0031-3998/89/2605-0448$02.00/0
PEDIATRIC RESEARCH

Vol. 26, No. 5, 1989
Printed in U.S.A.

Na^+-Dependent Alanine Transport in Plasma Membrane Vesicles from Late-Pregnant Rat Livers

ANTONIO FELIPE, XAVIER REMESAR, AND MARÇAL PASTOR-ANGLADA

Unitat de Bioquímica i Biologia Molecular B., Departament de Bioquímica i Fisiologia, Universitat de Barcelona, Diagonal 645, Barcelona 08028, Spain

ABSTRACT. Liver plasma membrane vesicles retaining Na^+-dependent L-alanine transport were purified from either virgin or 21-d pregnant rats. Kinetic analysis of L-alanine uptake data revealed that vesicles from pregnant rats show a slight increase in V_{max} without significantly affecting K_m. Plasma membrane marker recoveries were used to calculate alanine utilization indexes. These data showed that the overall liver capacity to take up alanine was increased 2-fold in late pregnant rats. When considering utilization indexes in the physiologic range of L-alanine concentrations in blood (0.1–2 mM), we found a highly significant enhancement of the liver capacity to take up this amino acid. It is concluded that, at late pregnancy, when amino acid requirements by the fetus are high, amino acid disposal by the maternal liver is not limited by a low transport capacity; thus, other mechanisms should be contributing to the nitrogen sparing at late pregnancy. (*Pediatr Res* 26: 448–451, 1989)

Abbreviation

EGF, epidermal growth factor

Human and rat pregnancies both are characterized by a nitrogen-sparing effect, mediated by a low ureagenic capacity, which has been suggested by others to be due to a low availability of amino acids (1, 2). We have recently shown, by direct estimations made on fed rats (3), that the availabilities of ureagenic substrates are not altered during late pregnancy and, for some amino acids, their hepatic balances are even enhanced. Amino acid supply to the liver is strongly dependent on the nutritional status. We reported indeed a lower availability of amino acids to the liver in pregnant than in virgin rats after a 24-h fast (3); nevertheless, the hepatic uptake was unchanged, thus suggesting that factors other than availability itself are controlling liver amino acid uptake at late pregnancy. Among them, two mechanisms are likely to exert strong modulation on amino acid uptake by liver: first, a short-term effect related to the hormonal status in the portal vein (in fact it has been proved that either glucagon (4) or EGF (5) exert a stimulatory action on alanine transport by means of membrane hyperpolarization) and, second, a long-term effect mediated by a stable change at the plasma membrane. Plasma membrane vesicles from rat liver retain the enhancement of alanine transport induced by either diabetes (6) or glucagon injection (7), and thus, they may be a good tool to ascertain which mechanism is contributing to the increase in substrate uptake *in vivo*, whether it is a circulating factor (*i.e.* hormones) or a stable change at the plasma membrane (*i.e.* insertion of more carrier molecules, posttranslational modification of transport proteins, or both). In our work we used plasma membrane vesicles purified from either virgin or late-pregnant rats to study this specific point. We used L-alanine as a substrate, for several reasons: first, because L-alanine preferentially enters the hepatocyte by means of systems A and ASC, and both are responsible for most of the neutral amino acid uptake by liver; second, because L-alanine transport across the hepatocyte plasma membrane seems to be the rate limiting step of its metabolism (8); third, because L-alanine contribution to the overall liver amino acid uptake is high either in fed virgin or late-pregnant rats (3); and finally, because L-alanine provision to the fetus is also high at this period of pregnancy (9).

Our results show that at late pregnancy, the liver capacity to take up L-alanine is enhanced, especially at the physiologic range of L-alanine concentrations in blood. A role for hepatic amino acid transport in mediating nitrogen sparing to support fetal growth should be discarded.

Received March 23, 1989; accepted July 11, 1989.
Correspondence and reprint requests, Dr. Marçal Pastor-Anglada, Departament de Bioquímica i Fisiologia, Unitat de Bioquímica i Biologia Molecular B, Universitat de Barcelona, Diagonal 645, Barcelona 08028, Spain.

MATERIALS AND METHODS

Animals. Albino rats of the Wistar strain were used. They were mated at 65 d of age (more than 200 g body wt). Impregnation was assessed by the presence of spermatozoa in vaginal smears. Animals were caged individually and maintained under controlled conditions of light (12 h on/12 h off light cycle) and temperature (20 ± 1°C). They were fed laboratory food (Panlab, Barcelona, Spain) *ad libitum*.

Preparation of Plasma Membrane Vesicles. Protein and enzyme assays. Plasma membrane-vesicles from livers of both virgin and 21-d pregnant rats were prepared using the method of Van Amelsvoort *et al.* (10). Protein in homogenate and membrane preparations was determined according to Reference 11. 5'Nucleotidase (E.C.3.1.3.5) activity was selected as a plasma membrane marker and assayed in both fractions according to Reference 12. Three enzyme markers of membranes pertaining to other subcellular organelles were assayed in both homogenate and plasma membrane preparations: cytochrome-oxidase (E.C.1.9.3.1.) (mitochondrial marker) following Reference 13, β-N-acetylglucosaminidase (E.C.3.2.1.30.) (lysosomal marker) according to Reference 14 and glucose-6-phosphatase (E.C.3.1.3.9.) (marker of endoplasmic reticulum) according to Reference 15. The results were the mean ± SEM of duplicate estimations on five different membrane preparations.

Transport assays. The procedure used was adapted from that of Sips *et al.* (16). Briefly, 10 μL of plasma membrane preparations were mixed with 40 μL of the incubation mixture to give the following concentrations: 0.25 M sucrose, 0.2 mM $CaCl_2$, 10

mM $MgCl_2$, 10 mM HEPES/KOH, pH 7.4, 100 mM either Na- or K-sulfocyanate and L-(2,3)-^{3}H-alanine (Amersham, Bucks, U.K.) from 0.1 to 20 mM. The specific activity of alanine was monitored so that in all cases the medium contained 1 μCi. The intravesicular medium was the same as the incubation mixture but without alanine, Na- and K-sulfocyanate. The reaction was stopped after 10 s (near linearity conditions) by adding 1 mL of a cold stop solution (0.25 M sucrose, 100 mM NaCl, 0.2 mM $CaCl_2$, 10 mM HEPES/KOH, pH 7.4) to the incubation tube. Then the whole content was quickly filtered through a nitrocellulose filter (Sartorius, Munich, Germany, pore size 0.45 μm) and washed with 4 mL cold stop solution. Dried filters were then counted for radioactivity. All the assays were done in triplicate. Pooled plasma membrane samples were used because the individual preparations were found to be highly homogeneous regarding their alanine transport rates (data not shown).

Calculations and statistics. The amounts of alanine taken up by plasma membrane vesicles were calculated from the radioactivity retained by the filters and the specific activity of the medium. The results were expressed in pmols of alanine taken up per μg of membrane protein and were referred to the incubation time. Kinetics of alanine transport were analyzed by Eadie-Hofstee plots (v *versus* v/s). Correlation coefficients of these plots were 0.93 and 0.94 for control and pregnant rats, respectively. To determine the whole hepatic capacity to take up alanine we referred V_{max} to 5′nucleotidase activity in the membrane fraction and then, we corrected it by the enzyme recovery and its original activity in the homogenate. This procedure leads to a simple equation to calculate alanine utilization indexes (AUI) as follows:

$$\text{AUI: } V_{max} \times A/B$$

where V_{max} should be given in μmol/min/mg protein, A is the total protein in the whole liver homogenate in mg, and B is the 5′nucleotidase enrichment in the membrane fractions. Thus, alanine utilization indexes calculated by this way are given in μmol of alanine taken up per min per whole liver. Significant changes induced by gestation were tested with the Student's *t* test.

RESULTS

Body and liver wt. Virgin and 21-d pregnant rats weighed 188 ± 4 and 362 ± 11 g, respectively. The liver weights were 6.9 ± 0.4 g for the former and 14.3 ± 0.8 for the latter.

Enzyme profile of preparations. The recoveries and the relative specific activities (enrichments) of the enzyme markers are shown in Table 1. Recovery is given in percentage of activity in the homogenate. Relative specific activity is the ratio of marker enzyme activity in the membrane preparation to the activity in the homogenate. A greater amount of 5′nucleotidase activity was recovered in the preparations from pregnant than in those from virgin rats. Nevertheless, the relative specific activities were similar in both experimental groups. It means that more protein was obtained from the livers of pregnant rats than from those of virgin animals; however, the level of purification was similar (8- to 9-fold) and was in the range of what has been previously described (7, 10, 17, 18). Glucose-6-phosphatase recovery and its relative specific activity amounted to 2 and 1%, respectively, for both experimental groups, however, this result is satisfactory because microsome contamination is usually elevated, as reflected by the 2- to 3-fold purification of glucose-6-phosphatase reported by others (7, 10). Contamination of plasma membrane preparations by mitochondria and lysosomes was very low and similar to previous reports (7, 10, 17, 18). The apparent vesicular volumes of all the preparations were similar (data not shown).

Kinetics of L-alanine transport. L-Alanine transport in the K^+ medium increased linearly with alanine concentrations. This was taken as simple diffusion because no saturability was observed. Coefficients of diffusion of 0.09 ± 0.008 and 0.08 ± 0.002/10 s/μg protein were obtained for preparations from virgin and pregnant rats, respectively. When the diffusion component was subtracted from the transport measured in the Na^+ medium, we evidenced a saturable Na^+-dependent agency. The kinetic parameters of this component are shown in Table 2. They were determined by means of the Eadie-Hofstee plot of the experimental data (Fig. 1). Pregnancy induced a higher capacity to take up L-alanine, without concomitant changes in K_m. When we calculated, as indicated in "Materials and Methods," the overall hepatic capacity to transport L-alanine, we found a 2-fold increase in this parameter (Table 2). This enhancement remained slightly significant when data were expressed either on a liver wt basis (0.23 ± 0.005 and 0.30 ± 0.009 μmol/min per g of liver for virgin and pregnant rats, respectively, $p < 0.01$) or per 100 g body wt (Table 2). Alanine utilization indexes, in the physiologic range of substrate concentrations, were found to be markedly higher in pregnant than in virgin rats, either when expressed by whole liver or by 100 g body wt (Table 3).

DISCUSSION

Plasma membrane vesicles have been proved to be a useful tool for the study of the energetics and kinetics of amino acid transport, but they have been used also more recently for the study of the hepatic alterations in amino acid transport capacity, induced by normal and pathologic situations such as short-term fasting (17), diabetes (6), glucagon injection (7), and suckling (19). The use of plasma membrane vesicles instead of intact cells allows a direct estimation of natural substrate transport without having to use substrate analogues or to inhibit amino acid metabolism, which is often done by unspecific inhibitors that might affect transport capacity itself.

In our study we have used liver plasma membrane vesicles from virgin and 21-d pregnant rats to ascertain if the hepatic capacity to take up L-alanine is increased at late-pregnancy by means of a persistent effect at the plasma membrane level. Our

Table 1. *Recoveries and enrichments of enzyme markers in plasma membrane preparations from either virgin or late-pregnant rats**

	Physiologic situation			
	Virgin		21-day pregnant	
Marker	Recovery	Enrichment	Recovery	Enrichment
5′Nucleotidase	11 ± 1	8 ± 1	20 ± 2†	9 ± 1
Glucose-6-phosphatase	2.1 ± 0.06	0.9 ± 0.07	2.1 ± 0.3	1.4 ± 0.16
β-N-acetyl glucosaminidase	0.3 ± 0.13	0.2 ± 0.05	0.6 ± 0.05	0.3 ± 0.03
Cytochrome oxidase	0.5 ± 0.13	0.3 ± 0.04	0.7 ± 0.16	0.3 ± 0.04

* Recoveries are expressed as percent of original activity in homogenate recovered in plasma membrane preparations. Enrichments are calculated as the ratio of the specific activities in plasma membrane preparations and homogenates. Results are the mean ± SEM of duplicate estimations on five membrane preparations.

† Statistical differences between groups, $p < 0.001$.

Table 2. *Kinetic parameters of alanine uptake**

Parameter	Physiologic situation	
	Virgin	21-day pregnant
Vmax		
(pmol/10 s μg protein)	1.7 ± 0.04	2.0 ± 0.06†
(μmol/min liver)	1.6 ± 0.04	4.4 ± 0.10‡
(μmol/min 100 g body wt)	0.9 ± 0.02	1.2 ± 0.02§
Km (mM)	2.3 ± 0.14	1.8 ± 0.15

* All the observations were made in triplicate on pooled homogeneous samples from five membrane preparations. Vmax when expressed either on a whole liver or on a body wt basis was calculated as indicated in methods.

Significant differences between groups, † $p < 0.05$, ‡ $p < 0.01$, § $p < 0.001$.

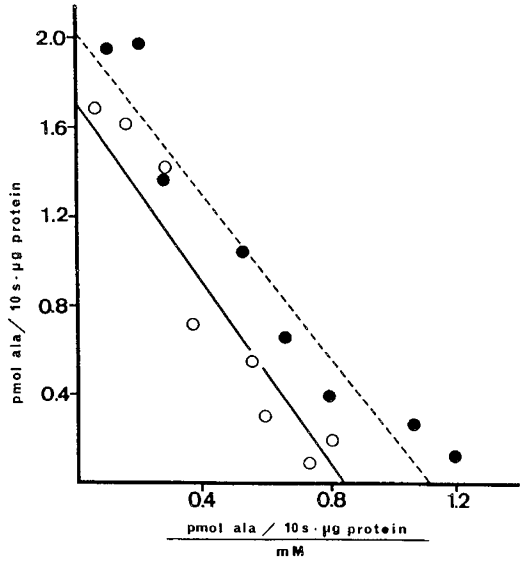

Fig. 1. Eadie-Hofstee linear transformation of the Na-dependent alanine transport in plasma membrane vesicles from virgin and late-pregnant rat livers. The saturable component of alanine transport (amount of alanine taken up in Na sulfocyanate medium minus amount taken up in K sulfocyanate medium) gave a linear plot when the Eadie-Hofstee transformation was used. Correlation coefficients: 0.93 and 0.94 for virgin (○) and late-pregnant (●) rats, respectively.

Table 3. *Alanine utilization indexes by liver of late-pregnant and virgin rats**

Alanine (mM)	Uptake rates (nmol/min)	
	Virgin	21-day pregnant
0.1	68 ± 1	238 ± 12†
	(36 ± 1)	(66 ± 3)†
0.25	191 ± 8	523 ± 26†
	(102 ± 4)	(144 ± 7)‡
0.5	274 ± 20	773 ± 23†
	(146 ± 10)	(213 ± 6)‡
1	523 ± 12	1299 ± 56†
	(278 ± 6)	(359 ± 15)‡
2	665 ± 58	2116 ± 20†
	(354 ± 31)	(584 ± 5)‡

* Results are expressed as nanomols/min either per whole liver or per 100 g body wt (in parentheses). Data were derived from kinetic measurements on plasma membrane vesicles and from the recovery of 5′nucleotidase activity. Mean ± SEM of three determinations on homogeneous preparations purified from control and pregnant rats.

Student's *t* test, † $p < 0.001$, ‡ $p < 0.01$.

calculated K_m were of the same magnitude than those reported previously for alanine transport into plasma membrane vesicles from rat liver (6, 16). Because K_m are in the high range of physiologic concentrations of alanine in portal vein (2mM), it is obvious that fluctuations in substrate availability may determine its hepatic uptake. This does not seem to be the case at late pregnancy when no changes in alanine availability have been reported to occur (3), but it may be important at midlactation when the availability of this amino acid is enhanced (20).

In our work it is shown that the alanine transport capacity increases along with the liver mass. Because the liver of pregnant rats is hypertrophic and hyperplasic, we can suggest that both factors contribute to the absolute increase in alanine transport activity. It means that not only are there more cells and consequently more carriers, but there are also more carriers per cell. Furthermore, the hepatic capacity to take up alanine remained enhanced when expressed on a body wt basis, especially when considering the physiologic range of L-alanine concentrations in blood. This apparently agrees with our previous observations made *in vivo*, showing a net increase in alanine uptake by the liver of late-pregnant rats when expressed per 100 g body wt (3). Nevertheless it seems rather unlikely that a 50% mean increase in this parameter (Table 3) could fully account for the net uptake found *in vivo*. Thus, we think that the hormonal status in the portal vein must be a key factor regulating amino acid uptake by liver *in vivo*. As pointed out previously, EGF and glucagon may exert a short-term stimulatory action on alanine uptake by isolated hepatocytes (4, 5). Furthermore plasma EGF levels are increased during pregnancy in the mouse (21) and some hepatic metabolic functions are resistant to insulin (22), thus modifying the normal response of the liver to the portal insulin/glucagon ratio. Obviously all these effects are lost after purifying plasma membrane vesicles.

The question now is, to what extent these changes might be assumed to be present in other species, such as humans. As recently discussed (23), some differences among species may be expected to exist. Indeed, alanine turnover and its contribution to gluconeogenesis are unaltered in late-pregnant women, although the fraction rate of alanine conversion into gluconeogenesis is decreased (24). Unfortunately, alanine kinetics has never been appropriately studied in pregnant rats at term. We have recently reported a significant increase in alanine turnover rates at midpregnancy (25), but no conclusions about what is happening at late-pregnancy can be drawn from these results, because many features are distinct in both stages of gestation. Thus, it is not known yet if similar adaptative responses occur in rats and humans. In any case, our results do not support the view that the liver capacity to take up amino acids is decreased at late pregnancy, but rather it is slightly enhanced. We suggest that intracellular events might be, at least partially, if not fully, responsible for the nitrogen sparing of pregnancy. One of the indirect evidences supporting this hypothesis has been reported in both species. This is the development of the orotic aciduria (26, 27), which can be explained as the result of heterogeneous partition of nitrogen between urea and pyrimidine biosynthesis.

A major conclusion of this work is that amino acid transport capacity by liver does not significantly contribute to the nitrogen sparing of pregnancy.

REFERENCES

1. Freinkel F, Metzger BE, Nitzan M, Hare JW, Shambaugh GE, Marshall RT, Surmaczynska BZ, Nagel TC 1972 "Accelerated starvation" and mechanisms for the conservation of maternal nitrogen during pregnancy. Isr J Med Sci 8:426–439
2. Kalhan SC, Tserng K, Gilfillan C, Dierker LJ 1982 Metabolism of urea and glucose in normal and diabetic pregnancy. Metab Clin Exp 31:824–833
3. Casado J, Remesar X, Pastor-Anglada M 1987 Hepatic uptake of amino acids in late-pregnant rats. Effect of food deprivation. Biochem J 248:117–122

4. Moule SK, Bradford NM, McGivan JD 1987 Short-term stimulation of Na^+-dependent amino acid transport by dibutyryl cyclic AMP in hepatocytes. Characteristics and partial mechanism. Biochem J 241:737–743
5. Moule SK, McGivan JD 1987 Epidermal growth factor, like glucagon, exerts a short-term stimulation of alanine transport in rat hepatocytes. Biochem J 247:233–235
6. Rosenthal NR, Jacob R, Barrett E 1985 Diabetes enhances activity of alanine transport in liver plasma membrane vesicles. Am J Physiol 248:E581–E587
7. Schenerman MA, Kilberg MS 1986 Maintenance of glucagon-stimulated system A amino acid transport activity in rat liver plasma membrane vesicles. Biochim Biophys Acta 856:428–436
8. Groen A, Sips HJ, Vervoorn RC, Tager JM 1982 Intracellular compartmentation and control of alanine metabolism in rat liver parenchymal cells. Eur J Biochem 122:87–93
9. Battaglia FC, Meschia G 1978 Principal substrates of fetal metabolism. Physiol Rev 58:499–527
10. Van Amelsvoort JMM, Sips HJ, Van Dam K 1978 Sodium-dependent alanine transport in plasma-membrane vesicles from rat liver. Biochem J 174:1083–1086
11. Peterson GL 1979 Review of the Folin phenol protein quantitation method of Lowry, Farr, and Randall. Anal Biochem 100:201–220
12. Aronson Jr NN, Touster O 1974 Isolation of rat liver plasma membrane fragments in isotonic sucrose. In: Fleischer S, Packer L (ed) Methods of Enzymology, Vol 31. Academic Press, New York, pp 90–102
13. Rafael J 1983 Cytochrome c oxidase. In: Bergmeyer HV (ed) Methods of Enzymatic Analysis, Vol III. Verlag Chemie, Weinheim, pp 266–273
14. Carroll M 1978 Characterization of proteins structurally related to human N-acetyl-β-D-glucosaminidase. Biochem J 173:191–196
15. DeDuve C, Pressman BC, Gianetto R, Appelmans F 1955 Tissue fractionation studies: six intracellular distribution patterns of enzymes in rat liver tissues. Biochem J 60:604–617
16. Sips HJ, Van Amelsvoort JMM, Van Dam K 1980 Amino acid transport in plasma membrane vesicles from rat liver. Characterization of L-alanine transport. Eur J Biochem 105:217–224
17. Bourdel G, Forestier M 1982 Circadian variations of A-mediated transport in rat-liver plasma membrane vesicles. FEBS Lett 143:81–85
18. Quintana I, Felipe A, Remesar X, Pastor-Anglada M 1988 Carrier-mediated uptake of L-(+)-lactate in plasma membrane vesicles from rat liver. FEBS Lett 235:224–228
19. Casado J, Felipe A, Pastor-Anglada M, Remesar X 1988 Glutamine as a major nitrogen carrier to the liver in suckling rat pups. Biochem J 256:377–381
20. Casado J, Pastor-Anglada M, Remesar X 1987 Hepatic uptake of amino acids at mid-lactation in the rat. Biochem J 245:297–300
21. Kurachi H, Oka T 1985 Changes in epidermal growth factor concentrations of submandibular gland, plasma and urine of normal and sialoadenectomized female mice during various reproductive stages. J Endocrinol 106:197–202
22. Leturque A, Hauguel S, Ferré P, Girard J 1987 Glucose metabolism in pregnancy. Biol Neonate 51:64–69
23. Pastor-Anglada M 1988 Letter to the Editor. Metab Clin Exp 38:290
24. Kalhan SC, Gilfillan CA, Tserng K, Savin SM 1988 Glucose-alanine relationship in normal human pregnancy. Metab Clin Exp 37:152–158
25. Pastor-Anglada M, Champigny O, Ferré P, Remesar X, Girard J 1988 Alanine turnover rate and its hepatic metabolism are increased in midpregnant rat. Biol Neonate 54:126–132
26. Wood MH, O'Sullivan WJ 1973 The orotic aciduria of pregnancy. Am J Obstet Gynecol 116:57–61
27. Milner JA, Visek WJ 1978 Orotic aciduria in the female rat and its relation to dietary arginine. J Nutr 108:1281–1288