase – Nitric oxide – Agmatine – Proline

The amino acid arginine has been known for more than a century and its role in the urea cycle has long been the subject of attention. Besides directly participating in the synthesis of urea, it appears to be a regulatory signal of protein uptake. This is thought to occur through activation of N-acetylglutamate synthase (Caldovic and Tuchman, 2003), the enzyme forming the activator of the first enzyme of urea synthesis, i.e. carbamylphosphate synthase I. Arginine is also of interest on account of its other functions. It participates in creatine synthesis and is the precursor of the polyamines putrescine, spermidine and spermine, and of nitric oxide (NO), agmatine and proline.

Arginine itself is formed from glutamate or proline (Fig. 1). Expression of all the enzymes necessary for its synthesis, however, is restricted to the liver and the intestinal mucosa, where citrulline is formed by the effect of pyrroline-5-carboxylate (P5C) synthetase and ornithine transaminase is immediately transformed into arginine (Wu, 1997) or released into the blood circulation. Citrulline is taken up by the kidney and transformed into arginine and this is supplied to other tissues via specific transporters. The major system in most cell types is system y^+, a high affinity, Na^+-independent transporter of basic amino acids. Three genes, *Cat-1*, *Cat-2* and *Cat-3* encode these transporters and *Cat-4* has recently been identified (Sperandeo et al., 1998). With the exception of the liver, *Cat-1* is expressed ubiquitously, whereas the expression of *Cat-2*, *Cat-3* and *Cat-4* is more restricted. Differential splicing of the CAT-2 mRNA results in two isoforms: CAT-2A, a low-affinity transporter, insensitive to trans-stimulation and mainly expressed in the liver, and high-affinity CAT-2B, characterized by trans-stimulation. Expression of the poorly efficient CAT-2A in the liver may protect arginine from entering the liver and being hydrolyzed by arginase. mRNA for CAT-2, however, is markedly induced in the liver following LPS administration, whereas arginase mRNA is decreased, pointing to down-regulation of the urea cycle (Tabuchi et al., 2000). CAT-1 mRNA is negligible in the liver unless it is induced to regenerate or following the injection of glucocorticoids and insulin (Liu et al., 1998).

These first results were obtained on mice. The properties of human CAT-1, CAT-2A and CAT-2B differ from those of the mouse (Closs et al., 1997). hCAT-1 has a 3-fold higher substrate affinity and is more sensitive to trans-stimulation than h-CAT-2B, while hCAT-2A has a much lower substrate affinity and is regulated by a pathway involving protein kinase C (Gräf et al., 2001).

In addition to the system y^+, mammal cells transport arginine by means of three other mechanisms: y^+L, $b^{o,+}$ (Na^+-indep.) and $B^{o,+}$ (Na^+-dep.). y^+L was first described as an exchange route that recognizes cationic amino acids in the absence of Na^+ and requires the cation to interact with neutral amino acids, such as leucine. It is a glycoprotein-associated amino acid heterodimeric trans-

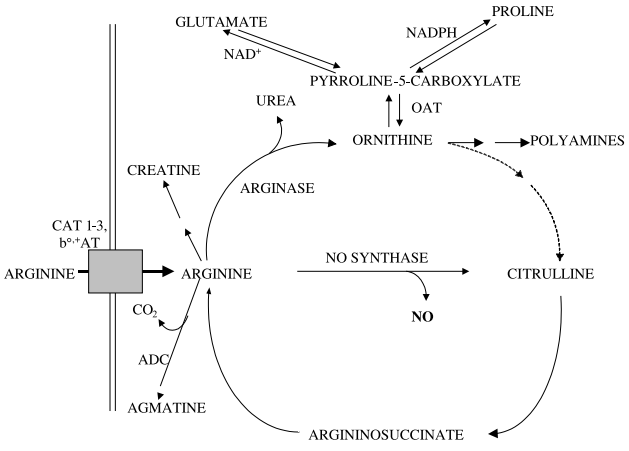

Fig. 1. Metabolism of arginine

porter (Pfeiffer et al., 1999), whose ''heavy chain'' is the glycoprotein 4F2hc, while the hydrophobic ''light chain'', y^+LAT-1 or y^+LAT-2, is responsible for the recognition and operational features of the transport process (Verrey et al., 1999). Expression of the y^+L isoform y^+LAT-1 is largely confined to the kidney (Torrents et al., 1998), suggesting that it is responsible for the release of arginine and its delivery to other tissues. Release by other tissues through the use of y^+LAT-2 may be supposed to secure the intercellular and interorgan transfer of arginine (Bröer et al., 2000).

Functional and genetic studies (Torrents et al., 1999; Borsani et al., 1999) have validated the suggestion (Torrents et al., 1998) that y^+L is responsible for the transport mechanism mutated in lysinuric protein intolerance (LPI). This transporter is thus responsible for the efflux of basic amino acids at the basolateral plasma membrane of epithelial cells, and therefore plays a crucial role in the intestinal absorption and renal reabsorption of basic amino acids. The efflux against membrane potential is probably achieved by exchange with neutral amino acids and simultaneous movement of sodium inside the cell. However, other unknown factors must also be responsible for the extrarenal manifestations of LPI. LPI-associated mutations, in fact, are not responsible for functional alterations in the transport of cationic amino acids, e.g. in human fibroblasts (Dall'Asta et al., 2000).

System $b^{o,+}$ is also a heterodimer. Its heavy chain is a 4F2hc-related glycoprotein (rBAT), with a single transmembrane helix and a large extracellular domain. The light chain is a typical membrane transport protein with 12 predicted transmembrane helices. This system has been detected in a ''renal proximal tubular'' cell line and in the apical membrane of the chicken jejunum. Mutations in the gene encoding rBAT have been held responsible for cystinuria. Very recently evidence has been obtained that mutations in the heavy chain alter the functional properties of this system (Pineda et al., 2003).

It has been suggested that cationic amino acids are also transported by amino acid transporter A3 (ATA), a subtype of the amino acid transport system A with an affinity higher than that shown for neutral amino acids. This system is expressed almost exclusively in the liver and may thus provide the major route for its uptake of arginine (Hatanaka et al., 2001).

Arginase

Before it can be used for the synthesis of polyamines or proline, arginine must be hydrolysed to urea and ornithine by arginase. This is a trimeric manganese metalloenzyme. Each of its subunits contains a binuclear manganese cluster required for full catalytic activity. One manganese ion can be reversibly dissociated to generate an enzyme retaining half of its catalytic activity. However, two isoforms of arginase are now known, the liver-type arginase I, expressed almost exclusively in the liver, and the non-hepatic type arginase II (Gotoh et al., 1997). Arginase I is located in the cytosol, arginase II in the mitochondrial matrix. The two isoforms are encoded by two different genes and differ in molecular and immunological properties and regulation of expression (Shi et al., 1998). Arginase mRNA II is strongly expressed in the adult kidney only, weakly in other tissues.

In the liver of ureotelic animals, ornithine formed in the soluble part of the cell is taken up by mitochondria and used for the urea cycle, though some is employed in loco for the synthesis of polyamines via a strictly regulated, well-characterized pathway. The role of arginase II must be different. Synthesis of glutamate and proline has been proposed as one of its functions, since ornithine formed in mitochondria could be used immediately by ornithine aminotransferase, which is also a mitochondrial enzyme. Moreover, as we shall see later, arginine is also used by other enzymes in the mitochondria. Regulation of NO synthesis by removal of arginine has been postulated for arginase in the nervous system and in immunological reactions. Arginine modulates NO production in activated mouse macrophages. However, by depleting intracellular arginine and by decreasing NO production, arginase prevents NO-dependent apoptosis (Gotoh and Mori, 1999). According to others, in the same cells endogenous levels

of arginase I are limiting for polyamine biosynthesis, but not for NO synthesis (Kepka-Lenhart et al., 2000).

It has, however, unexpectedly been shown that the inducible arginase in murine macrophages is arginase I, while arginase II is constitutively expressed in these cells (Louis et al., 1999; Munder et al., 1999). Th1 and Th2 cytokines may thus be supposed to regulate arginine metabolism. As a consequence, different amounts of ornithine, the precursor of polyamines, are formed. Spermine itself suppresses NO production in macrophages activated with LPS and inhibits the synthesis of proinflammatory cytokines in human mononuclear cells. As arginine is the precursor of proline, necessary for the synthesis of collagen, arginase I may also play a role during wound healing (Munder et al., 1999).

In bovine venular endothelial cells, basal levels of both arginase I and II are limiting for the synthesis of polyamines, proline and glutamate, and therefore for endothelial proliferation (Li et al., 2002).

Arginase activity is elevated in squamous cell and basal cell cancers of human skin and increases cell ornithine concentrations. It may thus be involved in the development of malignant skin tumours (Gokmen et al., 2001).

Synthesis of NO

In the last ten years, arginine has attracted attention as a precursor of NO, which is known to have important roles in many processes, including vasodilation, immune responses, neurotransmission and adhesion of platelets and leukocytes.

The supply of arginine is assured in several ways, depending on the cell type. Macrophages and bovine aortic endothelial cells synthesize arginine from citrulline. Expression of the rate-limiting argininosuccinate synthase increases, along with the synthesis of NO, when cells are stimulated with inflammatory mediators. However, mCAT-2 is also induced in macrophages by activation with LPS or IFN-γ (MacLeod, 1996). In cultured smooth muscle cells and in rats, CAT-1 and CAT-2B were also increased by LPS and IFN-γ (Hattori et al., 1999). The importance of this observation has been confirmed and stressed by the demonstration that arginine transport via the CAT-2 gene transporter is absolutely required for abundant synthesis of NO by macrophages (Nicholson et al., 2001). In T cells, too, system y^+ activity is increased upon activation. In cardiac myocytes, besides induction of the rate-limiting enzyme, an increase in arginine transport occurs. When these cells are exposed to IL-1β plus IFN-γ, this occurs by coinduction of CAT-1, CAT-2A and CAT-2B. By contrast, insulin augments arginine transport through CAT-1. In the liver, exogenous arginine alone promotes an increase of NO synthesis, whereas increased arginine synthesis is inefficient.

NO synthesis is also correlated with arginase activity. In murine macrophages, Th1 cytokines induce it and suppress arginase, whereas Th2 cytokines suppress it and induce arginase. A similarly regulated NO synthesis/arginase balance exists in murine dendritic cells (Munder et al., 1999). Moreover, as stated earlier, arginase I is upregulated, whereas arginase II is constitutively expressed.

Synthesis of NO is promoted by NO synthase. Different forms of this enzyme are now known: constitutive enzymes, neuronal NOS (nNOS or NOS 1) and endothelial NOS (eNOS or NOS 3) and the inducible NOS (iNOS or NOS 2), all encoded by separate genes and therefore differently regulated. The constitutive enzymes are calcium-calmodulin dependent, while iNOS activity does not depend on elevation in intracellular calcium levels. While nNOS and eNOS intermittently produce small amounts of NO, iNOS produces large amounts when it is expressed in response to various stimuli, and the NO thus formed displays both cytoprotective and cytotoxic effects. This occurs in numerous cell types. NO inhibits cell proliferation by a cGMP-dependent and a cGMP-independent mechanism. The latter occurs through nitrosylation by cysteine 360 of ornithine decarboxylase, thereby inactivating the enzyme and decreasing polyamine production (Bauer et al., 1999).

NO is synthesised by oxidation of arginine with the participation of NADPH. Only a small part of the intermediate N^G-hydroxy-L-arginine (NOHA) is released during the reaction, due to the fast rate of the second monooxygenation from NOHA to citrulline compared with the rate of dissociation, since NOS is a fusion protein of oxygenase and reductase domains (Iwanaga et al., 2000). NOHA inhibits arginase activity. Its concentration in human plasma is about 9 μM and higher in the serum of LPS-treated rats. Therefore when iNOS is induced by LPS, arginase activity is markedly inhibited in aortic endothelial cells This mechanism may ensure sufficient arginine for high-output production of NO.

The Km of eNOS for arginine is less than 10 μM. However, in cultured endothelial cells, arginine concentration is in the range of 0.1 to 0.8 mM. Consequently, eNOS should be saturated in these cells and an increase in extracellular arginine should not influence NO production. This, however, is not the case: NO production is increased by extracellular arginine, whereas its intracellular concen-

tration has no effect. The first attempt to explain this "arginine paradox" was based on the observation that CAT-1, the system y^+ protein present in endothelial cells and responsible for the uptake of most of their arginine, is colocalized with eNOS in plasma membrane caveolae and provides an efficient mechanism for delivery of substrate for NO synthesis (McDonald et al., 1997). This would also explain the observation that caveolar localization of eNOS is required for optimal NO production by eNOS. Caveolin may be supposed to be a protein of caveolae able to bind eNOS, though other proteins are probably involved. For maximal activation of NO release, caveolin may dissociate from eNOS, or other regulatory proteins may be recruited to relieve inhibition. Other proteins, in fact, co-precipitate with eNOS in some conditions. Moreover, it appears that not all of eNOS is co-localized with caveolin nor in caveolae, suggesting that eNOS in the cytoplasmic face of the Golgi produces NO (Garcia-Cardena et al., 1997). Preferential channeling of extracellular arginine to eNOS may thus be proposed.

An alternative explanation of the arginine paradox is that NOS isoforms in intact cells may be potently inhibited by endogenously produced compounds, both *in vivo* and *in vitro*. The methylarginines, N^G,N^G-dimethyl-arginine (asymmetric dimethyl arginine, ADMA) and N^G-methyl-arginine (NMA) are two potent endogenous NOS inhibitors derived by proteolysis when arginines have been methylated with S-adenosylmethionine: protein arginine N-methyltransferases (PRMT). Two and perhaps more types of these enzymes exist in mammalian cells: type I catalyze the formation of ADMA, type II give rise to symmetric dimethylarginine. Four type I isoforms with overlapping activities, but distinct regulation mechanisms and subcellular localization, have been described (Tang et al., 2000). Free methylarginines are found in cell cytosol, plasma and tissues: their concentration differs from one tissue to another. In rabbit endothelial cells, for example, it is 5 μM each (Masuda et al., 1999). Elevated plasma ADMA concentrations have been observed in some pathological conditions. NMA and ADMA are actively metabolized to citrulline and methylamines by dimethylarginine dimethylaminohydrolase (DDAH), a zinc-containing protein (Bogumil et al., 1998). Two DDAH isoforms with distinct tissue distribution and some relationship to NOS isoforms have been identified: DDAH I is found in tissues that express nNOS, whereas high levels of DDAH II are found in tissues with eNOS (Leiper et al., 1999). These results support the view that methylarginine concentration is actively regulated. Methylarginines are also transaminated and the enzyme responsible could also have a role in regulating their concentration. Moreover, NMA and ADMA inhibit arginine uptake (Tojo et al., 1997) and thus participate in the regulation of its metabolism.

Ki for ADMA, the more potent inhibitor, has been reported to be 0.4 to 2 μM for nNOS. As mentioned earlier, plasma ADMA levels are high in some pathological conditions, e.g. essential hypertension, atherosclerosis and hypercholesterolemia, whereas urinary excretion of the NO metabolites nitrite and nitrate, and of cGMP, a second messenger of NO, are reduced, and the arginine concentration is almost normal. However, i.v. or oral administration of L-arginine to healthy and ill humans as well as to animals increases plasma nitrite concentration and enhances urinary excretion of nitrate and cGMP. These observations induced Tsikas et al. to suggest that NOS inhibition by endogenous methylarginines, with ADMA as the most potent, is responsible for the arginine paradox.

Under physiological conditions the enzyme activities of NOS isoforms may be supposed to be reduced to a fraction of their maxima. Administration of arginine would thus increase its intracellular concentration and result in an exchange of intracellular inhibitors against extracellular arginine in NOS-producing cells. These effects would depend on the amount administered and last as long as the increase in the circulating arginine concentration.

Agmatine is another endogenous inhibitor of NO synthesis.

Agmatine

Synthesis, metabolism and function of agmatine in animal tissues have recently been reviewed (Grillo and Colombatto, 2004). However, it is important to observe that agmatine is thought to be formed in mitochondria. Arginase II is also a mitochondrial enzyme and a mitochondrial NOS (mtNOS), apparently different from the cytosolic forms (Lacza et al., 2003), has been demonstrated (Giulivi et al., 1998). The NO formed in mitochondria may be supposed to modulate the respiratory rate and ATP synthesis by inhibiting cytochrome c oxidase (Giulivi, 1998). As it is a Ca^{++}-dependent enzyme, upon Ca^{++} uptake by isolated mitochondria, mtNOS is stimulated and cytochrome c is released, due to the formation of $ONOO^-$ (Ghafourifar et al., 1999). Ca^{++}-induced apoptosis is therefore at least partially mediated via mtNOS. Mitochondrial pH, too, appears to be controlled by NO (Ghafourifar and Richter, 1999). Moreover, at different rates of endogenous NO production, the amount of

H_2O_2 formed is also changed, as NO modulates the rate of O_2 consumption at the cytochrome oxidase level. NO would thus seem responsible for the modulation of mitochondrial production of ROS (Sarkela et al., 2001) and cellular conditions that affect the availability of arginine or cofactors required for NOS activity would lead to varying amounts of NO and ROS.

The formation of agmatine thus requires the transport of arginine into mitochondria, whose uptake was studied long ago in kidney, and later in brain (Dolinska and Albrecht, 1998), where a y^+ system seems to be present. In rat liver mitochondria, arginine is taken up by an energy-independent mechanism that probably reflects membrane binding rather than transport into the matrix. In this way opening of the transition pore is modulated (Toninello et al., 2001). It is not known whether arginine transport is involved in the control of agmatine and NO synthesis in mitochondria.

Proline

Ornithine formed by arginase is metabolized by ornithine aminotransferase to give P5C and this is reduced to proline. Interconversion of P5C and proline constitutes a shuttle involved in transfer of redox potential across the mitochondrial membrane. However, little is known about the importance of the cellular transformation of arginine into proline other than in the small intestine and the mammary gland. Proline has recently attracted greater attention following its demonstration as the substrate of several enzymes important in cell growth, activation of the apoptotic cascade and regulation of oxygen homeostasis.

Proline oxidase, a mitochondrial enzyme, and its product P5C, inhibit cell growth and induce apoptosis in a lung carcinoma cell line (Maxwell and Davies, 2000) through the generation of reactive oxygen species (Donald et al., 2001). Proline oxidase is absent or reduced in several renal carcinomas, in some cases associated with mutant p53 (Maxwell and Rivera, 2003). Mutations in the proline dehydrogenase gene, sometimes associated with hyperprolinemia, have been postulated as the cause of schizophrenia (Chakravarti, 2002), although this has not been confirmed by others.

Proline-rich regions are frequently found in proteins, often as multiple tandem repeats and often of considerable length. In many cases, binding appears to be one of their main functions, in others they have a structural role. However, enzymes are now known, whose activity depends on the presence of proline, or act on proline present in proteins, or on amino acids near to proline, namely proline-rich tyrosine kinases, proline isomerases and proline hydroxylase.

The proline-rich tyrosine kinases are thought to be involved in platelet activation (Osada et al., 2001), in GLUT4 translocation/glucose transport (Sajan et al., 2002) and in collagenase-1 expression (Loeser et al., 2003).

Proline isomerases are important for the phosphorylation by tyrosine kinases of serine or threonine residues preceding proline (Ser/Thr-Pro), recognized as a major signaling mechanism, As some kinases phosphorylate the trans isomer only, cis/trans isomerization has to occur. Among the enzymes involved, Pin1 is extremely specific for phosphorylated Ser/Thr-Pro (pSer/Thr-Pro) residues. The resulting conformational changes have several profound effects, for instance on catalytic activity and on protein–protein interactions. It is suggested that this mechanism may play a role in cell growth control and diseases such as cancer and Alzheimer's (Lu et al., 2002).

Other enzymes acting on proline contained in a protein are HIF-1α prolyl hydroxylase 1, 2 and 3 (Bruick and McKnight, 2002). In normoxia, the hypoxia inducible factor-1α (HIF-1α) is hydroxylated at Pro 564 and Pro 402, which allows the von Hippel-Lindau protein (pVHL) binding required for ubiquitination and degradation by the 26S proteasome. Similarly, an asparaginyl hydroxylase modifies one of the carboxyl-terminal transactivation domains by blocking its interaction with transcriptional coactivator p300. Hypoxia blocks both hydroxylations, so that HIF-1α accumulates and binds to p300 and transcription of the gene is promoted (Bruick and McKnight, 2002). It has been shown that this occurs by selective increase of the expression of prolyl hydroxylase 2 mRNA levels (Berra et al., 2003), while it also seems that NO has an inhibitory role in controlling the regulation of hydroxylase activity (Metzen et al., 2003).

From all this it appears that proline is involved in intracellular signalling pathways, in cell growth control and in some diseases such as cancer and Alzheimer's. It would therefore be important to establish whether proline is also actively formed from arginine in tissues other than the small intestine and mammary gland in order to be able to control it when necessary.

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