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de Jonge, W.J.

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Arginine-metabolizing enzymes in the developing rat small intestine

Wouter J. de Jonge, Maria A. Dingemanse, Piet A.J. de Boer, Wouter H. Lamers* and Antoon F.M. Moorman

Department of Anatomy and Embryology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.
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Summary

Prior to weaning, arginine biosynthesis from citrulline most likely takes place in the small intestine rather than in the kidney. We studied the expression of ornithine cycle enzymes in the rat small intestine during perinatal development. The spatio-temporal patterns of expression of ornithine aminotransferase (OAT), carbamoylphosphate synthetase (CPS), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) mRNAs were studied by Northern-blot analysis and in situ hybridization. In addition, the expression of carbamoylphosphate synthetase and argininosuccinate synthetase protein was studied by immunohistochemistry. Before birth, the developmentally more mature proximal loops of the intestine expressed the mRNAs at higher concentrations than the more distal loops. After birth, this difference was no longer obvious. The mRNAs of argininosuccinate synthethase and argininosuccinate lyase, the enzymes that metabolize citrulline to arginine, were only detectable in the upper part of the villi, whereas the other mRNAs were concentrated in the crypts. The distribution of argininosuccinate synthetase protein corresponded with that of the mRNA, whereas carbamoylphosphate synthetase protein was present in all enterocytes of the crypts and villi. Hepatic arginase mRNA could not be detected in the enterocytes. The spatial distribution of the respective mRNAs and proteins along the villus axis of the suckling small intestine indicates that the basal enterocytes synthesize citrulline, whereas the enterocytes in the upper half of the villus synthesize arginine.
Abbreviations:
ARG [EC 3.5.3.1] : Arginase
ASL [EC 4.3.2.1] : Argininosuccinate lyase
ASS [EC 6.3.4.5] : Argininosuccinate synthetase
CPS [EC 6.3.4.16] : Carbamoylphosphate synthetase
OAT [EC 2.6.1.13] : Ornithine aminotransferase
OTC [EC 2.1.3.3] : Ornithine transcarbamoylase
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Introduction

Arginine, an intermediate metabolite of the ornithine cycle, is considered to be a dispensable amino acid for most healthy adult animals [1]. Renal arginine biosynthesis, the main source of endogenous arginine in the adult [2], does not vary with fluctuations in dietary arginine intake [3-5], but depends on the vascular supply of its precursor citrulline [6]. Intestinal citrulline synthesis from glutamine is, in turn, the main source of circulating citrulline [7,8]. The liver is not considered to contribute to circulating citrulline or arginine levels because of its high levels of ASS, ASL and ARG. In contrast to the situation in adults, arginine is regarded as a semi-essential nutritional compound in many adolescent animals, including rats, because it is synthesized by the kidney at rates that are inadequate to support rapid growth [9]. This discrepancy between the requirement for arginine and its endogenous biosynthetic capacity may even be aggravated in suckling rats, since the enzymes that convert citrulline to arginine in the kidney only rise to adult levels towards weaning [10,11]. Nevertheless, the finding that the ratio of the amino acid composition of rat body and milk is approximately 1 for essential amino acids, whereas it is more than 2 for arginine (and glycine) [12], clearly suggests a high degree of endogenous arginine biosynthesis during the suckling period.

The ornithine cycle enzymes except arginase are, expressed in the small intestine of suckling rats [7,10,13,14], but data on their cellular distribution are not yet available. By analysing the expression and localisation of ornithine-cycle enzyme mRNAs and protein by Northern blotting, in situ hybridization, and immunohistochemistry, we provide evidence that newborn rats have the capacity to synthesize citrulline and arginine at the base and the upper part of the villus of the small intestine, respectively.
Materials and Methods

Animals. Adult Wistar rats were obtained from the HSD animal farm in Zeist (The Netherlands). Timed matings were used for the study of fetal animals. The day of copulation was taken as day 0 of pregnancy (ED 0). Birth normally occurred at the beginning of day 22 of pregnancy. The animals were weaned at three weeks of age.

Preparation of tissue sections. Serial tissue sections were prepared exactly as described previously [15]. Transverse sections in the abdominal region of prenatal animals were used, while of postnatal animals the proximal part of the jejunum, approximately 5 cm distal to the ligament of Treitz, was dissected free and processed further.

cRNA probes. The following cDNA fragments were cloned into the pBluescript vector to generate cRNA probes for the detection of the different ornithine cycle mRNAs: carbamoylphosphate synthetase I (CPS), the 564 bp BamHI-Smal fragment of the rat cDNA clone pBR-CPS5 [16]; ornithine transcarbamoylase (OTC), the 870 bp Xbal-HindIII fragment from the rat cDNA clone pOTC1 [17]; argininosuccinate synthetase (ASS), the 1300 bp PstI fragment from the rat cDNA clone rAS [18]; argininosuccinate lyase (ASL), the 580 bp EcoRI-HindIII fragment from the rat cDNA clone pALr-3 [19]; arginase (ARG), the 768 bp PstI-StuI fragment from the rat liver cDNA clone pARGr-2 [20,21]; ornithine aminotransferase (OAT), the 1850 bp from the OAT cDNA clone pROLT10 [22]. Probes were prepared by in vitro transcription of the appropriate DNA strand and had a specific activity of 1.5 *10^9 cpm/μg. Onto each section 10 μL probe containing 40,000 cpm per μL was applied.

In situ hybridization. A comprehensive protocol to detect mRNA molecules in tissue sections using radioactively labelled cDNA probes has been described.
previously [15]. At variance with this protocol the temperature of hybridization and washes was raised to 55 °C as cRNA rather than cDNA was used.

Northern-Blot Analysis. Total RNA was isolated as described [23]. Ten micrograms of total RNA, denatured by heating for 10 minutes at 65 °C in the presence of 2.2 M formaldehyde, was electrophoresed on an agarose gel, containing formaldehyde. After transfer onto a nylon membrane (Amersham International plc., Little Chalfont, Buckinghamshire, U.K.), the RNA was hybridized to \( \alpha^{32}P \)-cytidine triphosphate (CTP)-labelled (Amersham) cDNA probes derived from the same clones used for the cRNA labelling in the in situ hybridizations. Hybridization was performed in 50% formamide, 5 times Denhart's solution (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin (fraction V)), 0.5% SDS and 5 times SSC (0.75 M NaCl, 75 mM Na\(_3\)Citrate). cDNA probes were labelled using the random primed labelling method. After washing, the blots were exposed to phosphor screens for approximately 15 hs, and signals were quantified using the Phosphorimager software (Molecular Dynamics, Sunny Vale, CA).

Immunohistochemistry. After deparaffination and rehydration, endogenous peroxidase activity in the sections was eliminated by incubation for 30 min in PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH=7.4) and 50 % methanol, containing 3 % (wt/vol) hydrogen peroxide. Non-specific protein binding sites were blocked by incubation for 30 min in TENG-T buffer (10mM Tris, 5 mM EDTA, 150 mM sodium chloride, 0.25 % gelatin, 0.05 % Tween-20, pH=8.0. Serial sections were incubated overnight with an appropriate dilution of rabbit polyclonal antibodies against CPS [24], and ASS [25,26]. The indirect unconjugated peroxidase-anti-peroxidase technique [27] was used to visualise binding of the primary antibodies, with 3,3-diaminobenzidine (0.5 mg/ml) as a substrate, dissolved in imidazole buffer (30 mM imidazole, 1 mM EDTA, pH 7.0), to which 0.01 % hydrogen peroxide was added.
Arginase activity measurements. Arginase activity was measured by a modification of the assay described by Adlung et al [28]. Fifty mg of tissue was homogenised in 2 mL of a 30 mM sucrose buffer containing 5 mM MnCl₂, 30 mM imidazole (pH 7.5), and 0.05 % Triton X-100. This homogenate was sonicated for 30 s and activated at 55 °C for 15 min. The mixture was diluted with 1 vol H₂O and centrifuged. The assay mixture (200 μL) contained 20 μL of the enzyme extract, 25 mM (guanido-¹⁴C)-labelled L-arginine (pH 9.5, 1.0 Ci.mol⁻¹; Dupont NEN), and 75 mM glycine (pH 9.5). The reaction was stopped after 6 min of incubation at 25 °C by addition of 100 μL 0.5 M L-arginine (pH 9.5) and 800 μL 30% (wt/vol) phosphotungstic acid (P₂O₅; Malinckrodt Baker, Deventer, The Netherlands). After centrifugation, 100 μL of the supernatant was counted.
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Results

Developmental changes in mRNAs. Figure 1 shows the developmental changes of CPS, OTC, ASS, ASL and OAT mRNA levels in the small intestine of the rat. The levels are expressed per µg of total RNA and relate to the corresponding mRNA levels in the adult liver, demonstrating that all mRNAs except ASL were highly expressed in the suckling small intestine. mRNA levels of all genes were highest during the suckling period. ASS and ASL mRNAs declined to hardly detectable levels in the second postnatal week, whereas mRNA levels of CPS and OAT declined to adult levels in the third postnatal week. OTC mRNA level did not change with development. However, it should be acknowledged that the mRNA levels were determined per µg of total RNA, so that our data do not take into account the increase in epithelial surface as a result of the growth of the intestine. The mRNA concentration of CPS and OAT was approximately twofold higher in the ileum than in the jejunum, whereas concentrations of the other mRNAs studied were approximately equal in both parts of the small intestine. After birth, the horizontal gradient was either no longer detectable (OTC, ASS, ASL), or had even become reversed, with the highest concentration in the distal (ileal) parts of the small intestine (Figure 1, CPS and OAT).

The cellular distribution of the mRNAs pre- and postnatally was investigated using in situ hybridization. The earliest stage investigated was ED 16. Even though the small intestine is still immature at this age, the enterocytes expressed CPS, GDH, ASS and OAT mRNAs, but not yet OTC and ASL (not shown). Two days later in development (Figure 2), the presence of intestinal villi could be observed in the proximal loops of the small intestine, but not yet in the distal ones, in line with the cranio-caudal gradient of development. The expression of CPS, OTC, ASS and OAT mRNAs was stronger in the more mature, proximal part of the small intestine, compared to the distal part, as can be seen from the difference between loops where intestinal villi are present and loops with a simple epithelial lining of the lumen (Figure 2). While the morphogenesis of the villi continued towards birth, the expression of CPS, OTC, ASS, and OAT mRNAs
remained essentially unchanged (shown for ASS and CPS, Figure 3). ASL mRNA was undetectable prenatally.
Figure 2. Expression of the mRNAs encoding CPS (a), OTC (b), ASS (c), ASL (d), ARG (e) and OAT (f) in transverse serial sections, showing the abdominal part of an ED18 rat fetus. The difference in development and expression of tissue-specific mRNAs between the proximal (pi) and distal (di) intestinal loops is very pronounced. Liver (li). Bar: 250 µm.

After birth, the developmental changes in the intensity of the hybridization signals in the proximal part of the small intestine (Figures 4, 5), reflected that of the Northern blots: the expression of CPS, ASS, ASL and OAT was highest at 7 days,
and had clearly declined at 17 days, whereas the expression of OTC remained fairly constant.

![Image of tissue sections showing expression of mRNAs encoding CPS and ASS](image)

**Figure 3.** Expression of the mRNAs encoding CPS (a) and ASS (b) in transverse serial sections of an ED20 rat fetus, showing liver (li) and proximal (pi) and distal (di) intestinal loops. Note that the expression of CPS mRNA is distributed along the entire villus, including its base, whereas ASS mRNA is absent from the villus-base. ASS mRNA is expressed in the enteric neurons of the myenteric plexus (arrowhead). Only very weak hybridization was seen in the distal parts of the intestine. Bar 100 μm.

In the adult jejunum CPS, OTC and OAT mRNAs were still expressed, but the expression of ASS and ASL mRNAs was no longer detectable, except in the neurons of the myenteric plexus (Figure 6). GDH mRNA continued to be expressed in the adult small intestine (not shown).

**Zonation of expression along the crypt-villus axis.** Heterogeneity along the axis of the villus evolved parallel with villus development. At ED 18 the expression of
CPS, OTC and OAT mRNAs was seen in the epithelium along the entire villus (Figure 2), whereas ASS levels were weaker at the intervillus region than at the upper part of the villi.

Figure 4. Expression of CPS (a), OTC (b), ASS (c), ASL (d), ARG (e) and OAT (f) mRNAs in serial sections of the jejunum of 7 day-old neonatal rats (ND7). The difference in distribution along the villus of CPS, OTC and OAT mRNAs on the one hand and ASS and ASL mRNA on the other is very pronounced. Note the silver grains in panel e, which were found not to be due to the presence of ARG mRNA (see text). Bar: 100 μm.
Figure 5. Expression of CPS (a), OTC (b), ASS (c), ASL (d), ARG (e) and OAT (f) mRNAs in serial sections of the jejunum at ND17 of rat development. Note the reduced expression of ASS and ASL mRNA compared to Figure 3. Bar: 100 μm.
Figure 6. Expression of CPS (a), OTC (b), ASS (c), ASL (d), ARG (e) and OAT (f) mRNAs in serial sections of the jejunum of an adult rat. CPS, OTC and OAT mRNAs have become confined to the crypts and lower part of the villus. Note the expression of ASS mRNA in the neurons in the myenteric plexus. Bar: 250 µm.

Figure 7. Presence of CPS (a, c, e, g, i) and ASS (h, d, f, h, j) protein in the rat jejunum of ED 20 (a, b), ND 1 (c, d), ND 8 (e, f), ND 18 (g, h) and adult (i, j). ASS protein is only present at the upper half of the villi, whereas CPS protein is seen in the developing crypts and the enterocytes along the entire villus. In the adult jejunum, ASS protein is only detected in the enteric neurons (arrowheads). Bar: 100 µm.
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At ED 20 the concentration of CPS, OTC and OAT mRNA was higher at the intervillus region, whereas ASS mRNA was absent from this part of the epithelium (shown for CPS and ASS, Figure 3). After birth, this difference became more pronounced: ASS and ASL were only detectable in the upper part of the villi, whereas CPS, OTC and OAT were present mainly in the basal part of the villi and in the developing crypts (Figures 3-5).

Antibodies against two key enzymes, CPS and ASS were applied to investigate to what extent mRNA and protein colocalize (Figure 7). Whereas CPS mRNA was concentrated at the basal part of the villi and in the developing crypts, CPS protein was present in all enterocytes. ASS protein, on the other hand, colocalized with ASS mRNA, and was confined to the upper part of the villi. ASS protein was, except for the enteric neurons, no longer detectable in the adult intestine, while CPS protein remained present.

Arginase. In order to demonstrate that the small intestine of the suckling rat indeed has the capacity to synthesize arginine, arginase activity should be absent. Arginase mRNA was undetectable at all ages investigated, both by in situ hybridization and Northern-blot analysis. The only exception was a highly reproducible in situ hybridization signal at ND 7 (Figure 4, panel e). This signal could not be eliminated by RNAase treatment of the section before hybridization (not shown).

Moreover, arginase mRNA was not detectable on Northern blots (Figure 8, panel a) and arginase activity was virtually undetectable in jejunal extracts before ND 18 (Figure 8, panel b). Therefore, the in situ hybridization signal observed at ND7 was judged to be non-specific. After ND18, arginase activity in the jejunum showed an abrupt increase. The adult values were found to be $8.7 \pm 0.5\%$ (n=5) of the activity measured in the adult rat liver.
Figure 8 panel a. Northern-blot analysis showing the absence of hepatic arginase mRNA in rat jejunum. The blot is representative for five separate experiments, run in parallel with those shown in Figure 1, panels a-e. Panel b. Development of arginase activity in homogenates of rat jejunum. On the X-axis the age of rats is given, on the Y-axis the enzyme activity in nmol/min/mg protein. Values are means ± SEM; n = 5 animals per age group.
Discussion

The metabolic demands and capacities of organs can change during development. An intriguing example is found in the small intestine. In adult mammals, the intestine produces citrulline from glutamine. After release of citrulline into the circulation, the kidney converts citrulline into arginine. However, the newborn kidney acquires the capacity to synthesize arginine from citrulline only gradually, with adult levels being established around weaning [11,29]. Prior to weaning, arginine biosynthesis most likely takes place in the gut rather than the kidney [10], because the activities of the enzymes involved in arginine biosynthesis are high, and those that utilize arginine or its precursors are low.

In the small intestine of mice, rats, and pigs, the activities of the enzymes involved in citrulline biosynthesis (CPS, N-acetylglutamate synthetase (AGAS) and OTC), as well as those involved in the conversion of citrulline into arginine (ASS and ASL) peak shortly after birth and gradually decline to adult levels at weaning [10,30-33]. Similarly, enzymes that convert glutamine to ornithine (phosphate-dependent glutaminase (PDG), pyrroline-5-carboxylate synthase (P5CS) and OAT all show activities well above adult levels in the period before weaning [13,14,31,33-36]. Since neonatal transgenic mice, in which the OAT gene was disrupted, have very low circulating arginine levels, it is plausible that the activity of OAT in the small intestine is directed towards the synthesis of ornithine in the neonatal period [37]. On the other hand, the activity of pyrroline-5-carboxylate reductase (P5CR), the enzyme that can divert pyrroline-5-carboxylate towards proline, is low during the suckling period in rat and hamster [35,38]. Furthermore, ornithine decarboxylase (ODC) activity, which diverts ornithine towards polyamine biosynthesis, is very low during the suckling period, but shows a sharp, transient burst in activity at the onset of weaning [14,39]. Finally, we and others [10,14,38,40] found that intestinal arginase activity is virtually absent in the intestine until it appears abruptly at weaning.
The absence of a hybridization signal with our ARG probe in gut tissue at a time when arginase activity is easily measurable, i.e. after weaning, shows that this arginase activity arises from an arginase isoform that is different from that in liver [41-43]. Indeed, an immunologically distinct arginase in rat mammary gland and kidney has been isolated [44], and cloned [45,46]. The cellular distribution of this non-hepatic arginase gene-product in the gut remains to be established.

The ASS mRNA and protein in the neurons of the myenteric plexus of the adult small intestine colocalises with neuronal nitric oxide synthase mRNA (unpublished results), 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 [47,48] and macrophages [49].

Perhaps the most intriguing aspect of the present study is why the mRNAs for glutaminase [13,50], CPS, OTC and OAT mRNAs are concentrated at the base of the villi and in the developing crypts, whereas ASS and ASL can only be demonstrated in the upper part of the villi. This difference in distribution can already be detected at ED 18, is easily visible at ED 20, and persists until weaning. The question arises in what respect the enterocytes occupying the upper half of the villus in the fetal and neonatal intestine, differ from those occupying the same position after weaning. In this respect, it might be of relevance that dramatic maturational changes occur in the rat small intestine in the third postnatal week, including an acceleration of the turnover rate of enterocytes [51]. It is tempting to relate the disappearance of ASS and ASL gene-expression from the enterocytes in the third week to this higher turnover rate of the enterocytes. ASS and ASL can apparently only be expressed in the enterocytes with a long lifetime. Another conspicuous property of suckling enterocytes is the presence of a giant supranuclear lysosome. These highly typical cells disappear at weaning and, prematurely, upon treatment with glucocorticoids or polyamines.
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Metabolic zonation, that is, the spatial separation of the metabolic pathways among otherwise similar cells, potentially exists in two directions of the gut. The first direction, i.e. heterogeneity of the enterocytes along the proximal-distal axis of the organ, is pronounced during prenatal development, but is less obvious postnatally. The zonation in the second direction, i.e. along the crypt-villus axis, appears to be a characteristic of the citrulline/arginine biosynthetic pathway. In view of the fact that CPS, OTC, ASS, ASL and OAT should all be present within one enterocyte in order to sustain arginine biosynthesis, it may seem surprising, at first glance, that ASS and ASL mRNA and protein are absent from the enterocytes in the crypts and at the base of the villi. This finding implies that the "young" enterocytes, at the base of the villus, can only synthesize citrulline, whereas those near the top of the villus can not only synthesize citrulline, but can also convert it into arginine. 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. This metabolic zonation is reminiscent of that in hepatocytes along the porto-central radius of the liver lobule (the smallest metabolic unit of that organ [52-54]).

We hypothesize that the capacity to produce arginine in the small intestine of sucklings is beneficial for several reasons. First, during this developmental period, the intestine has a very high growth rate and, thus, a high demand for amino acids. We already cited the study by Davis et al [12], implying a high net synthesis of arginine during the suckling period. Second, the surface area of the intestinal epithelium has a high potential not only for digestion and absorption of nutrients, but also for damage by noxious dietary substances and micro-organisms. To deal with the latter, the intestinal mucosa contains both physical barriers and immunological defences. During development, the need for a mucosal means of defence arises abruptly at birth. However, the specific mucosal immune system matures during suckling and becomes fully functional only after weaning [55]. Arginine has been identified as a potential immunomodulatory substance [56], partly because it is a substrate for nitric oxide synthase. Adult
enterocytes have been shown to express this enzyme [57,58], but a relation with a local intestinal arginine production in the neonate remains to be established.
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