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REVIEW ARTICLE

Expression and function of arginine-producing and consuming-enzymes in the kidney

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Abstract The kidney plays a key role in arginine metabolism. Arginine production is controlled by argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) which metabolize citrulline and aspartate to arginine and fumarate whereas arginine consumption is dependent on arginine:glycine amidinotransferase (GAT), which mediates creatine and ornithine synthesis. Histological and biochemical techniques have been used to study the distribution and activity of these enzymes in anatomically dissected segments, in isolated fragments of tubules and in whole tissues. ASS and ASL mRNAs and proteins are expressed in the proximal tubule. Within this nephron segment, the proximal convoluted tubule has a higher arginine synthesis capacity than the proximal straight tubules. Furthermore, this arginine-synthesizing portion of the nephron matches perfectly with the site of citrulline reabsorption from the glomerular filtrate. The kidney itself can produce citrulline from methylated arginine, but this capacity is limited. Therefore, intestinal citrulline synthesis is required for renal arginine production. Although the proximal convoluted tubule also expresses a significant amount of GAT, only 10% of renal arginine synthesis is metabolized to guanidinoacetic acid, possibly because GAT has a mitochondrial localization. Kidney arginase (AII) is expressed in the cortical and outer medullary proximal straight tubules and does not degrade significant amounts of newly synthesized arginine. The data presented in this review identify the proximal convoluted tubule as the main site of endogenous arginine biosynthesis.

Keywords Citrulline · Kidney · Microdissected tubules · Proximal convoluted and straight tubules · Mammals · Argininosuccinate synthetase and lyase · Arginase · Tubular and subcellular localization

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History of renal arginine synthesis

The ability of the kidney to synthesize arginine was discovered 70 years ago when Borsook and Bubnoff (1941b) observed that rat kidney slices could produce guanidinoacetic acid from citrulline and glycine. This finding suggested to them that citrulline could be metabolized to arginine. Borsook and Bubnoff (1941a) subsequently confirmed the ATP-dependent formation of arginine from citrulline and either glutamic acid or aspartic acid in rat and guinea pig kidneys. In addition, the amino acids proline, ornithine, lysine, and hydroxyproline support arginine synthesis in rat kidneys (Borsook and Dubnoff 1941a; Dubnoff and Borsook 1948). Arginine formation in respiring pig kidney homogenates was reported later (Ratner and Petrack 1953).

Arginine biosynthesis from citrulline requires two consecutive enzymatic reactions. The first reaction is controlled by argininosuccinate synthetase (ASS, EC 6.3.4.5) and consists of two steps. In the first step, citrulline is activated by ATP under the formation of adenylocitrulline (Schuegraf et al. 1960) and pyrophosphate (Petrack and Ratner 1958). In the second step, adenylocitrulline is transferred to the amino group of aspartate to form argininosuccinic acid (Ratner and Pappas 1949; Ratner et al.

1953a, b, Rochovansky and Ratner 1967). The second reaction, hydrolysis of argininosuccinic acid to arginine and fumarate, is catalyzed by argininosuccinate lyase (ASL, EC 4.3.2.1) (Ratner et al. 1953a).

Databases and molecular weights for ASS and ASL gene products

The National Center for Biotechnology Information (NCBI) and ExPASy Proteomics Server databases provide access to ASS and ASL genes, mRNAs, and proteins of mouse, rat, human, and several other species. The accession numbers of ASS, ASL genes and their products are provided in Supplemental Table 1. Detailed data concerning the protein structure of ASS and ASL can be found at UniProtKB/Swiss-Prot (http://www.uniprot.org/). Native ASS is a homotetramer ($M_w \approx 185$ kDa). In mouse, rat, and human, the polypeptide subunit of ASS consists of 412 amino acid residues with a molecular weight of approximately 46.3 kDa. Native ASL is a homotetramer of approximately 202 kDa. The polypeptide subunit of ASL is composed of 464 (mouse and human) or 461 (rat) amino acid residues ($M_w \approx 52$ kDa).

Expression of ASS and ASL in the kidney

ASS and ASL are expressed in the kidney of rats (Aperia et al. 1979; Dhanakoti et al. 1990; Goutal et al. 1999; Levillain and Wiesinger 2011; Mistry et al. 2002; Ratner 1973, 1976; Ratner and Murakami-Murofushi 1980; Yu et al. 1995), mice (Morris et al. 1991; Natesan and Reddy 2001), pigs (Wakui et al. 1992), dog (Levillain et al. 2008), ferrets (Deshmukh and Rusk 1989), and human (Kimball and Jacoby 1980). ASS and ASL expression in the kidney is readily detectable in the fetus (Goutal et al. 1999; Husson et al. 2003; Morris et al. 1991).

The kidney is composed of several zones: the cortex (C), divided into the superficial (Cs) and the deep layer (Cd), and the medulla, composed of the outer (OM) and the inner medulla (IM) (Fig. 1). The outer medulla is subdivided into the outer stripe (OS) and the inner stripe (IS). The inner medulla is composed of four equal zones: IM1, IM2, IM3, and IM4. The IM3 and IM4 zones are also known as the papilla (Pap1 and Pap2, respectively) (Fig. 1). The smallest structural unit in the kidney is the nephron. The most proximal part of the nephron is Bowman's capsule, which forms an envelope around the glomerulus (Glom). Adjacent to Bowman's capsule is the proximal tubule, which can be subdivided into three unequal parts: the proximal convoluted tubule (PCT), the cortical proximal straight tubule (CPST), and the outer medullary proximal straight tubule (OSPST). The proximal part of the PCT is composed of the so-called S1-type cells while the distal part of the PCT comprise the S2 cell types. The CPST also consists of S2-type cells, whereas the OSPST is exclusively composed of S3-type cells (Kaissling and Kriz 1979). The proximal tubule leads to the descending thin limb (DTL), the ascending thin limb (ATL), the medullary thick

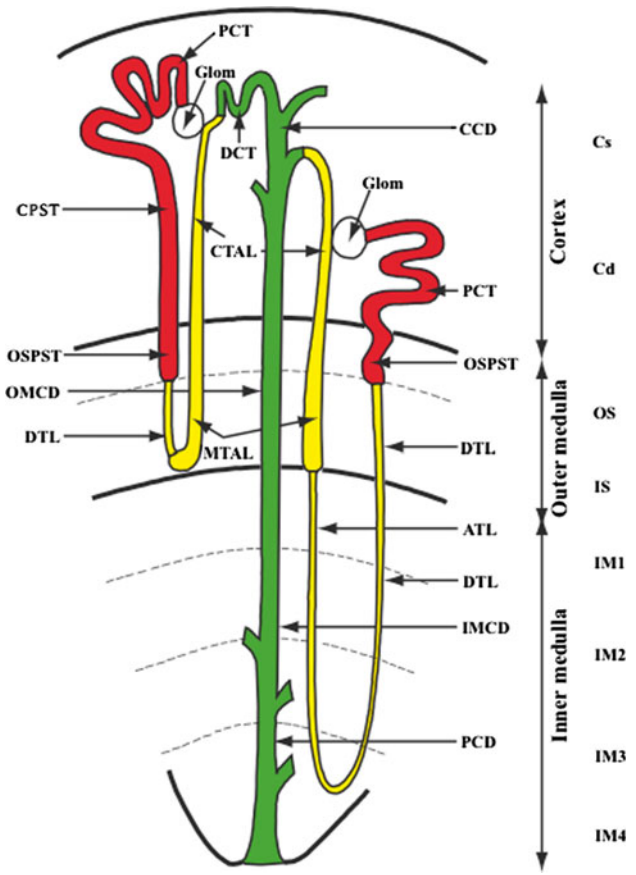

Fig. 1 Scheme localizing the tubules in superficial and deep nephrons. The kidney is composed of several zones: the cortex (*C*) is divided into the superficial (*Cs*) and the deep layer (*Cd*), while the medulla is composed of the outer (*OM*) and the inner (*IM*) medulla. The OM is subdivided into the outer stripe (*OS*) and the inner stripe (*IS*). The inner medulla is composed of four equal zones: IM1, IM2, IM3, and IM4. The smallest structural unit in the kidney is the nephron which is preceded by the glomerulus (*Glom*). The nephron consists of several morphologically and functionally different segments. The first segment is the proximal tubule (*red*), which is subdivided into three unequal parts: the proximal convoluted tubule (*PCT*), the cortical proximal straight tubule (*CPST*), and the outer medullary proximal straight tubule (*OSPST*). The proximal tubule is followed by the Henle's loop (*yellow*) composed of the descending thin limb (*DTL*), the ascending thin limb (*ATL*), the medullary thick ascending limb (*MTAL*), and the cortical thick ascending limb (*CTAL*). The connection between the thick limb and the collecting duct (*CD, green*) is ensured in the distal convoluted tubule (*DCT*). The collecting duct runs through the cortex (*CCD*), the outer medulla (*OMCD*), the inner medulla (*IMCD*), and the papilla (*PDC*). Superficial nephrons differ from deep nephrons in the length of their Henle's loop (color figure online)

ascending limb (MTAL), and the cortical thick ascending limb (CTAL). The distal convoluted tubule (DCT) connects the thick limb and the collecting duct (CD). The CD runs through the cortex (CCD), the outer medulla (OMCD), the inner medulla (IMCD), and the papilla (PDC) (Fig. 1) (Bankir et al. 1987; Kriz and Bankir 1988). Three types of nephron can be found in the kidney: superficial, intermediary, and deep nephrons, which differ in the length of their Henle's loop (Fig. 1).

Because of the complex anatomy of the mammalian kidney (Kriz and Bankir 1988), the identification of the renal structures that are involved in arginine synthesis requires the use of subtle and elegant techniques. In addition, a complex network of vascular structures is present around the tubules (Dworkin and Brenner 2000). The simplest approach to localize the arginine-synthesizing enzymes in the kidney is to separate the cortex from the medulla and measure the enzymatic activities in each zone. Szepesi et al. (1970) were the first to assay «arginine synthetase» activity in the kidney and reported that approximately 86% of the activity was present in the medulla and only about 14% in the cortex. Three years later, the same group confirmed this finding in sheep kidneys (Rabinowitz et al. 1973). A threefold increase in protein content of the food caused a threefold increase in «arginine synthetase» activity (Rabinowitz et al. 1973). Szepesi et al. further found that «arginine synthetase» activity in dog, cat, and horse kidneys also localized predominantly in the medulla (Rabinowitz et al. 1973). Taken together, these early results all pointed to the renal medulla as the main site of arginine synthesis.

The development of molecular biological tools allowed new experimental approaches. Morris et al. (1989) found that in Sprague–Dawley rats, ASS and ASL mRNAs were expressed almost exclusively in the renal cortex. Only faint traces of both mRNAs were seen in the medulla and none in the papilla (Morris et al. 1989). Recently, our group observed that the expression of 1.6 kb ASS mRNA decreased sharply from the cortex towards the papilla in both male and female mouse kidneys (Levillain et al. paper in preparation). More than 65% of ASS mRNAs was present in the superficial or outer cortex, whereas 30% was detected in the outer stripe of the outer medulla. Small amounts of ASS mRNA were observed in the inner stripe of the outer medulla, and in the inner medulla and papilla.

More than 90% of ASL activity has been localized in the renal cortex of rats, whereas only little activity was found in the medulla and papilla (Dhanakoti et al. 1990, 1992; Morris et al. 1989). In a more detailed follow-up study, ASS and ASL activities were assayed in five renal zones, along with the activities of marker enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), which is restricted to the cortex, and hexokinase, which is mainly localized in the medulla and papilla (for references, see Dhanakoti et al. 1992). The distribution of ASS and ASL closely resembled that of PEPCK. More than 90% of their activities was found in the cortex, of which 71% was in the outer cortex and 20% in the inner cortex. Only very low ASS and ASL activity was detected in the outer (6%) and inner medulla (3%) (Dhanakoti et al. 1992).

Renal metabolism has also been studied in isolated renal tubules which were separated from their environment with collagenase (Guder et al. 1971). The quality of isolated tubules was assessed by the higher level of oxygen uptake and glucose formation than cortical slices or an isolated perfused kidney. The preparation of renal cortical tubules was further improved by applying a Ficoll gradient to eliminate lysed cells, cellular debris, and glomeruli (Balaban et al. 1980).

Dhanakoti et al. (1992) also used isolated cortical tubules to localize ASS and ASL activity in rat kidney. Given that a suspension of cortical tubules consists of PCTs, proximal straight tubules (PST), distal straight tubules, CDs, and glomeruli, the authors fractionated them on a Percoll gradient (Vinay et al. 1981). ASS and ASL enzymes were found predominantly in the fraction which exhibited the highest PEPCK activity, that is, in the PCT (S1 and S2 portions) (Dhanakoti et al. 1992). These tubules also exhibit the highest rate of arginine synthesis when compared to the other fractions.

More recently, our group used Western-blotting to analyze the distribution of ASS and ASL proteins (Levillain and Wiesinger 2011). In both male and female rat kidneys, ASS protein was detected in the entire cortex and the outer medulla, but not in the inner medulla and papilla. Almost 90% of ASS protein was present in the cortex, of which two-thirds were in the outer cortex. ASL protein, in contrast, was expressed along the entire corticopapillary axis, with the highest levels in the outer (77%) and inner (16%) cortex, and low, but significant levels in the inner medulla and papilla. In mice of both sexes, the distribution of ASS and ASL proteins resembled that reported in rats.

Taken together, the early and later reports contradict each other with respect to the intrarenal localization of arginine synthesis. At present, it is still a mystery why Rabinowitz et al. (1973) and Szepesi et al. (1970) found almost all «arginine synthetase» activity in the renal medulla rather than the cortex. Informations that could explain this contradiction could not be found in the literature.

Identification of the renal tubules involved in arginine production

Burg and Orloff (1962) pioneered the dissection of single viable nephron segments from kidneys of New Zealand rabbits. Tubules from collagenase-treated kidneys were dissected by hand at room temperature using a stereo-

microscope. Isolated cortical tubules were judged viable if they accumulated para-aminohippuric acid (PAH), consumed oxygen, and maintained concentration gradients for Na^+ and K^+ for 2 h. Microdissected tubules afford an excellent means to explore and characterize the metabolism in a single piece of tubule. However, the amount of tissue protein which can be isolated by this way is extremely limited for some tubules (e.g. thin limbs, DCT, IMCD). Therefore, new sensitive microtechniques were needed to determine enzymatic activities in such tubules. Kobayashi (1963) measured histidine decarboxylase activity by incubating a tubular fragment with [U-^{14}C]-histidine in a rubber-capped glass scintillation counting vial or Warburg flask. The reaction was terminated with acid, which also cleaved $H^{14}CO_3^-$ into $^{14}CO_2$. The $^{14}CO_2$ formed was trapped in hyamine hydroxide and radioactivity was counted in a liquid scintillation counter. The sensitivity of the method was sufficient to measure enzyme activity in less than 1 µg of tissue (Beaven et al. 1978). Based on this approach, a new microtechnique was developed to measure $^{14}CO_2$ production during oxidation of [U-^{14}C]-substrates in single nephron segments during four successive 30 min periods (Hus-Citharel and Morel 1986; Le Bouffant et al. 1984). A closed chamber is formed by the opposed sunk glass slides tightly sealed with vaseline. The cover glass slide contains a KOH droplet (2 µL) to trap newly produced $^{14}CO_2$, while the other slide contains a droplet of 1 µL with the tubular fragment (Fig. 2). The tubules are photographed for the subsequent determination of their length and the results are expressed per millimeter tubular length.

To determine which of the renal structures and cell types of the nephron and/or vessels produce arginine, we took advantage of these techniques and incubated single or pooled viable microdissected nephron segments with [ureido-^{14}C]-citrulline (Fig. 2). In tubular cells expressing ASS and ASL genes, the [ureido-^{14}C]-citrulline is metabolized into [guanidino-^{14}C]-arginine. Commercial arginase and urease were added in excess to the incubating medium, so that newly formed [guanidino-^{14}C]-arginine was metabolized to ornithine and [^{14}C]-urea, and [^{14}C]-urea to two molecules of NH_3 and one molecule of $^{14}CO_2$. The $^{14}CO_2$ was continuously trapped by diffusion into KOH (Fig. 2) (Levillain et al. 1990). The technique is specific, reproducible, and very sensitive, since PCT less than 0.5 mm long produced enough arginine to be detected by liquid scintillation counting (Levillain et al. 1993). With this technique, arginine synthesis was assayed in glomeruli and almost all nephron segments, including proximal tubules, thin limbs, distal tubules, and CDs. In all species studied, including rat, mouse, rabbit, cat, and gerbil, arginine synthesis was found to be restricted to the whole proximal tubule (Hus-Citharel et al. 1995; Levillain et al. 1990, 1993, 1996). When the concentration of citrulline was fixed at 108 µM, the three portions of the proximal tubule displayed a different capacity to synthesize arginine (Fig. 3).

The PCT always exhibited the highest capacity to synthesize arginine, whereas the OSPSTs produced the lowest amounts of arginine. The CPSTs produced intermediate levels of arginine in rat, cat, and gerbil (Hus-Citharel et al.

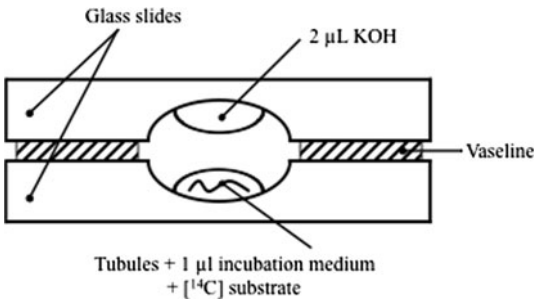

Fig. 2 Closed chamber for metabolic incubation with radiolabeled substrates. The metabolic chamber is closed during the incubation period. The nephron segments are in incubation medium which contains the appropriate ^{14}C-labelled substrate and the commercially obtained enzymes (arginase and/or urease). The newly formed ^{14}C-arginine is hydrolyzed into ^{14}C-urea and ^{14}C-urea urea is split into $2NH_3$ and $^{14}CO_2$ which is continuously trapped in isoosmotic KOH. The KOH is collected and the radioactivity counted in a liquid scintillation counter

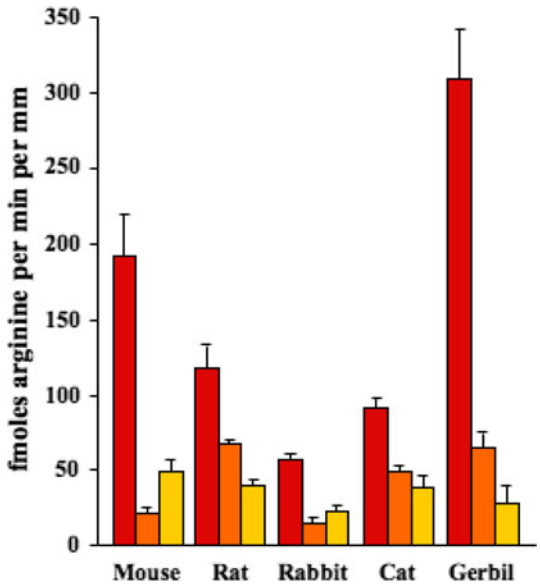

Fig. 3 Rate of arginine synthesis in dissected proximal tubules from five mammalian species. *Red bars* proximal convoluted tubules, *orange bars* cortical proximal straight tubules, and *yellow bars* outer medullary proximal straight tubules. The *bars* represent the mean arginine synthesis values ± SE ($n = 6$ mice and rats, 5 rabbits and cats, and 4 gerbils). Modified with permission (Levillain et al. 1990, 1993, 1996) (color figure online)

1995; Levillain et al. 1990, 1993, 1996), and low levels in mouse and rabbit. Moreover, arginine synthesis was heterogenously distributed within the PCT. The production of arginine was highest in the PCT portion closest to the glomerulus (S1) and dropped steeply in the next few millimeters of this tubule (Fig. 4) (Levillain et al. 1993).

The amounts of arginine produced by the S1–S3 subsegments of the proximal tubules vary from species to species. Per unit tubular length, murine PCT always exhibited the highest capacity to synthesize arginine, whereas rabbit PCT had the lowest (Fig. 3). Intermediate levels were found in the cat and rat PCT. Interestingly, isolated proximal tubules of cat incubated with 108 μM citrulline produced significant amounts of arginine (Levillain et al. 1996). Although to our knowledge, neither ASS nor ASL activities have been reported in cat kidney, this result strongly suggests that feline proximal tubular cells express both ASS and ASL at a comparable level as rat kidney. Given that renal arginine production mainly depends on the arterial citrulline concentration (Bouby et al. 1993; Dhanakoti et al. 1990; Levillain et al. 1990) and that the plasma citrulline concentration in the cat is extremely low (6–10 μM) (Levillain et al. 1996), one can expect that the cat kidney produces only small amounts of arginine. The very small increase in arginine concentration in the renal vein compared to the renal artery (Levillain et al. 1996) supports this prediction.

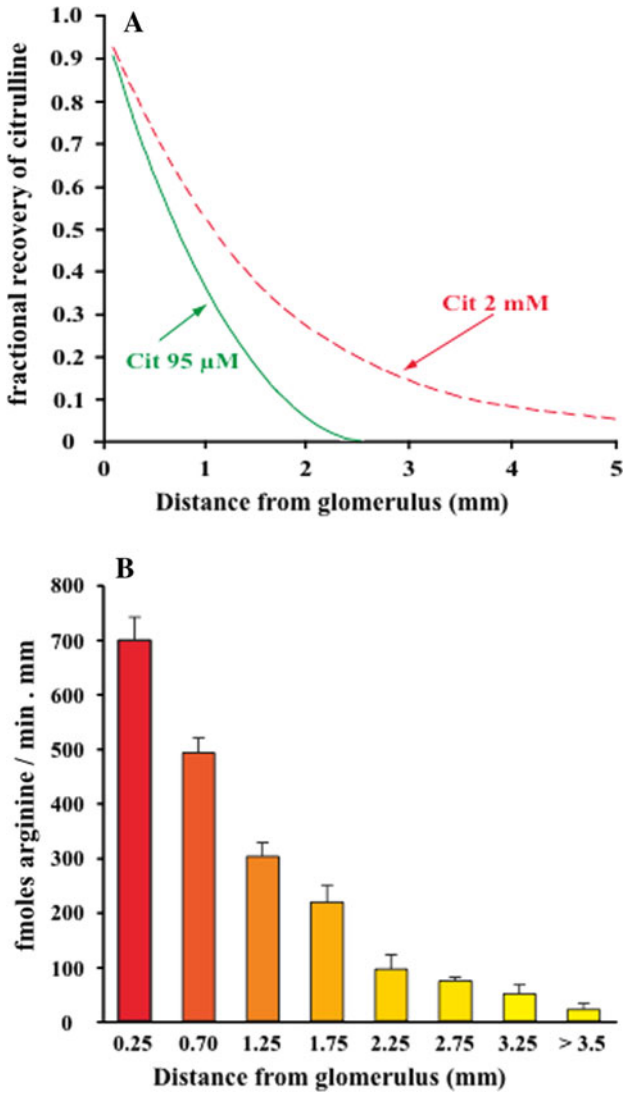

Fig. 4 Citrulline reabsorption and arginine synthesis along the mouse proximal convoluted tubules. **a** Predicted concentration profiles of citrulline along the proximal convoluted tubule with two plasma concentrations. The physiological plasma concentration of citrulline is approximately 95 μM in rats and mice. Modified with permission (Kettner and Silbernagl 1985). **b** Arginine synthesis along the mouse proximal convoluted tubules. The longest proximal convoluted tubules were collected, subdivided into successive sub-segments of 0.5–1 mm, and incubated individually to quantify their capacity to produce arginine. The *bars* represent the mean arginine synthesis values ± SE. Modified with permission (Levillain et al. 1993)

The constant rate of hydrolysis of arginine due to exogenous arginase in our experimental closed chamber model (Fig. 2) suggests that arginine is transported out of the tubular cells by basolateral carriers. This observation supports the idea that, in vivo, the newly synthesized arginine is transported out of the cells from the basolateral side rather than the apical side to facilitate its entry into the blood stream. The renal vascular anatomy facilitates the recycling of solutes that are filtered and/or metabolized within the PCT. Indeed, the efferent arterioles feed a dense capillary network that surrounds the PCT and allows arginine to enter the blood stream and become available for the other organs of the body (Dhanakoti et al. 1990; Perez et al. 1978).

Precise localization of ASS and ASL proteins and mRNAs in the kidney

The first attempt to localize ASS and ASL protein in kidney by immunohistochemistry was carried out in young adult female Swiss-Webster mice. ASS and ASL were detected in the proximal tubules and colocalized with γ-glutamyltranspeptidase (γ-GTP), a marker of these nephron segments (Morris et al. 1991). Immunoreactivity for these enzymes was more intense near the luminal membrane of the proximal tubule. The renal expression of ASS and ASL appeared at 15 days of gestation and increased throughout fetal and postnatal development (Morris et al. 1991). The selective expression of ASS and ASL in the proximal tubule was confirmed in male Wistar rat kidneys (Miyanaka et al. 1998). As expected, ASS and ASL immunoreactivities were strong in the cortex, much lower in the medulla, and absent in the papilla and

glomeruli. However, it is noteworthy that a faint labeling of ASL was also present in the medulla and papilla. The whole cytoplasm stained diffusely for ASS. Staining for ASS was the most intense in the portion of the proximal tubules that connect directly to the glomerulus (Miyanaka et al. 1998). The high concentration of ASS in the initial portion of the proximal tubule explains why this portion of the nephron produces the highest amount of arginine (Hus-Citharel et al. 1995; Levillain et al. 1990, 1993).

The production of a highly specific rabbit antibody against amino acids 196–222 of murine hepatic ASS (Schmidlin et al. 1997) allowed our group to localize ASS protein by indirect immunofluorescence. Briefly, in both male and female rats, the PCT stained intensely, while the CPST in the medullary rays stained weakly (Fig. 5). A more detailed analysis of the fluorescent staining revealed uneven cellular distribution of that ASS protein. Although it was present throughout the cells of the PCT, the strongest staining was near the apical membrane (Levillain and Wiesinger 2011). These results are in a good agreement with those of Miyanaka et al. (1998).

The renal subcellular localization of ASS and ASL was studied in isolated PCTs that were purified on a Percoll gradient. Specific markers of the nuclear, mitochondrial, microsomal, and cytosolic fractions were used. The relative specific activities of ASS and ASL followed the distribution of lactate dehydrogenase (LDH), demonstrating that ASS and ASL are cytosolic enzymes (Dhanakoti et al. 1992). If proteins in the cytosolic fraction of rat kidney cortex or medulla were, instead, separated by two-dimensional electropheresis and identified by mass spectrometry, ASS was found in a severalfold higher concentration in the cortex than in the medulla (Witzmann et al. 1998). Recently, we analyzed the renal expression of ASS and ASL in dog cortex (Levillain et al. 2008). The cytosolic and mitochondrial fractions were isolated and purified by differential centrifugation and proteins were analyzed by Western blot. Aconitase (EC 4.2.1.3), ornithine aminotransferase (OAT, EC 2.6.1.13), α-F1-ATPase, and arginase type II (EC 3.5.3.1) were used as mitochondrial markers. The results clearly demonstrated that both ASS and ASL proteins are present exclusively in the cytosolic fraction (Fig. 6).

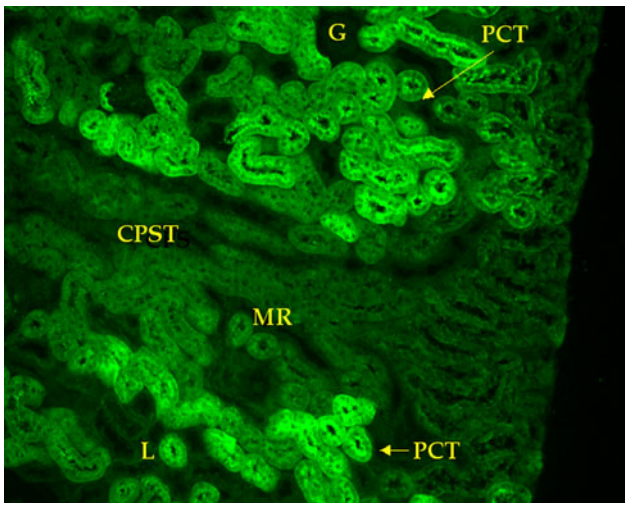

Fig. 5 Localization of argininosuccinate synthetase in the cortex of the rat kidney. Kidney sections of thickness 4–7 μm were prepared. Nonspecific sites were blocked with PBS-Triton-BSA, incubated with rabbit anti-ASS primary antibody (dilution 1:100), rinsed, and then incubated with the Alexa fluor 488-conjugated goat anti-rabbit IgG. *G* glomerulus, *PCT* proximal convoluted tubule, *CPST* cortical proximal straight tubule, *MR* medullary rays, *L* labyrinth (magnification ×50)

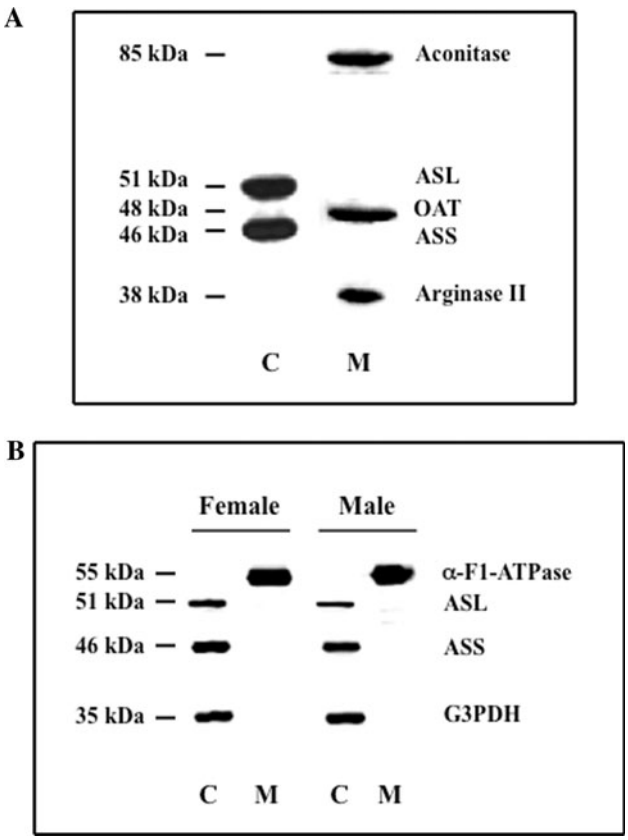

Fig. 6 The subcellular distribution of argininosuccinate synthetase, argininosuccinate lyase, and arginase II proteins in the cortex of dog and rat kidneys. Western blots of dog (**a**) and rat (**b**) extracts. The mitochondrial fraction was obtained by differential centrifugation at 12,000*g* and the cytosolic fraction at 128,0000*g*. Proteins were separated for SDS–PAGE. *C* cytoplasm, *M* mitochondria, *ASS* argininosuccinate synthetase, *ASL* argininosuccinate lyase, *OAT* ornithine aminotransferase, and *G3PDH* glyceraldehyde-3-phosphate dehydrogenase. Aconitase, OAT, and α-F1-ATPase were used as mitochondrial markers. G3PDH was used as a marker of the cytosolic fraction. The molecular size of each protein is given on the left. **a** Reproduced with modification and permission (Levillain et al. 2008)

Sources of citrulline in the kidney

The complete dependence of renal arginine synthesis on exogenous citrulline was demonstrated in vivo, in isolated PCT (Levillain et al. 1990) and by measuring the arterio-venous concentration differences of citrulline and arginine in the renal vessels (Dhanakoti et al. 1990). The source of citrulline remained an open question for a long time because it proved impossible to synthesize citrulline from ornithine, bicarbonate, and ammonia in renal cells, slices, or tubules. It is now well established that the kidney does not express the necessary mitochondrial enzymes *N*-acetyl-glutamate synthase (N-AGS, EC 2.3.1.1), carbamylphosphate synthetase I (CPS-I, EC 6.3.4.16), and ornithine transcarbamylase (OTC, EC 2.1.3.3) (Caldovic et al. 2002; Mizutani 1968) for this route of citrulline synthesis. The arterio-venous changes in the concentration of citrulline and arginine strongly suggest that the blood stream is an important source of citrulline for the kidneys. The main source of plasma citrulline are the enterocytes of the small intestine (Windmueller and Spaeth 1974).

In the last 20 years, it has become clear that citrulline can also be produced from arginine by nitric oxide synthase (NOS) and from mono- and dimethylarginine by dimethylarginine dimethylaminohydrolase (DDAH) (Palm et al. 2007). However, as we will show, the quantitative importance of these "new" sources of citrulline, relative to the enteral synthesis of citrulline, is small.

Nitric oxide synthase (NOS, EC 1.14.13.39) metabolizes arginine to citrulline and nitric oxide (NO) (Moncada et al. 1989). The newly produced citrulline can be recycled to arginine, if ASS and ASL are co-expressed with NOS and aspartate is available. All three NOS isoenzymes (NOS1-3) are expressed in the kidney. For further details, the reader is invited to refer to reviews focused on this subject.

The second pathway that can mediate renal production of citrulline originates in protein catabolism. Methylation on the guanidino nitrogen of arginine residues in proteins produces *N*G-monomethylarginine (NMMA), *N*G,*N*G-dimethylarginine (asymmetrical dimethylarginine: ADMA), and *N*G,*N*′G-dimethylarginine (symmetrical dimethylarginine: SDMA) (Kakimoto and Akazawa 1970). These arginine derivatives are released after proteolysis and were first identified in human urine (Kakimoto and Akazawa 1970). When rats were injected with [^{14}C]-ADMA, radioactivity was recovered mainly in the form of citrulline in tissue samples of the kidney and the pancreas (Ogawa et al. 1987b). The responsible enzyme, *N*G,*N*G-dimethylarginine dimethylaminohydrolase (DDAH, EC 3.5.3.18), was purified from rat kidney (Ogawa et al. 1987a). DDAH consists of a single polypeptide [$M_W \approx 32$ kDa, isoelectric point (pI): 5.2, maximum activity at pH 6.5] and metabolizes either ADMA or MMA into L-citrulline and dimethylamine or monomethylamine, respectively (Ogawa et al. 1989). The K_M values are 180 μM for ADMA and 360 μM for MMA. DDAH is expressed in kidney, pancreas, liver, and brain of rats (Ogawa et al. 1989) and humans (Kimoto et al. 1995; Leiper et al. 1999). DDAH was cloned from rat kidney (Kimoto et al. 1997) and human liver (Kimoto et al. 1998). Because DDAH activity and DDAH protein expression did not always correlate, a new isoform was searched for and found. DDAH II is expressed predominantly in heart, kidney, placenta, pancreas, lung, but also in skeletal muscles, liver, immune cells, and brain (Leiper et al. 1999; Palm et al. 2007). Accession numbers of DDAH I and DDAH II and their related products are given in Supplemental Table 1.

Because the kidney expresses the highest level of DDAH I and DDAH II, the hypothesis that these enzymes supplied citrulline for arginine synthesis was investigated. Immunostaining demonstrated DDAH I in the apical vesicles of PCT cells and in the cytoplasm of PST cells, while the brush border membrane, mitochondria, and nuclei remained unstained (Tojo et al. 1997). When the distribution of DDAH I and DDAH II was directly compared, the highest concentration of DDAH I was found in the cytoplasm of the OSPST (S3) tubular cells, followed by that of the PCT (S1 and S2) cells (Onozato et al. 2008). In contrast, DDAH II was expressed in vascular structures, thick ascending limbs, DCT, cortical and inner medullar CDs, and macula densa, but not in proximal tubules (Onozato et al. 2008). The high concentration of DDAH I in S3 segments may locally enhance the concentration of citrulline and facilitate the production of arginine. However, in addition to local enzyme concentration, substrate supply is important. Plasma levels of ADMA in healthy humans, mice, and rats are approximately 0.4, 1.2, and 0.7 μM, respectively (Al Banchaabouchi et al. 2000; Marescau et al. 1997; Tran et al. 2003). The intracellular concentration of ADMA in mouse and rat renal cortex is estimated to be about tenfold higher than in plasma (Al Banchaabouchi et al. 2000). The substrate data therefore indicate that the contribution of DDAH in supplying citrulline for arginine synthesis is modest at best.

Citrulline was first detected in blood in 1944 (Archibald 1944), but did not generate much interest until Windmueller and Spaeth (1974, 1978, 1980) observed that approximately 5% of the glutamine that was taken up by the intestine was converted into citrulline. The use of labeled precursors subsequently confirmed a direct conversion of glutamine into citrulline and of citrulline into arginine (Boelens et al. 2005). Furthermore, the enterocytes of the small intestine were identified as the primary site of citrulline synthesis in the body (Windmueller and Spaeth 1974, 1975, 1978, 1981). Glutamine uptake was also found in the intestine of dog, cat, hamster, and monkey, but not in rabbit and guinea pig (Windmueller and Spaeth 1974).

Physiological experiments underscored the essential role of the intestine in citrulline synthesis. The administration (via the drinking water) of the powerful and specific OTC inhibitor Gly-Gly-PALO [*δ*-*N*-(phosphonacetyl)-L-ornithine] in rats fed an arginine-free diet blocked citrulline synthesis in the enterocytes and caused growth retardation and a 1.9- and 4.6-fold decrease in serum citrulline and arginine concentration, respectively (Hoogenraad et al. 1979, 1985; Mori et al. 1977). The addition of 1% citrulline restored serum citrulline and arginine levels and completely prevented growth inhibition by PALO (Hoogenraad et al. 1985).

Partial resection of the small intestine also demonstrated the fundamental role of intestine in citrullinogenesis and its contribution to renal arginine synthesis. Circulating plasma citrulline levels declined commensurate with an increasing length of resection, with resection of proximal parts having bigger impact than resection of distal parts. Feeding an arginine-free diet further aggravated the effect (Wakabayashi et al. 1995; Windmueller and Spaeth 1981), whereas supplementation of citrulline compensated for the loss of intestinal function (Osowska et al. 2004). In human patients with destructive proximal and distal small-intestinal mucosal lesions, plasma citrulline concentration was threefold lower than in patients with only proximal lesions (Crenn et al. 2003). Plasma citrulline concentration is now considered as a simple and reliable marker of functional enterocyte mass and serves as a quantitative parameter of intestinal functionality after, e.g., and small bowel transplantation (Crenn et al. 2003; Curis et al. 2005, 2007).

The intestinal capacity to synthesize citrulline varies according to species. The cat intestine cannot metabolize glutamate to ornithine and citrulline because of the very low expression of ornithine aminotransferase (OAT) and pyrroline-5-carboxylate synthetase (P5C-S) (Baker and Czarnecki-Maulden 1991; Rogers and Phang 1985). As a result, cats have a very low circulating concentration of citrulline (6–10 μM), or approximately 10% of the circulating citrulline concentration in rats (Levillain et al. 1996), mice (Cremades et al. 2004; Hallemeesch et al. 2001; Luiking et al. 2004), dogs (Levillain et al. 2008), and rabbits (Levillain et al. 1996). Consequently, arginine is an essential amino acid for cats and other strict carnivores (Morris and Rogers 1978), but supplementation with citrulline can replace arginine (Anderson et al. 1979; Morris et al. 1979; Morris and Rogers 1978). Chickens (*Gallus gallus domesticus*) resemble carnivores in this respect (Wu et al. 1995). Enterocytes of chickens express neither P5C-S nor OTC, while glutaminase and OAT activities represent only 17 and 3% of that found in pig enterocytes (Wu et al. 1995). Although recently, CPS-I, OTC as well as other enzymes of the ornithine cycle have been recently assigned to chicken chromosomes, it remains to be examined whether these genes function together in a physiological process (Shimogiri et al. 2004).

Enterocytes produce and release citrulline into the venous blood. Citrulline transport across the rat intestine increases by threefold from the pyloric end towards ileocaecal junction (Vadgama and Evered 1992). It is commonly stated that citrulline either does not or poorly enters the hepatocytes, so that the bulk of intestinal citrulline remains available for the kidney (Windmueller and Spaeth 1981). However, the reality is more complex (Curis et al. 2007). In cats, blood citrulline concentration slightly increases from the portal to the hepatic veins (Levillain et al. 1996), whereas a small net uptake of citrulline by the liver was reported in other animals (Windmueller and Spaeth 1981).

The kidney is the main site of citrulline uptake from the circulation. More than 80% of the citrulline produced by the intestine is taken up by kidneys (Cohen and Hayano 1946; Windmueller and Spaeth 1981). Using in vivo microperfusion of rat PCTs with different concentrations of [^{14}C]-citrulline (Kettner and Silbernagl 1985), reabsorption of citrulline into tubular cells proved the existence of unidirectional and saturable transport systems. The identity of the transporter remains unknown, although the inhibition of citrulline uptake by phenylalanine suggested involvement of a neutral amino-acid carrier (Kettner and Silbernagl 1985). If present at physiological concentrations, citrulline was reabsorbed within the first 2–3 mm of the PCT (S1) (Fig. 4). However, if the tubular load of citrulline was increased, the late PCT and PST also participated in citrulline reabsorption (Fig. 4) (Kettner and Silbernagl 1985). These data show that the highest rates of citrulline reabsorption and arginine synthesis co-localize in the same portion of the PCT. In addition, a peritubular uptake of citrulline has been characterized in *Xenopus laevis* and strongly resembles that of the luminal uptake of citrulline in rat kidney (Gekle and Silbernagl 1991; Kettner and Silbernagl 1985). It is highly likely that such a peritubular uptake of citrulline also occurred in our metabolic chamber when citrulline was added to the incubating medium to quantify arginine synthesis in isolated tubules (Fig. 2) (Levillain et al. 1990, 1993). Therefore, both apical and basolateral carriers of citrulline, together with ASS and ASL, optimize the renal synthesis of arginine.

In 1973, Featherston et al. showed that intravenously injected [^{14}C-ureido]-citrulline in rats was consumed by the kidney and that [^{14}C-guanidino]-arginine was produced and incorporated into proteins (Featherston et al. 1973; Perez et al. 1978). When the kidney was excluded from the circulation, plasma citrulline levels increased and the recovery of arginine in protein dropped dramatically, but when the liver was excluded from circulation, intravenous citrulline still enabled the production of arginine and its

incorporation into proteins (Featherston et al. 1973). Several groups have since quantified renal citrulline–arginine metabolism and reported a net renal uptake of citrulline from the blood and a net release of arginine in rats (Bouby et al. 1993; Dhanakoti et al. 1990; Hallemeesch et al. 2001; Luiking et al. 2004; Prins et al. 2002), mice (Boelens et al. 2005), and dogs (Levillain et al. 2008; Yu et al. 1996). As expected, the molar relationship was 1:1. The near 1:1 stoichiometry between citrulline uptake and arginine release strengthens the model that arginine that is synthesized in the kidney is primarily used for export to extrarenal tissues rather than for intrarenal utilization.

Metabolic fate of arginine in the kidney

A small, but significant amount of arginine (approx. 10%) is, nevertheless, metabolized within the proximal tubular cells (Funahashi et al. 1981). This arginine is either transamidinated by arginine:glycine amidinotransferase (GAT, EC 2.1.4.1), hydrolyzed by arginase type II (AII, EC 3.5.3.1), or metabolized by arginine decarboxylase (ADC, EC 4.1.1.19) or NOS (Fig. 7).

Arginine:glycine amidinotransferase

GAT uses arginine as a donor to transamidinate glycine and form guanidinoacetic acid and ornithine (Perez et al. 1978). The GAT gene encodes a 423 amino-acid protein with a *N*-terminal mitochondrial targeting peptide of 37 amino acid residues (4.2 kDa) that targets the protein to the inner mitochondrial membrane. The subcellular localization of GAT was confirmed experimentally (Magri et al. 1975). Native GAT is a dimer in rats (McGuire et al. 1980; Gross et al. 1988), pigs (Conconi and Grazi 1965), and humans (Gross et al. 1986). The M_W of the native GAT polypeptide is approx. 48 kDa. However, in rat kidney, two GATs (α- and β-GAT) with M_Ws of approx. 44 and 42 kDa and pIs between 7.0–7.2 and 6.9–7.3, respectively, and a similar substrate affinity were found (McGuire et al. 1980). Accession numbers of the GAT and its related products are given in Supplemental Table 1.

In rat and human, GAT is highly expressed in the kidney, pancreas and, to a lesser extent, in brain, liver, spleen, and testes (Van Pilsum et al. 1972). In adult male rat kidney, GAT activity is 60% higher than in female kidney, because renal GAT activity decreases in female rats after puberty (Tormanen and Sutter 1985). In mice, the highest GAT activity is also found in pancreas and kidney, followed by brain, lung, testis and in a lesser extent skeletal muscle and heart (David and Reddy 1986). GAT protein is only found in the renal cortex, with most prominent staining seen in the PCTs (McGuire et al. 1986). Using a micromeasurement of GAT activity in pooled isolated nephron segments, guanidinoacetic acid, the product of the reaction, was only detected in PCTs and PSTs, with a fourfold higher activity in PCTs than PSTs (Takeda et al. 1992).

The PCT is the site of reabsorption of citrulline, arginine, and glycine. Most of the ASS and ASL activity is also found in this nephron segment. Despite a relatively high concentration of substrates for GAT, only a limited fraction of arginine (approx. 10%) is converted into guanidinoacetic acid. This paradox has been explained by the mitochondrial localization of GAT compared to the cytosolic expression of ASS and ASL. The guanidinoacetic acid produced in the proximal tubule is released into the blood stream because the kidney does not express the guanidinoacetate *N*-methyltransferase (GAMT, EC 2.1.1.2) needed to catalyze the formation of creatine from guanidinoacetate and *S*-adenosylmethionine (Brosnan and Brosnan 2004).

Arginase II

Kochakian et al. (1940) first reported arginase activity in mouse kidney. Since that time, extra-hepatic arginases were characterized and cloned in mouse (Iyer et al. 1998; Shi et al. 1998), human (Gotoh et al. 1996; Morris et al. 1997), rats (Iyer et al. 1998; Gotoh et al. 1996), and other species (for a review, see Jenkinson et al. 1996). The AII gene encodes a 354 amino acid protein with a *N*-terminal mitochondrial targeting peptide of 22 amino acid residues (2.5 kDa) that targets the protein to the mitochondria (Iyer et al. 1998; Gotoh et al. 1996; Shi et al. 1998). In the kidney, AII has been localized in the mitochondrial matrix of rats, dogs, and mice (Levillain et al. 2005a, b, 2008; Skrzypek-Osiecka et al. 1983; Spector et al. 1994). The predicted M_W of the native AII polypeptide is about 39 kDa. Accession numbers of the AII and its related products are given in Supplemental Table 1.

AII activity was measured in six zones of sheep, goat, dog, and rat kidneys (Robinson and Schmidt-Nielsen 1963). In sheep, AII activity increased from the outer cortex to the papilla. In goat, AII activity was lower in the outer medulla than in the other zones. In dog, almost all AII was found in the cortex, with 63% in the outer cortex. Immunohistochemical localization of AII in male rat kidney (Miyanaka et al. 1998) revealed a strong staining in the medullar rays, especially in the OSPSTs. In the same study, the distribution of ASS and ASL proteins along the proximal tubule has been shown to be complementary with distribution of AII (Miyanaka et al. 1998), demonstrating that arginine synthesis and degradation do not occur in the same cell. These results were confirmed by in situ hybridization of AII performed in the mouse kidney (Yu et al. 2003).

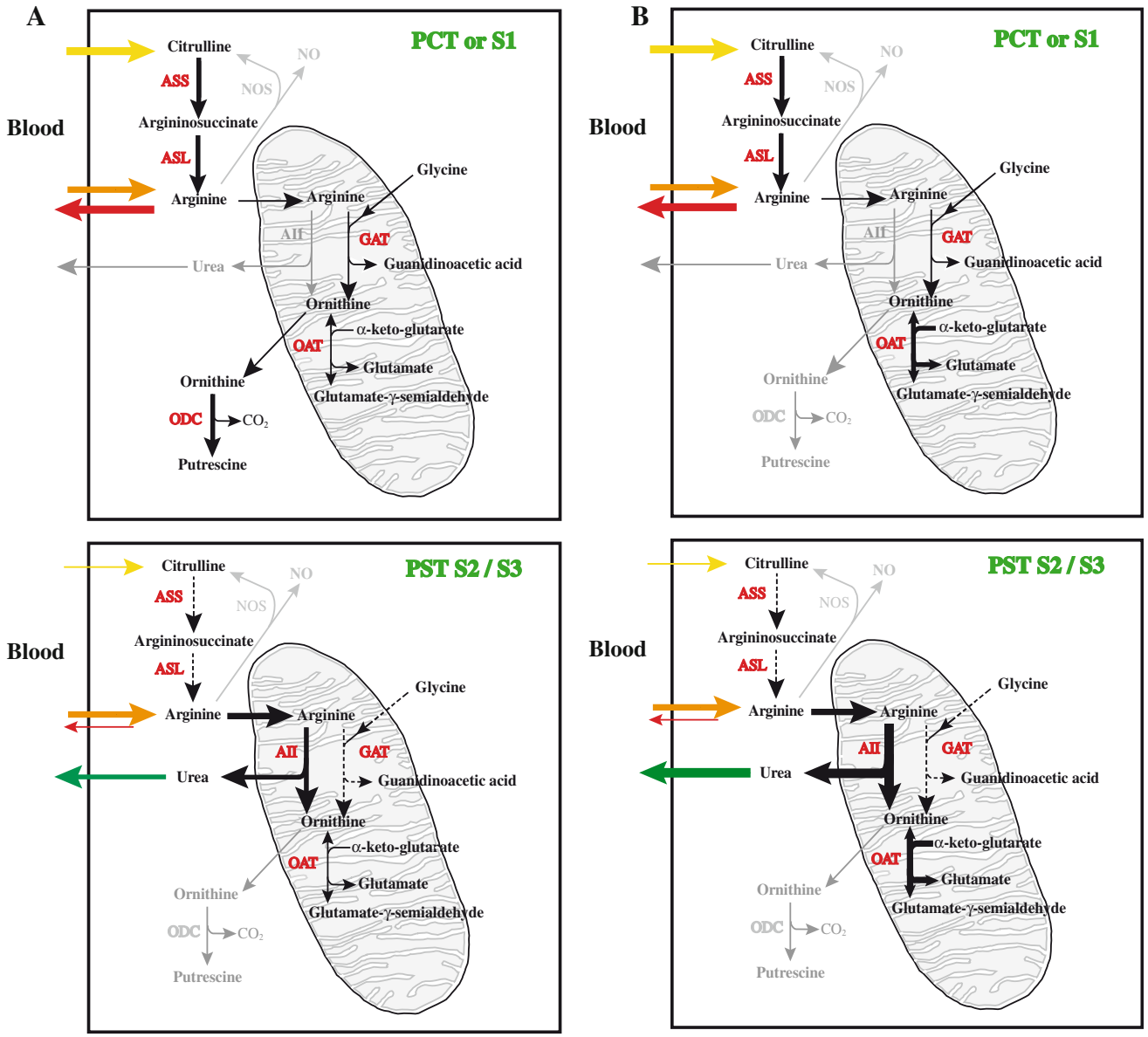

Fig. 7 Expression and localization of arginine-producing and consuming-enzymes along the proximal tubule of the mouse. **a** Male and **b** female. In the S1 cell type of the early proximal convoluted tubule (*PCT*), argininosuccinate synthetase (*ASS*) and lyase (*ASL*) are highly expressed and produce large amounts of arginine (*thick arrow*) from filtered citrulline (*medium arrow*). A small fraction of arginine is metabolized by arginine:glycine amidinotransferase (*GAT, thin arrow*). Note that arginase II is not expressed. In the S2 and S3 cell types of the proximal straight tubules, on the other hand, ASS and ASL are hardly expressed (*dotted line*) and arginine originates mainly from the blood. This arginine is catabolized by arginase II (*AII*) which is highly expressed in these cell types (*thick arrow*). Urea is released from the cells. In contrast, GAT activity is barely or not expressed (*dashed arrow*). Pathways coded in grey are not expressed under control physiological conditions. The thickness of the *arrow/line* represents the intensity of an enzymatic activity. Cytosolic ornithine decarboxylase (*ODC*) is expressed only in the male mouse PCT (**a**). Mitochondrial ornithine aminotransferase (*OAT*) is more highly expressed in the PCT and PST of the female (**b**) than the male mouse (**a**). Mitochondrial arginase II (*AII*) is more highly expressed in PST of the female (**b**) than the male mouse (**a**)

We developed a radiolabeled enzymatic micromethod to quantify urea production in single or pooled microdissected nephron segments (Fig. 2). If AII is expressed in tubular cells, [^{14}C-guanidino]-arginine is hydrolyzed into ornithine and ^{14}C-urea and quantified as detailed in "Identification of the renal tubules involved in arginine production" (Fig. 2) (Levillain et al. 1989). The technique is specific, reproducible, and sufficiently sensitive to measure arginase activity in less than 0.5 mm PCT tubules of rat, mouse, rabbit, cat, guinea pig, and gerbil (Fig. 8). AII activity increased from the CPST to the OSPST, where it was highest in all species (Fig. 8) (Hus-Citharel et al. 1995;

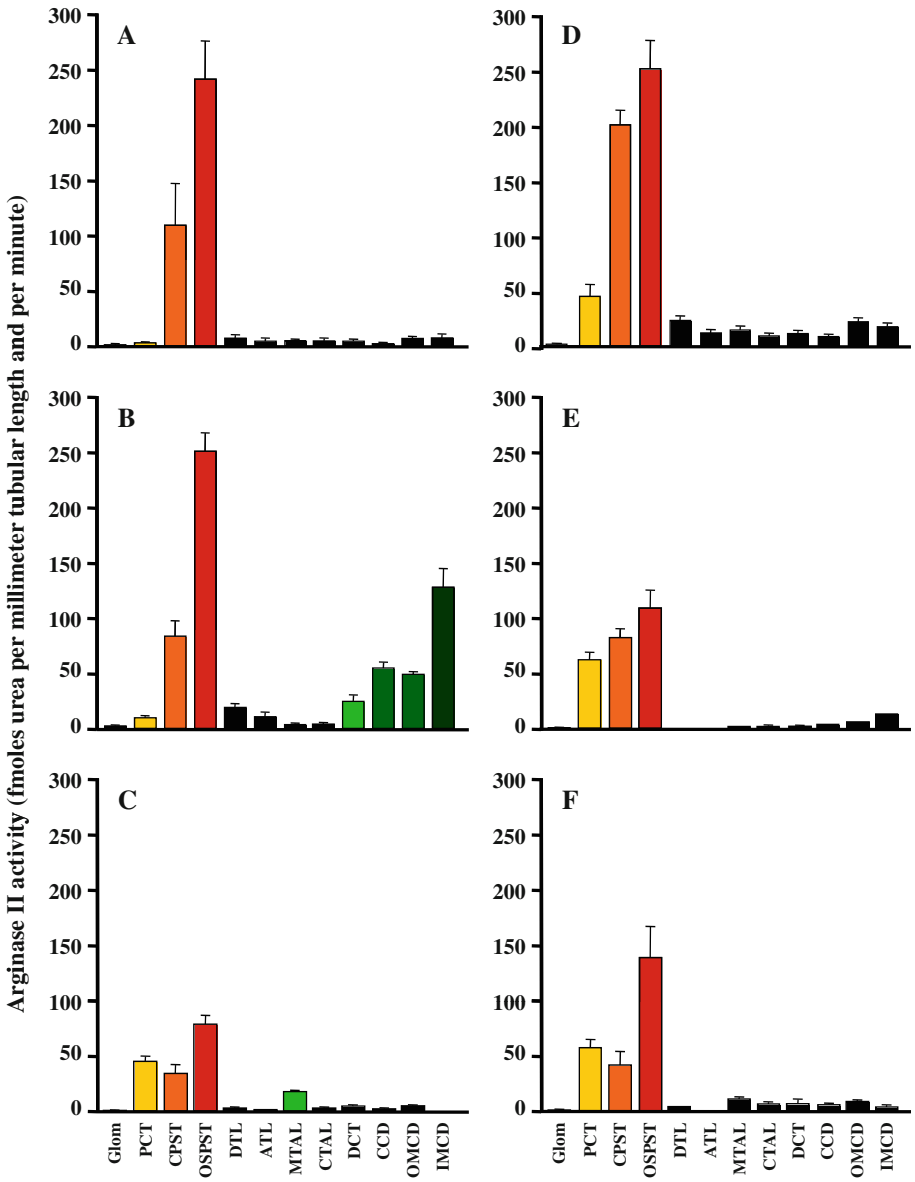

Fig. 8 Distribution of arginase II and production of urea from arginine along the nephron of six mammalian species. **a** Mouse, **b** rat, **c** rabbit, **d** gerbil, **e** cat, and **f** guinea pig. Viable tubules were microdissected and incubated with 216 μM L-[^{14}C-guanidino]-arginine to quantify arginase II activity. *Glom* glomerulus, *PCT* proximal convoluted tubule, *CPST* cortical proximal straight tubule, *OSPST* outer medullary proximal straight tubule, *DTL* descending thin limb, *ATL* ascending thin limb, *MTAL* medullary thick ascending limb, *CTAL* cortical thick ascending limb, *DCT* distal convoluted tubules, *CCD* cortical collecting duct, *OMCD* outer medullary collecting duct, *IMCD* inner medullary collecting duct. The *bars* represent the mean ± SE urea production per mm tubule per min, $n = 9$ rats, 6 gerbils, and 5 mice, rabbits, guinea pigs, and cats. Reproduced with modification and permission (Hus-Citharel et al. 1995; Levillain et al. 1990, 1992, 1996)

Levillain et al. 1989, 1992, 1996, 2005a). Rabbit (Levillain et al. 1992), gerbil (Hus-Citharel et al. 1995), cat (Levillain et al. 1996), and guinea pig PCT exhibited a high AII activity compared to those of the rat and mouse (Fig. 8). Assuming that arginine uptake is similar in the three subsegments of the proximal tubule of the respective animal species, AII activity is clearly higher in rat, mouse, and gerbil than in cat, guinea pig, and rabbit. The rat CDs express a high AII activity, which increases from the cortex to the inner medulla (Levillain et al. 1989, 2005b). In rabbits, medullary thick ascending limbs express AII (Levillain et al. 1992). All tubules of the Henle's loop, distal tubule, and CDs of the gerbil exhibit a low but significant amount of AII activity (Hus-Citharel et al. 1995). AII was also found in a suspension of cortical proximal tubules isolated from the dog kidney (Levillain et al. 2008).

We finally have determined the fraction of the newly synthesized arginine that is immediately hydrolyzed by AII in proximal tubular cells. Tubules were incubated with [^{14}C-ureido]-citrulline and commercial urease was added in the incubation medium (Fig. 2). In PCTs of rat, mouse, rabbit, and gerbil, only 3.4, <1, 8, and 0.008%, respectively, of newly synthesized arginine was hydrolyzed by AII (Hus-Citharel et al. 1995; Levillain et al. 1990, 1993). These experiments support that almost all arginine produced in PCT is released into the blood stream and becomes available for absorption by other tissues. The high percentage of arginine hydrolyzed in rabbit tubules is explained by a high AII activity (Levillain et al. 1992). In the PST of rat, mouse, rabbit, and gerbil, AII activity is high and increases from the cortex to the medulla. In agreement with this, 40, 66, 39, and 65% of arginine are

immediately hydrolyzed in the CPST (S2) of these four species. In the OSPST (S3) of the same species, 64, 81, 68, and 66% of newly formed arginine is cleaved by AII. Although AII is located into the mitochondria (Fig. 6), its efficiency to catabolize arginine seems greater than that of GAT (Fig. 7). The coexpression of ASS, ASL, and AII in the PST strongly suggests that ornithine and/or urea are necessary for other physiological events in this nephron segment. It should be recalled that the highest levels of ASS and ASL proteins are present in the early portion of the PCT, whereas the reverse is true for AII (Figs. 3, 4, 7, and 8) (Morel et al. 1996). The spatial dissociation of the arginine anabolizing and catabolizing enzymes along the proximal tubule constitutes an excellent protective system against futile cycling of arginine (Fig. 7).

Arginine decarboxylase

A decade and a half ago, ADC cDNA was cloned from the cortex of the rat kidney (Morrissey et al. 1995). The nucleotide sequence of the rat ADC shares significant similarity with that of *E. coli*, *Azotobacter vinelandii* and *Pseudomonas sp.* (Coleman et al. 2004). ADC enzymatic activity was found only in the cortex, OS, and IS of the rat kidney and was associated with the mitochondrial fraction (Morrissey et al. 1995). Another group measured ADC activity in homogenized rat tissues using 1-[^{14}C]-arginine as a substrate (Lortie et al. 1996). However, this protocole mixed the renal AII, GAT, OAT, and ornithine decarboxylase (ODC, EC 4.1.1.17) activities leading to arginine decarboxylation which may simulate an arginine decarboxylase activity. In contrast, our group compared both 1-[^{14}C]-arginine and 1-[^{14}C]-ornithine decarboxylation in the presence or the absence of specific inactivators (DFMO and DFMA) in isolated mouse nephron segments (Soulet et al. 1997). Although monitoring $^{14}CO_2$ production suggested arginine decarboxylation, no ADC activity could be found. In male mice, we proposed that GAT catabolized arginine to ornithine which was further decarboxylated by ODC. In female mice, this ornithine was catabolized by OAT since ODC is not expressed (Soulet et al. 1997). The inability of hepatic and renal mitochondria to produce agmatine confirmed the absence of hepatic and renal ADC in rats and mice (Coleman et al. 2004).

Nitric oxide synthetase

The constitutive or endothelial (cNOS or eNOS), neuronal (nNOS) and inducible (iNOS) are expressed in the nephron and the vascular structures of the kidney. nNOS has been reported in the macula densa, Bowman's capsule and IMCD (Tojo et al. 1997). The expression of nNOS in the macula densa has been confirmed in mice, rats, rabbits, pigs, guinea pigs, and humans (Bachmann et al. 1995). eNOS was found in vascular endothelial cells and the glomerulus (Tojo et al. 1997). In the female rat kidney, iNOS mRNA is highly expressed in isolated MTAL and glomerulus. Low amounts of iNOS mRNA were found in the vasa recta bundle, OMCD, and cortical tubules whereas OSPST did not express iNOS. LPS induced a ten- and threefold increase in iNOS mRNA in glomeruli and outer medulla, respectively (Morrissey et al. 1994). In contrast, Tojo et al. (1997) reported the expression of iNOS in PCT, PST, DCT, TAL, CCD, OMDC, and IMCD of male rats. None of the three NOS activities could be measured in isolated tubules. Therefore, it is difficult to estimate the contribution of the NOS activity in catabolism of the newly synthesized arginine. Nevertheless, when iNOS expression is induced, larger amounts of arginine may be catabolized but also recycled.

Our assays demonstrate unequivocally that neither GAT nor AII can deplete the arginine formed in the PCT and that this segment is responsible for the production of arginine for the body. Consequently, this nephron segment is essential to prevent animals and humans from becoming arginine auxotrophs. Drugs and diseases that impair the proximal tubular cell functions and viability will therefore cause dramatic effects in these organisms.

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