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Ontogeny of L-Alanine Uptake in Plasma Membrane Vesicles from Rat Liver

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ABSTRACT

Alanine uptake into liver plasma membrane vesicles was studied at different stages of postnatal rat development. Before weaning, alanine hepatic uptake showed lower values for the global K_M than after weaning (0.34, 0.77, 1.45, and 1.61 mM for 1-, 15-, and 28-d-old and adult rats, respectively). Alanine uptake capacity increased progressively until reaching maximum values in the adult state (values for V_{max}: 0.078, 0.199, 0.317, and 0.613 nmol alanine/mg protein/3 s for 1-, 15-, and 28-d-old and adult rats, respectively). These results seem to point to a prevalence of a high affinity, low capacity alanine transport component (traditionally assumed to be attributable to system A) in newborn and suckling rats, in agreement with our previous results on isolated hepatocytes (Martínez-Mas JV, Casado J, Felipe A, Marin JJG, Pastor-Anglada M: Biochem J 293: 819–824, 1993). The suckling-weaning developmental transition seems to play a role in establishing the pattern of adult hepatic alanine transport characterized by a higher capacity but a lower affinity (because most alanine is taken up by system ASC) inasmuch as K_M values show a 100% increase after weaning, although V_{max} values continue to increase steadily until the adult age. (***Pediatr Res* 38: 81–85, 1995**)

Abbreviation

MeAIB, *N*-(methylamino)isobutyric acid

Amino acid transport and utilization during postnatal development have not been extensively studied in spite of their role in sustaining growth. Amino acids are mainly used for anabolic purposes, such as protein accretion (1) with rates even higher than in the adult (2–4) or purine and pyrimidine biosynthesis (5). Moreover, typical gluconeogenic amino acids such as alanine play only a minor part as substrates for glucose production along the whole suckling period (1, 6). This lack of information is particularly noticeable regarding the suckling-weaning developmental transition, a period of trascendental metabolic changes in which carbohydrate and lipid metabolism are very well studied (7–9). Furthermore, most of the studies on amino acid transport during development have been performed on cultured hepatocytes from protease-treated livers (10–12), with some controverted results. Different approaches applied to amino acid transport during development are quite scarce (13). Recently, we have reported the kinetic study of alanine transport in freshly isolated hepatocytes from rat fetuses and neonates by a less injurious methodology (14). In that report, it was shown that, in fetal and neonatal hepatocytes, there is a preeminence of alanine transport by a high affinity, low capacity agency that seems well established at these developmental stages. In the present study, we use liver plasma membrane vesicles to characterize the kinetics of alanine uptake during postnatal development, specially stressing the suckling-weaning developmental transition. Our conclusions corroborate that our previous results (14) are the reflection of the stable effects on the membrane.

METHODS

Animals. Female Wistar rats (200 g) from the Animal House of the University of Barcelona were kept under standard conditions and individually mated. Neonates were used between 12 and 24 h after birth, without discriminating between sexes. All other animals used were females. Suckling pups were used 15 d after birth, and weaned animals were used 28 d after birth (weaned on d 21). Sixty-day-old female rats were used as adult controls. Body and liver weight and liver protein contents of all four groups are presented in Table 1.

Purification of plasma membrane vesicles. Liver plasma membrane vesicles from neonatal, suckling, weaned, and adult animals were purified by a Percoll density gradient method as previously described (15). All preparations were assayed for proteins (16) and different enzyme markers to assess the degree of purification of plasma membrane fractions and the contamination by other subcellular membranes: 5′-nucleotidase (EC

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Table 1. *Body and liver weights and hepatic protein contents*

Rat	Body weight (g)	Liver weight (g)	Liver protein (mg/g liver)
Newborn	6.9 ± 0.4	0.26 ± 0.01	230 ± 18
Suckling	29.9 ± 0.9	0.87 ± 0.03	220 ± 11
Weaned	66.8 ± 2.0	2.61 ± 0.11	192 ± 11
Adult	194.9 ± 3.1	7.98 ± 0.32	225 ± 9

Body weight, liver weight, and liver protein contents in all four experimental groups. Results are mean ± SEM of six to 10 animals. Body and liver weights are the result of individual measurements. Hepatic protein contents are mean values for every litter, except for adult animals that are also individual values.

3.1.3.5) as plasma membrane marker, assayed as described by Lin and Morales (17); glucose-6-phosphatase (EC 3.1.3.9) as endoplasmic reticulum membrane marker, assayed as in Baginski *et al.* (18); *N*-Acetyl-β-glucosaminidase (EC 3.2.1.52) as lysosomal membrane marker, determined as in Caroll (19); and succinate dehydrogenase (EC 1.3.99.1), a mitochondrial marker, assayed as shown by Bonner (20).

Uptake measurements. Amino acid transport into the inner vesicular space was measured by a filtration procedure adapted from Sips *et al.* (21) and Pastor-Anglada *et al.* (15). The incubation medium consisted of 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid/KOH, pH = 7.4, 100 mM NaSCN or KSCN and L-[2,3-^{3}H]alanine (Amersham Corp.) at different concentrations and specific activities depending on the characteristics of each experiment. Transport was measured at 15°C, to improve measurements of initial velocities over the classical method at 25°C (data not shown). Alanine uptake inhibitable by excess concentrations of MeAIB was determined as a measure of system A activity.

Calculation of the kinetic parameters. Kinetic constants of the Na^+-dependent uptake of alanine were derived mathematically from the experimental data by nonlinear regression analysis using FigP software (FigP Software Corp., Durham, NC). Kinetic analysis was always done in triplicate on plasma membrane fractions from four to six single purifications. The results are mean ± SEM of the kinetic parameters calculated for every rat liver.

Blood alanine levels. In 15- and 28-d-old and adult female rats, portal alanine levels were also measured. Four to six animals per group were anesthetized by an intraperitoneal pentobarbital injection (60 mg/kg body weight), and blood samples were withdrawn from the portal vein using heparinized syringes. Alanine was determined by a standard enzymatic spectrophotometric method (22), and results are given as mean ± SEM.

RESULTS

Characterization of plasma membrane preparations. The recoveries and relative specific activities (enrichments) of 5'-nucleotidase, the plasma membrane enzyme marker, are shown in Table 2. Recoveries are given as the percentage of initial homogenate activities found in final preparations. Enrichments are the ratios of enzyme specific activity in the membrane preparation to the specific activity in the homogenate. 5'-Nucleotidase enrichments were similar in all groups except in neonatal preparations in which it was significantly higher. Contamination by other subcellular membranes (microsomal, mitochondrial, lysosomal) were in the range previously described (23–26). Apparent vesicular volumes were 530 ± 65, 320 ± 73, 255 ± 12, and 220 ± 11 nL/mg protein for newborn, suckling, weaned, and adult animals, respectively. Statistical differences were found between newborn and adult rats ($p < 0.001$, Student's *t* test).

Table 2. *Characterization of plasma membrane vesicle preparations*

Rat	5'-Nucleotidase	
	Recovery	Enrichment
Newborn	7.3 ± 1.2	9.3 ± 2.0*
Suckling	5.0 ± 0.8	4.2 ± 0.7
Weaned	8.8 ± 0.7	4.5 ± 0.4
Adult	6.6 ± 1.2	4.3 ± 0.5

5'-Nucleotidase (plasma membrane marker) recoveries and enrichments. Recoveries are given as percentage of initial homogenate activities. Enrichments are the ratio of specific activity in the plasma membrane fraction to the activity in the initial homogenate. Each point is the mean ± SEM of four to six independent preparations. Statistical comparison by a *t* test.
* $p < 0.05$ *vs* adult.

Kinetics of L-alanine transport. In the absence of an inward transmembrane sodium gradient (K^+ medium), L-alanine uptake by plasma membrane vesicles in all groups increased steadily with alanine concentrations, and it was assumed to represent simple diffusion, because no saturability was observed. Diffusion coefficients (K_d) were quite similar in all groups and only slightly higher in the neonatal preparations (Table 3). When the diffusion component was substracted from the whole L-alanine transport in the Na^+ medium, a Na^+-dependent, saturable component emerged. This component is presented in Figure 1, showing a classical hyperbolic behavior of initial rates as alanine concentrations increase. The kinetic parameters of this component (K_M and V_{max}) for all the groups are given in Table 3 and their evolution along development is presented in Figure 2. Plasma membrane vesicles from newborn rats showed much lower values of both K_M and V_{max} than the adults. The evolution of K_M during development is strictly paralleled by the evolution of portal blood alanine concentrations (Fig. 3), showing a perfect linear correlation coefficient between them ($r = 1.00$, Fig. 4). Both parameters showed a sharp increase around weaning (Figs. 3 and 4). On the other hand, V_{max} values seemed to be a simple function of time, showing no particular feature around the suckling-weaning

Table 3. *Kinetic parameters of hepatic alanine transport*

Rat	V_{max}	K_M	K_d
Newborn	0.078 ± 0.003***	0.34 ± 0.07**	0.146 ± 0.017
Suckling	0.199 ± 0.009***,‡	0.77 ± 0.13*,†	0.092 ± 0.10
Weaned	0.317 ± 0.025**	1.45 ± 0.25	0.075 ± 0.013
Adult	0.613 ± 0.069	1.61 ± 0.33	0.110 ± 0.030

Kinetic parameters for the Na^+-dependent component (K_M and V_{max}) and diffusion (K_d) of alanine transport. K_M is given in millimolar, V_{max} is given in nanomoles of alanine per mg of protein · 3 s and K_d is given in nanoliters/3 s/μg of protein. Each point is the mean ± SEM of four to six independent preparations. Statistical comparisons by a *t* test are as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs* adult. † $p < 0.05$; ‡ $p < 0.01$, suckling *vs* weaned.

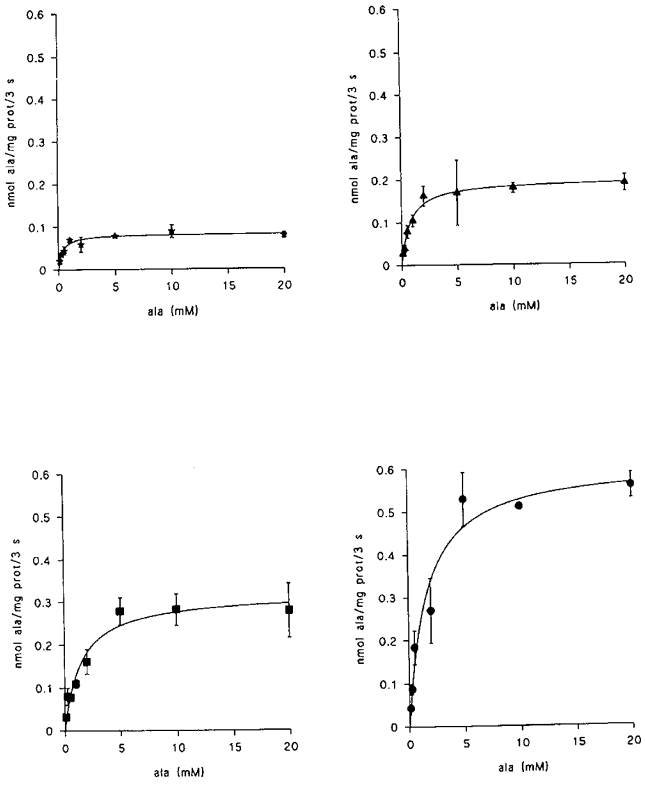

Figure 1. Substrate concentration dependence of the Na^+-dependent component of alanine transport: *upper left*, newborn rats; *upper right*, suckling rats; *lower left*, weaned rats; *lower right*, adult rats. Each point is the mean ± SEM of four to six independent preparations. All panels are drawn at the same scale for easy comparison between groups.

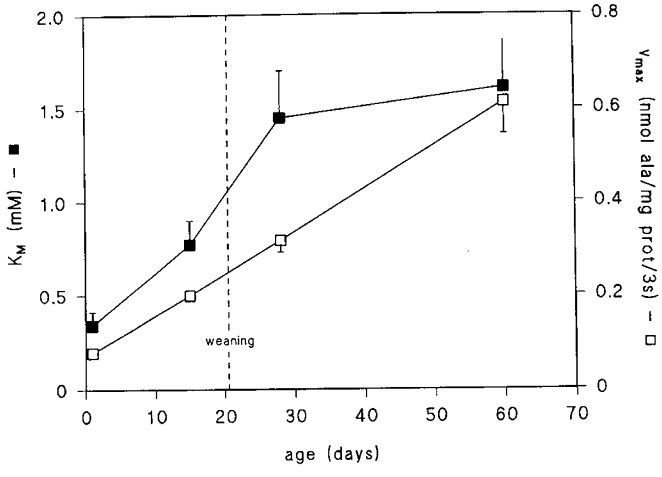

Figure 2. Evolution of kinetic parameters (K_M, ■; V_{max}, □) during development. Although V_{max} values seem to be only a function of time, K_M adult values have already been reached immediately after weaning.

transition (Fig. 2) and a much lower lineal correlation coefficient with portal alanine levels (r = 0.82, Fig. 4). The contribution of transport systems A and ASC to total sodium dependent alanine uptake in suckling, weaned and adult rats is shown in Table 4. At all conditions tested, the contribution of system A (MeAIB-sensitive, Na^+-dependent alanine transport) to the overall alanine uptake was much higher in rat pups that in the adults, although we could not detect significant differences in

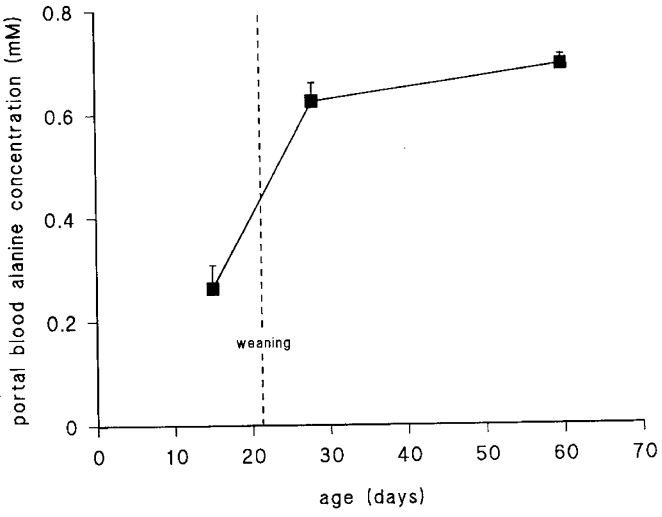

Figure 3. Evolution of portal blood alanine concentrations during development. Results are mean ± SEM of four to six individual determinations.

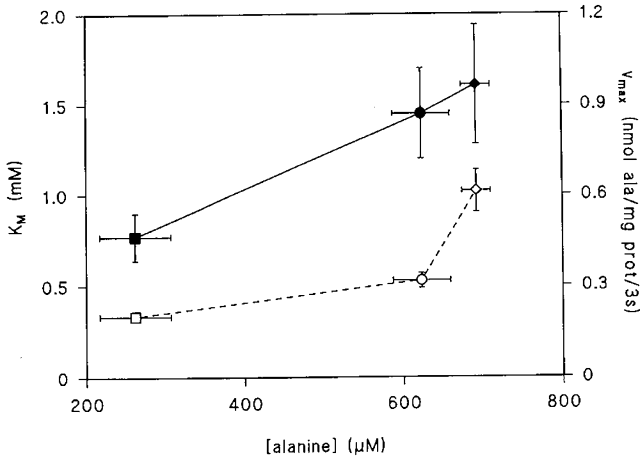

Figure 4. Correlation between alanine portal blood levels and either K_M (*closed symbols*) or V_{max} (*open symbols*) values during development (□,■, suckling; ○,●, weaned; ◇,◆, adult). Lineal correlation coefficients: r = 1.00 for K_M and alanine concentrations; r = 0.82 for V_{max} and alanine concentrations.

absolute terms. System ASC activity (MeAIB-resistant, Na^+-dependent alanine transport), however, was higher in adult than in suckling and weaned rats.

DISCUSSION

Plasma membrane vesicles are useful in studies comparing different physiologic situations provide that physical properties of vesicles are similar among the various situations. Thus, they have been used to study such situations as short-term fasting (24), pregnancy (26), diabetes (27), suckling (28), or obesity (29). In the present work, all groups of vesicles had similar physical properties, with the exception of plasma membrane vesicles from neonates. Vesicles from this group presented a higher 5′-nucleotidase enrichment, a slightly higher (although not statistical) diffusion coefficient and also a higher intravesicular volume, in accordance with other reports (13); all these data taken together point toward a somehow different composition of the plasma membranes of rat liver parenchymal cells

Table 4. *Contribution of transport systems A and ASC to total Na^+-dependent alanine uptake*

	L-Alanine concentration			
	0.5 mM		1 mM	
Rat	A	ASC	A	ASC
Suckling	76 ± 8 (88%)	10 ± 1 (12%)	119 ± 21 (98%)	3 ± 0 (2%)
Weaned	59 ± 11 (82%)	13 ± 1 (18%)	102 ± 7 (94%)	7 ± 0 (6%)
Adult	66 ± 10 (59%)	45 ± 9 (41%)	108 ± 30 (66%)	56 ± 10 (34%)

System A activity (MeAIB-sensitive, Na^+-dependent alanine uptake) and system ASC activity (MeAIB-resistant, Na^+-dependent alanine uptake) were measured to assess the contribution of each transport agency to total Na^+-dependent alanine uptake at three physiologic alanine concentrations. MeAIB was used at a concentration of 25 mM. Results are expressed as picomoles of alanine/3 s/mg of protein. Values are means ± SEM of triplicate measurements from three to four different vesicle preparations.

shortly after birth compared with the adult state. These differences would make difficult to compare the results of plasma membrane vesicles from rat neonates with those from the other groups. Nevertheless, they do not imply a loss of significance in the statistical comparisons because all the differences in the plasma membrane physical properties suggest a certain overestimation of L-alanine transport rates in the newborn preparations. Vesicles with larger volumes will show a higher alanine concentrative capacity, which will result in apparent higher transport kinetic parameters, especially if there is also a greater diffusion. So, the actual differences with the adult preparations would be even higher than those here reported. Besides, these differences on alanine transport capacity are to be due to intrinsic differences between alanine transport agencies in neonates and those in the other groups, because sodium gradient dissipation rate seems not to be increased in neonatal plasma membrane vesicles (13).

Alanine is mainly transported into the parenchymal liver cell by two different agencies: a high affinity, low capacity system (assimilated to system A) and a low affinity, high capacity one (assumed to be system ASC) (30). Our results suggest that the high affinity agency would be already established by the time of birth, in concordance with our previous report on isolated hepatocytes (14) and with the results of Leoni *et al.* (12), who found a high fetal system A activity in rat liver that progressively declined during the perinatal period to the lower values of the adult. This could be understood as an adaptation to secure that the high hepatocyte amino acid requirements will be met in a situation in which portal amino acid concentrations are relatively low (31). Not only system A shows a higher affinity for amino acid transport but it is also responsive to the drop in blood amino acid levels occurring after birth that would release hepatocytes from any *trans*-inhibiting effect allowing a higher alanine transport rate (10, 32). This postnatal system A, unlike the one in the adult rat, is mostly unresponsive to insulin (32, 33), in agreement with the generalized state of insulin resistance of this age (34, 35). So liver amino acid supply would not be altered by changes in insulin concentrations.

The suckling-weaning nutritional and developmental transition is characterized by important nutritional (36), hormonal (7), and metabolic changes affecting both carbohydrate (8, 9, 37) and lipid metabolism (8, 9, 38). This nutritional breakpoint seems to play a key role in the evolution of alanine transport into the parenchymal liver cell, because the apparent K_M for this uptake is sharply increased immediately after weaning. This result can be considered as reflecting the moment in which the high capacity, low affinity agency described in hepatocytes (14) would become the main alanine transport system in these cells. This would be physiologically consistent, because blood alanine concentration after weaning has reached normal adult values and hepatic amino acid requirements are lower and more easily met. However, the functionality of this system is not perfectly established immediately after weaning, because V_{max} values grow steadily until the adult state. In agreement with this point, system ASC activity is lower in weaned than in adult rats at the concentrations tested (all in the physiologic range), but there is a tendency, although not statistically significant, to be higher than in suckling rats. An alternative explanation for the different ASC activities found between adult and developing rats might be a different degree of canalicular membrane contamination in the preparations from each group. Thus, because ASC activity has been localized mostly in the canalicular membrane (39), a higher ASC activity in the adult preparations could reflect only a higher contamination by the canalicular membrane fraction. Although canalicular contamination is present to some extent in our preparations (40), such an explanation does not seem likely, because differences in high capacity, low affinity alanine uptake among these three developmental stages have been reported also in isolated hepatocytes (14), where this problem would not be present.

In conclusion, hepatic alanine uptake shows a lower capacity and higher affinity right after birth compared with the adult state. Such a metabolic feature seems appropriate for the developing liver to ensure its amino acid supply in a context of relatively low portal availability. The suckling-weaning developmental transition reflects a sharp increase in the K_M value for hepatic alanine uptake, which could be considered as the shift toward the adult system for neutral amino acid transport in the liver.

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