L-Alanine uptake by rat liver parenchymal and haematopoietic cells during the perinatal period

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Alanine disposal by liver parenchymal and haematopoietic cells from 21-day fetuses, newborns and adult rats was studied. Preparations selectively enriched in either haematopoietic cells or hepatocytes were obtained by direct perfusion of fetal- and neonatal-rat livers. L-Alanine transport into liver parenchymal cells was best fitted to two Na⁺-dependent saturable systems. The high-affinity system showed a much higher activity (V_{max}) in hepatocytes from fetuses and newborns than in those from adult rats (2.4, 4.3 and 0.3 nmol/8 min per 10⁶ cells for fetuses, newborns and adults respectively). V_{max} for the low-affinity component was slightly lower during the perinatal period than in

the adult (about 30 nmol/8 min per 10⁶ cells for hepatocytes from fetuses and newborns, versus 48 nmol/8 min per 10⁶ cells for adult rat parenchymal cells). Haematopoietic cells from fetal-rat livers showed significant Na⁺-dependent L-alanine uptake which was completely abolished after birth. These results show that the transport systems involved in L-alanine uptake by liver parenchymal cells are fully developed before birth. This probably contributes to fulfilling the high requirement for neutral amino acids for protein synthesis during development. Haematopoietic cells may play an important role in liver amino acid metabolism during fetal life.

INTRODUCTION

During fetal and neonatal development, amino acids are mainly used for protein accretion and purine and pyrimidine biosynthesis (Snell, 1982; Jones and Rolph, 1985). The high amino acid requirements for anabolic purposes in the growing fetus are met by the mother, who increases food intake and decreases amino acid catabolism (Beaton et al., 1954; Freinkel et al., 1972). At this time, the placenta plays a crucial role in the active transfer of amino acids towards the fetal blood, where many of these compounds reach higher concentrations than those found in maternal blood (Palou et al., 1977; Battaglia and Meschia, 1978; Battaglia, 1989). After birth, the maternal supply disappears and circulating amino acid concentrations fall, although the protein-synthesis rate remains higher than that in adults (Goldspink and Kelly, 1984; Goldspink et al., 1984; Lewis et al., 1984). Amino acids play only a minor role as substrate for glucose production during this period and thus, they are mainly used anabolically (Snell, 1981, 1982). Some 20 years ago, Christensen (1973) reviewed experimental evidence that indirectly pointed to an induction of the amino acid concentrative capacity *in vivo* of the liver at birth, which was attributed to an interesting case of adaptive control induced by the presence of hypoaminoacidaemia. Besides the little available information on this topic, the method traditionally used for transport studies in fetal- and neonatal-rat isolated hepatocytes consists of incubation of the whole organ with solutions containing proteolytic enzymes (Bellemann, 1981a,b; Handlogten and Kilberg, 1982; Vadgama and Christensen, 1983; Miller et al., 1986; Leoni et al., 1987). The cells obtained by this procedure were usually cultured for several hours before carrying out the experiments. The results of the studies on amino acid transport in neonatal-rat hepatocytes were somehow different from those reported by other authors using 'in vivo' models. Furthermore, those studies did not consider the uptake of the natural substrates taken up prefer-

entially by liver (i.e. alanine). In the present work, fetal- and neonatal-rat parenchymal liver cells were obtained by an improved method based on collagenase perfusion and subsequent disaggregation of the hepatic tissue. Freshly isolated haematopoietic cells and hepatocytes were then used to investigate the kinetic characteristics of L-alanine uptake at different stages of rat development.

MATERIALS AND METHODS

Animals

Female Wistar rats (200 g) were purchased from the Animal House of the University of Barcelona. They were individually mated, and impregnation was assessed by the presence of spermatozoa in vaginal smears. That was considered to be day 0 of pregnancy. The 21-day fetuses were obtained by Caesarean section, and newborns were used between 12 and 24 h after birth. Female Wistar rats (200 g) were used as adult controls.

Isolation of liver parenchymal and haematopoietic cells

Liver parenchymal cells from fetuses at term and newborns were isolated by a modification of the method described by Burgaya et al. (1988). Briefly, fetuses and pups were anaesthetized by a sodium pentobarbital injection (20 mg/kg body wt., intraperitoneally), laparotomized, and the liver was retrogradely perfused (0.8 ml/min) with a Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution containing 0.5 mM EGTA, buffered at pH 7.4 with 20 mM Hepes. After washing the organ for 10 min, a 5 mM CaCl₂/collagenase (0.2 mg/ml)-supplemented buffer was used for perfusion. After 20 min, the liver was excised and immediately washed with the same buffer. At this point, the organ was incubated at 37 °C for 15 min more in a rotatory shaker bath to disaggregate the tissues fully and give isolated cells. The whole preparation was then filtered through a double-layered nylon

Abbreviation used: AIB, α -aminoisobutyrate.

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100-mesh gauze and centrifuged for 3 min at 50 *g*. The pellet obtained was a mixture of hepatocytes and haematopoietic cells. After low-speed differential centrifugation of the resuspended cells (Burgaya et al., 1988), two different fractions were collected. Fraction 1 was enriched in liver parenchymal cells, as calculated from regular counting with a Neubauer haemocytometer. Fraction 2 was selectively enriched in haematopoietic cells. By this technique we usually obtained fractions highly enriched in the cell type of interest. Fraction 1 usually contained more than 85 % hepatocytes, and fraction 2 usually contained more than 95 % of haematopoietic cells. Both cell types were easily identified by their morphological characteristics. To differentiate transport rates accounted for by hepatocytes from those accounted for by haematopoietic cells, we resolved the following equations:

$$J_1 = (LPC_1 \times J_{lpc}) + (HC_1 \times J_{hc})$$

$$J_2 = (LPC_2 \times J_{lpc}) + (HC_2 \times J_{hc})$$

where J_1 and J_2 are the uptake rates determined in both fractions, LPC_1 , LPC_2 , HC_1 and HC_2 are the percentage of liver parenchymal (LPC) and haematopoietic cells (HC) in fractions 1 and 2 respectively, and J_{lpc} and J_{hc} are the actual transport rates accounted for by pure preparations of hepatocytes and haematopoietic cells. The same procedure was used to calculate the protein content of both cell populations.

Hepatocytes from fed adult rats were isolated by the classical anterograde collagenase perfusion method described elsewhere (Moreno et al., 1989). Viability of the cells was assessed on the basis of their ability to exclude 0.2 % Trypan Blue. Mean viability of liver parenchymal cells was above 90 % in all the experimental groups. Similar results were obtained for haematopoietic cells. The mean yields of fetal and neonatal liver preparations were over 15×10^6 and 18×10^6 cells respectively. Since this is not enough for a complete kinetic analysis, we made pools of liver cells from the whole litter (generally 8–10 rats) to perform every individual set of experiments.

Transport measurements

L-Alanine uptake rates in liver parenchymal and haematopoietic cell preparations were determined as previously described (Moule et al., 1987; Moule and McGivan, 1987). Briefly, fresh suspensions of either hepatocyte- or haematopoietic-cell-enriched preparations were incubated at 37 °C for the indicated times in a medium containing 1.3 mM $CaCl_2$, 5.4 mM KCl, 0.44 mM KH_2PO_4 , 0.98 mM $MgCl_2 \cdot 6H_2O$, 0.81 mM $MgSO_4 \cdot 7H_2O$, 136.8 mM NaCl, 1.33 mM $Na_2HPO_4 \cdot 2H_2O$, 1 % BSA (fatty-acid-free), 20 mM Hepes, 5.55 mM glucose and the uniformly labelled substrate ($[U-^{14}C]$ alanine; Amersham International, Amersham, Bucks., U.K.) at the indicated concentrations (specific radioactivity in the range 9.6–720 $\mu Ci/mmol$). Choline chloride was used instead of NaCl to differentiate Na^+ -dependent transport from simple diffusion. Amino-oxyacetate (1 mM) was added 15 min before the uptake measurements to block alanine metabolism. The mean cell concentration used during the incubation was 1×10^6 cells/ml. When the decided incubation times had elapsed, samples (0.25×10^6 cells) were collected and added to ice-cold Eppendorf tubes, containing an upper buffer phase, an intermediate oil layer [dibutyl phthalate/bis-(3,5,5-trimethylhexyl)phthalate (3:1, v/v)] and a lower glycerol-supplemented 10 % $HClO_4$ solution. The tube was then immediately centrifuged (14000 *g* for 60 s), and the cells were separated from the incubation medium and pelleted in the $HClO_4$ layer. $[^3H]$ Mannitol (0.38 $\mu Ci/ml$) was included in the incubation media to estimate the amount of extracellular medium trapped in the acid layer. The contents of the tube were aspirated, except for the

bottom layer, which was used for radioactivity counting. The net cell-associated radioactivity was corrected by the specific radioactivity of the substrate, and all the results were expressed as L-alanine (in nmol) taken up during a certain incubation time by 10^6 cells. The rate of L-alanine uptake was not modified by cell density in the range tested $[(0.5-2) \times 10^6$ cells/ml]. This method gives accurate estimations of Na^+ -dependent L-alanine transport. Indeed, the mannitol space was very small (around 4.5 $\mu l/10^6$ cells) and unchanged in all the preparations tested, whether they had been incubated with Na^+ or choline. Furthermore, the pool of intracellular L-alanine is not markedly modified by resuspending the cells in either a NaCl or a choline chloride medium. Using a spectrophotometric method (Williamson, 1986), we measured the L-alanine content of adult rat hepatocytes that had been resuspended in any of those media and lysed with 6 % $HClO_4$. The L-alanine content was 12 ± 1 and 10 ± 0.5 nmol/mg of protein ($n = 3$) for hepatocytes incubated for 30 min in a NaCl and a choline chloride medium, respectively.

Analysis of the data

All the calculations were performed with an Apple-Macintosh SE microcomputer. Basically, we analysed the Na^+ -dependent component of transport, since this is the major and, perhaps, only route for carrier-mediated transport of L-alanine into liver parenchymal cells. Then saturation curves were solved by applying a non-linear regression analysis that includes an *F* test (Van Melle and Robinson, 1981; Brot-Laroche et al., 1986). To do so, we used the software kindly provided by Professor Francisco Alvarado (C.N.R.S., Meudon-Bellevue, France). Where indicated, the statistical significance of the differences between the experimental groups was established by Student's *t* test.

RESULTS

The time course of 0.25 mM L-alanine uptake into liver parenchymal and haematopoietic cells from 21-day fetuses, newborns and adult rats showed that transport was linear up to 10 min in all the experimental groups and for all the preparations tested. Results for hepatocytes are shown in Figure 1. Even at high substrate concentrations (20 mM L-alanine) we showed that

Figure 1 Time course of L-alanine uptake into liver parenchymal cells

Hepatocytes isolated from 21-day fetuses (■, □), newborns (●, ○) and adult rats (▲, △) were incubated, as indicated in the Materials and methods section, with 0.25 mM L-alanine, either in a NaCl medium (■, ●, ▲) or in a choline chloride (□, ○, △) medium. Results are means $\pm$ S.E.M. of triplicate estimations made on three independent cell preparations.

Figure 2 Relationship between substrate concentration and transport rate of L-alanine by liver parenchymal cells

Hepatocytes isolated from adult rats (a), newborns (b) and 21-day fetuses (c) were incubated for 8 min, as indicated in the Materials and methods section, with L-alanine concentrations in the range 0.1 to 20–30 mM. The Na^+ -dependent L-alanine uptake was calculated by subtracting the uptakes in the presence of choline chloride from those measured in the NaCl medium. The values shown are means $\pm$ S.E.M. of triplicate estimations made on 5–7 independent cell preparations.

L-alanine uptake remained linear up to 10 min. At those concentrations, transport rates in the choline chloride medium only represented 9%, 6% and 12% of the rates found in the NaCl medium for fetal-, newborn- and adult-rat preparations respectively (results not shown). Thus we chose 8 min as the incubation time for further transport studies. L-Alanine uptake rates measured at low L-alanine concentrations (0.1 mM) were higher in those liver parenchymal cell preparations from fetuses (2.39 ± 0.14 nmol/8 min per 10^6 cells; $P < 0.001$) and neonates (1.66 ± 0.33 ; $P < 0.05$) than in those from adult rats (0.68 ± 0.07). With high concentrations of L-alanine, uptake rates were no longer significantly different in fetal and neonatal preparations compared with adults (results not shown). This can be interpreted on the basis of the kinetic study discussed below.

Figure 3 Woolf-Augustinson-Hofstee plot of Na^+ -dependent L-alanine uptake into liver parenchymal cells

Uptake rates from Figure 2 are represented according to the Woolf-Augustinson-Hofstee plot. For details see the legend to Figure 2.

L-Alanine uptake by haematopoietic cells from fetal liver was also linear for up to 10 min, but was slower than that in fetal hepatocytes (0.87 versus 2.39 nmol/8 min per 10^6 cells). Interestingly, the rate of L-alanine uptake by neonatal-rat haematopoietic liver cells was negligible.

The concentration-dependence of the Na^+ -dependent L-alanine uptake in the different cell preparations was monitored by determining the uptake rates at different substrate concentrations in the incubation medium (from 0.1 to 20–30 mM). L-Alanine uptake into liver parenchymal cells proved to be almost saturated in the range of substrate concentrations tested (Figure 2). Furthermore, the total Na^+ -dependent uptake is in the range of what has been described in the literature (for review see Meijer et al., 1990). The Wolf-Augustinson-Hofstee linear transformation of the Na^+ -dependent L-alanine transport is shown in Figure 3.

Table 1 Kinetic parameters for alanine uptake in isolated rat hepatocytes from 21-day fetuses, newborns and adult rats

L-Alanine uptake was determined as indicated in the Materials and methods section. The kinetic parameters were derived from data of Figure 2 by a non-linear regression analysis to an equation involving two saturable components and one non-saturable component. The *F* test includes the degree of freedom (df) in parentheses.

Group	$V_{\max,1}$ (nmol/8 min per 10^6 cells)	K_{m1} (mM)	$V_{\max,2}$ (nmol/8 min per 10^6 cells)	K_{m2} (mM)	<i>F</i> (df)
Fetus	32.3	6.1	2.4	0.03	1.1 (2,16)
Newborn	29.8	15.3	4.3	0.24	0.4 (4,40)
Adult	48.4	8.2	0.35	0.09	0.3 (4,34)

The results suggest that more than a single carrier is involved in the transport of L-alanine by isolated hepatocytes. Indeed, the best fit of our results yielded evidence of two saturable systems, and those liver parenchymal cells isolated from fetuses at term and from newborns showed a much higher contribution of the high-affinity low-capacity system. The kinetic parameters for L-alanine uptake in isolated hepatocytes are shown in Table 1. When we fitted the uptake data to two saturable systems plus a diffusion component of transport, we obtained the same kinetic parameters, because in all cases simple diffusion was negligible, giving K_d values ranging from 10^{-6} to 10^{-16} min $^{-1}$. This proves that subtraction of uptake rates measured in the choline media from those measured in the Na $^{+}$ media appropriately corrected for the non-saturable component of transport. When we tried to fit our data to a single saturable system, we were able to obtain a rather good fit only for those uptake rates of adult rat liver parenchymal cells. Kinetic parameters were $V_{\max}$ 44.8 nmol/8 min per 10^6 cells, K_m 6.6 mM, with an *F* of 0.746 for 6 and 34 degrees of freedom. The addition of a diffusion component of transport to the mathematical analysis did not make any difference, because, again, the K_d was negligible. When we fitted the kinetic data from fetuses and newborns to a single saturable system, we obtained *F* values far beyond those determining statistical significance, and then the possibility that a single system could be responsible for L-alanine uptake into isolated hepatocytes from fetal and newborn rats was considered to be highly unlikely.

The $V_{\max}$ of the low-affinity component was slightly lower in hepatocytes from developing rats than in those from adults; however, the expression of the high-affinity component seems to be higher during the perinatal period, because its activity is 12-fold greater in the newborns (Table 1). When these results were calculated on a protein-content basis, the magnitude of the difference was even greater (3.33, 7.1 and 0.27 nmol/min per mg of protein for fetuses, newborns and adult rats respectively). Similar differences are found when results are expressed on the basis of the reported size and surface area of developing rat hepatocytes (Blázquez et al., 1987). This adaptation allows hepatocytes from developing rats to take up much larger amounts of L-alanine in the physiological range of substrate concentration. For instance, uptake of 0.25 mM L-alanine was 3.56 ± 0.48 , 2.74 ± 0.39 and 1.6 ± 0.17 nmol/8 min per 10^6 cells, for fetuses, newborns and adult rats respectively.

The dependence on the substrate concentration of L-alanine uptake in haematopoietic cells from livers of 21-day fetuses is shown in Figure 4. L-Alanine is transported according to

Figure 4 Relationship between substrate concentration and transport rate of L-alanine by haematopoietic cells

Haematopoietic cells isolated from 21-day fetuses were incubated for 8 min, as indicated in the Materials and methods section, with L-alanine concentrations in the range 0.1–30 mM. The Na $^{+}$ -dependent L-alanine uptake was calculated by subtracting the uptakes in the presence of choline chloride from those measured in the presence of NaCl. The resulting data are plotted against the substrate concentration. The results shown are means $\pm$ S.E.M. of triplicate estimations made on 5 independent cell preparations.

Michaelis–Menten kinetics, with a $V_{\max}$ of 6.44 nmol/8 min per 10^6 cells and an apparent K_m of 1.24 mM. Obviously, the fraction that we call neonatal-rat haematopoietic cells may contain a heterogeneous cell population, probably showing different transport activities; thus we cannot completely rule out the possibility of having more than one system involved in L-alanine uptake. In any case, it is clear from our data that this cell population can take up L-alanine with high affinity at a value of $V_{\max}$, which is in the range of that calculated for the high-affinity component of transport for this amino acid in fetal liver parenchymal cells.

DISCUSSION

L-Alanine uptake by liver parenchymal cells

L-Alanine gets into the hepatocyte through two major Na $^{+}$ -dependent systems, i.e. systems A and ASC (Kilberg, 1982). When Na $^{+}$ -dependent L-alanine uptake is monitored in the presence of increasing concentrations of amino acid and the results are plotted according to the Woolf–Augustinson–Hofstee transformation, a biphasic pattern is observed. Similar results are obtained when α -aminoisobutyrate (AIB) is used instead of L-alanine as a substrate (Fehlmann et al., 1979a,b; Fafournoux et al., 1982, 1983; Kristensen et al., 1983). These kinetics have been sometimes solved according to a non-linear regression analysis, giving two components of transport, one of high affinity and low capacity and the other of low affinity and high capacity for the substrate. In classical studies on the regulation of amino acid uptake into liver parenchymal cells, using either L-alanine or AIB as substrate, the high-affinity component, which is supposed to be accounted for by system A, is significantly induced. This has been reported to occur in either starved or diabetic rats (Fehlmann et al., 1979a; Samson et al., 1980; Kristensen et al., 1983), as well as in rats fed on a high-protein diet (Fafournoux et al., 1982, 1983, 1990). Furthermore, similar results were obtained in primary cultures of rat hepatocytes when they were treated with insulin, glucagon, glucocorticoids or catecholamines (Fehlmann et al., 1979b; Canivet et al., 1980), thus reflecting up-regulation of system A activity. This kind of analysis does not imply that system A activity is negligible in basal conditions. Indeed, when we analysed the ability of methyl-AIB to inhibit

uptake of 1 mM L-alanine into liver parenchymal cells from adult rats, we found that nearly 60% of Na⁺-dependent L-alanine transport was inhibited at methyl-AIB concentrations greater than 60 mM (results not shown). This proves the presence of system A in the adult rat liver parenchymal cells and is in agreement with previous reports showing that methyl-AIB-sensitive L-alanine uptake is concomitant with biphasic kinetic behaviour solved on the basis of two components of transport differing in their apparent affinities for the substrate (Kristensen et al., 1983).

In the present paper, a similar induction to that reported in the physiological situations cited above is found in hepatocytes isolated from either 21-day rat fetuses or 1-day newborns. This strongly suggests that liver parenchymal cells from fetuses and newborns share carrier systems with higher affinity for afferent L-alanine than those from adult rats. This gives hepatocytes from developing rats a better ability to transport L-alanine when the amino acid is present in the medium at physiological concentrations. Interestingly, our results show that the pattern of development of both systems is different and, indeed, the total capacity (apparent $V_{\max}$ or the sum of the $V_{\max}$ of both components) is markedly lower in fetal and neonatal than in adult rat hepatocytes, at least when expressed per number of cells. This could probably explain why the concentrative ability of neonatal-rat hepatocytes can be either over- or underestimated, depending both on the substrate selected and the range of concentrations tested. From single experiments by Vadgama and Christensen (1983), it is known that, in primary cultures of fetal hepatocytes, system A is able to respond to amino acid starvation by increasing its activity, a phenomenon which is known as derepression or adaptive regulation. Moreover, the amino acid concentrative ability of the liver *in vivo* is markedly modified after parturition (Christensen, 1973). This has been interpreted as a consequence of adaptive control due to the prompt decrease in circulating amino acid levels occurring at birth. Despite the slight increase in the $V_{\max}$ of the high-affinity agency (when expressed on a cell-number basis), our results show that the development of the systems responsible for L-alanine uptake in liver parenchymal cells is extremely rapid and programmed to occur before birth.

In a previous report by Bellemann (1981b), studying AIB uptake in cultured hepatocytes from lactating rats, similar results were found. Kinetics of AIB transport gave a biphasic pattern, and the high-affinity component showed a much higher $V_{\max}$ in terms of specific activity. In that study, adult values were not reached even after weaning. Interestingly, parallel changes were found for the ouabain-inhibitable Rb⁺ uptake, which was considered to cause membrane hyperpolarization, thus contributing to enhance Na⁺-dependent amino acid transport in liver parenchymal cells. Concomitant changes in both activities during development can be explained on the basis of the suggested co-ordinate regulation of system A and Na⁺,K⁺-ATPase-subunit gene expression reported in cultured CHO cells (Qian et al., 1991). It is not known whether this applies to fetal development. Indeed, messengers coding for both α and β subunits of the Na⁺,K⁺-ATPase accumulate in livers from lactating rats, but are not detected in 18-day fetal livers (Emanuel et al., 1987).

L-Alanine uptake by liver haematopoietic cells

Haematopoietic cells are another major cell type in liver during fetal and early postnatal development. Our results indicate that Na⁺-dependent L-alanine uptake by haematopoietic cells is quite important in the 21-day fetal liver. This characteristic disappears

after birth, which suggests firstly that haematopoietic cells are a main site of liver amino acid metabolism during fetal development, and secondly that birth is a key landmark for the maturation of transport processes in this cell type. Similarly, in adults of all the mammalian species so far studied, a classical feature during the maturation of red-cell precursors is the loss of Na⁺-dependent transport for most amino acids (Tucker and Young, 1980; Fincham et al., 1984; Blostein and Grafova, 1987; Felipe et al., 1990, 1992).

Physiological implications and conclusions

An interesting finding of this study is that a high-affinity component of L-alanine transport shows an increased activity in fetal- and neonatal-rat liver parenchymal cells. Similar results have been found in another situation associated with liver hyperplasia, i.e. liver regeneration after partial hepatectomy (LeCam et al., 1979). Also, during pregnancy, when liver DNA is significantly increased, a co-ordinate induction of system A and ASC activities has been reported (Pastor-Anglada et al., 1987, 1990; Felipe et al., 1989). It is likely that the induction of this high-affinity component of transport for L-alanine is a consequence of gene programming during tissue hyperplasia, because, as suggested by our results, it is not a consequence of adaptive control caused by birth-associated hypoaminoacidemia. Moreover, our results confirm that cell differentiation is accompanied by changes in transport activity of several carriers, and hence the lack of Na⁺-dependent transport in haematopoietic cells from neonatal-rat livers presumably reflects a late step in the conversion of these precursors into mature red cells.

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