Amino Acid Uptakes in Normal and Small-for-Date Placentas, with Special Reference to Activity of Transport ATPase in Placental Slices and Isolated Trophoblastic Membrane

RYUJI YAMAGUCHI, TETSUNORI MATSUDA and TOMOKO AIZAWA

Department of Obstetrics and Gynecology, Nara Medical University, Kashihara, Nara 634

YAMAGUCHI, R., MATSUDA, T. and AIZAWA, T. *Amino Acid Uptakes in Normal and Small-for-Date Placentas, with Special Reference to Activity of Transport ATPase in Placental Slices and Isolated Trophoblastic Membrane.* Tohoku J. exp. Med., 1978, 125 (4), 367-376 — In our previous report, we presumed that there is a depressed transport of amino acids in the small-for-date (SFD) placenta. To substantiate this presumption, we conducted in vitro experiments with normal and SFD placental slices and compared their uptakes of amino acids, serine and lysine. Activity of Na-K ATPase in the trophoblastic membrane was also studied. (1) In both normal and SFD placentas, uptakes of amino acids were about two to three times as great for neutral amino acid serine as for basic amino acid lysine. (2) In the SFD placenta, amino acid uptake became significantly slower than in the normal after 30 min of incubation. After 90 min the uptake was lower than the normal by some 32% for serine and 42% for lysine. (3) Serine uptake began to be interrupted by the addition of 10^{-3} M ouabain at 30 min, and showed about 31% decrease at 90 min, revealing an amino acid uptake pattern similar to that of the SFD placenta. But lysine uptake was not noticeably affected by ouabain. (4) As for the release of intracellular amino acids into the incubation medium, no differences could be observed either between serine and lysine or between the normal and the SFD placenta. (5) Activity of transport ATPase of the isolated trophoblastic membrane was lower in the SFD than in the normal placenta. The findings tend to corroborate the suggestion that the low f/m ratio of amino acids in SFD pregnancy accounts for a decreased placental transport of amino acids corresponding with depression in the activity of its major promotor — membrane-bound transport ATPase. — placenta; amino acid transport; ATPase

In our previous clinical study of the placental transport of amino acids in search of the cause of small-for-date (SFD) pregnancy, we found that the concentration of amino acids was lower in the umbilical vein blood than in the

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Reprint request: Ryuji Yamaguchi, M.D., Department of Obstetrics and Gynecology, Nara Medical University, Kashihara, Nara 634, Japan.

maternal vein blood in SFD pregnancy, implying a reduced ability of the SFD placenta to transport amino acids (Matsuda et al. 1977). In this report, we newly conducted a study to find a clue to understanding the SFD identity, with in vitro experiments carried out with intracellular uptakes of marker amino acids in the human placenta and on the activity of trophoblastic membrane-bound transport ATPase (Na-K ATPase), which is generally considered to concern the active transport of amino acids.

MATERIALS AND METHODS

Placentas used in the experiments were from 37 cases of normal and 19 of maternal-disease-free SFD pregnancy delivered at our Obstetric Clinic.

Uptake and release of amino acids

Uptake of amino acids

Preparation of placental specimens. Immediately after delivery, the placenta was promptly taken out and washed thoroughly in physiological saline at 0°C, and then saline was removed by filter-paper. After blood vessels were removed as far as possible, 3~4 g of the placental pieces were subjected to a preincubation at 37°C for 10 min in a solution of Krebs-Ringer bicarbonate (pH 7.4) under an aerobic condition (95% O₂+5% CO₂). Then water was removed by filter-paper, and placental specimen thus processed was sliced at about 100 mg wet weight apiece.

Incubation. As marker amino acids were used serine, L-[³H(G)] specific activity 1 Ci/mmole, and lysine, L-[³H(G)] specific activity 1 Ci/mmole. Each marker amino acid was adjusted by 0.01 N HCl to approximately 100,000 cpm per 100 µl, and to this was added some 100 mg of placental slice and 5 ml of Krebs-Ringer solution. The mixture was incubated at 37°C under an aerobic condition for a scheduled time. Measurement of intracellular and extracellular spaces was made employing the inulin space method (Dancis et al. 1968). Inulin, [³H(G)] specific activity 500 mCi/g, was adjusted to about 100,000 cpm per 100 µl and was incubated for 1 hr under the same condition as for the marker amino acids. The calculation was made using the following formula:

$$\text{Concentration in ICS} = \frac{\text{total cpm in tissue} - \text{cpm/ml in medium ECS}}{\text{total tissue water} - \text{ECS}},$$

where ICS and ECS are intracellular and extracellular spaces. Total tissue water was obtained by subtracting the dry weight (dried at 50°C for 96 hr) from the wet weight.

Release of amino acids from the placental tissue to the incubation medium

After incubation for 60 min under the described condition, the placental slice was washed for a short time in a solution of 0.154 M NaCl at 0°C. Then water was removed by filter-paper, and the placental slice was incubated in an amino acid-free Krebs-Ringer solution for 15 and 30 min under the aerobic condition. The proportion of release into the medium was expressed by percentage of cpm in the medium to the total of that in the medium and tissue.

Radioactivity count

The placental tissue was processed with a Packard tri-carb sample oxidizer, and 100 ml of dioxane scintillator (PPO 4.5 g, POPOP 4.5 mg and naphthalin 75 g in a blend composed of 363 ml of ethanol, 330 ml of dioxane and 330 ml of xylene, totaling 1,023 ml) were added to 0.5 ml of the incubation medium. Radioactivity was counted by employing the Packard tri-carb-liquid scintillation spectrometer.

Experiment on the trophoblastic membrane

Preparation of trophoblastic membrane specimens

Immediately after the placenta was taken out, the umbilical arteries and vein were

cannulated to let 100,000 units of heparin sodium solution flow through the vessels. Then some 4 liters of 0.154 M NaCl were perfused through the vessels for 30 min to remove blood. After that, the placenta on the maternal side was taken off and cut into small pieces by scissors in an ice bath. These placental pieces were left in place for 30 min in a solution of 1 mM of NaHCO_3 containing 2 mM of CaCl_2 . Then the suspension was homogenized using the Dounce homogenizer (clearance 0.15~0.2 mm) to obtain trophoblastic membrane specimens by employing a modification of the method of Emmelot et al. (1964) (Fig. 1).

Fig. 1. Isolation scheme for the trophoblastic plasma membrane of the human term placenta (Emmelot et al. 1964).

Determination of transport ATPase (Na-K ATPase)

100 μl of the membrane preparation blend were added to 0.8 ml of 20 mM Tris-HCl (pH 7.4) containing 3 mM Tris-ATP, 5 mM MgCl_2 , 140 mM NaCl and 14 mM KCl, and were incubated at 37°C for a scheduled time. 30 min after the reaction was stopped by adding 1 ml of 10% trichloroacetic acid, the blend was filtered. To 1 ml of the filtrate were added 0.5 ml of 10 N H_2SO_4 , 0.5 ml of 2.5% $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ and 2 ml of distilled water, and further 0.05 ml of a mixture composed of 0.2 g of 1-amino-2-naphthol-4-sulfonic acid, 1.2 g of NaHSO_3 and 1.2 g of Na_2SO_4 per 100 ml. After the mixture was let alone for 30 min, inorganic phosphorus (Pi) in it was quantitated using Fiske-Subbarow's method (Fiske and Subbarow 1925). The quantity of Pi determined in the Mg^{++} -free incubation medium was subtracted from that in the Mg^{++} -added medium, and the difference was decided as Na-K ATPase. As the standard Pi, NaH_2PO_4 was used.

Determination of glucose-6-phosphatase

To examine comparative purity of the isolated trophoblastic membrane specimen, glucose-6-phosphatase (G-6-Pase), which is a marker of contamination of organelle components, was determined using Nordlie's method (Nordlie and Arion 1966). The chemical reaction in the blend of trophoblastic membrane specimens was stopped by adding,

to 100 μ l of the blend, 0.6 ml of 0.1 M cacodylate buffer (pH 6.5), 200 μ l of 0.15 M sodium glucose-6-phosphate, and 0.6 ml of distilled water. Then inorganic phosphorus (Pi) was quantitated using Fiske-Subbarow's method. Quantitation of protein was made by Lowry's method (Lowry et al. 1951).

Electron-microscopic observation

In order to assess the purity of trophoblastic membrane specimen, the membrane preparation was fixed in 2% glutaraldehyde in phosphate buffer (pH 7.4) and subsequently fixed in 1% aqueous osmium tetroxide solution. Thus processed sections were stained with uranyl acetate and lead, and observed with a Hitachi HU-11D electron microscope.

RESULTS

Amino acid uptake in the placental slice

Fig. 2 shows the uptakes of neutral amino acid serine and basic amino acid lysine in the normal and the SFD placenta. In both placental slices, serine uptake was two to three times greater than lysine uptake at each scheduled time of measurement.

The uptakes of two marker amino acids were only slightly lower in the SFD placenta than in the normal 15 min after incubation was initiated, but at 30 min and later the uptakes turned significantly lower in the SFD placenta ($p < 0.01$), showing a decrease rate of 32% for serine and 42% for lysine at 90 min.

Fig. 2

Fig. 2. Difference of amino acid uptake between the normal and SFD placentas in vitro. ●—●, normal ($n=26$); ○—○, SFD ($n=9$).

Fig. 3

Fig. 3. Effects of ouabain blocking on intracellular uptakes of serine and lysine. ●—●, normal ($n=7$); ○—○, ouabain ($n=7$).

Changes in amino acid uptake of the normal placenta with and without addition of 10^{-3} M ouabain are shown in Fig. 3. No noticeable differences were brought about by the addition of ouabain at 15 min of incubation. But later, serine in the ouabain-added group showed a distinctly lower rate of uptake than in the control, indicating a significant difference ($p < 0.025$) at 60 min and as much as some 31% decrease at 90 min. In contrast to serine, the uptake of lysine was not affected significantly by ouabain.

Amino acid release from the placental slice to the medium

Table 1 shows distinctive features of amino acid release from the placental slice into the incubation medium. The release of serine increased with time, but

TABLE 1. *Release rate of amino acids from placental slices into medium*

	³ H-serine		³ H-lysine	
	15 min	30 min	15 min	30 min
Normal placenta (n=10)	33.7±5.3	45.7±7.2	42.2±9.8	41.2±8.4
SFD placenta (n=5)	37.2±6.6	46.6±5.2	46.0±10.0	46.7±14.1

$$\text{Release rate (\%)} = \frac{\text{cpm in medium}}{\text{cpm in medium and tissue}}$$

Fig. 4. Electron microscopic finding of isolated trophoblastic membrane. Bar shows 1 μ m.

that of lysine attained a plateau 15 min after the initiation of incubation. The release rate was slightly higher for lysine than for serine at 15 min, though the difference was not significant; at 30 min release rates of two amino acids were almost equivalent. Compared with the normal placenta, the SFD placenta showed slightly higher release rates of both serine and lysine at 15 and 30 min, though either difference was not significant.

Transport ATPase activity of the isolated trophoblastic membrane specimen

The comparative purity of the trophoblastic membrane material was examined electron-microscopically (Fig. 4) and biochemically (Table 2). The ratio in ATPase activity (Pi released per minute calculated from 10 min incubation) of the membrane to the whole tissue was 3.2 in the normal placenta and 2.7 in the

TABLE 2. *Enzyme activities of isolated membrane specimen*

		Na-K ATPase	G-6-Pase
Normal placenta	M	10.6±2.5	10.2±7.0
	W	3.7±1.4	6.8±3.7
	M/W	3.2±1.1*	1.6±0.8*
SFD placenta	M	5.5±2.3	7.4±4.5
	W	2.7±1.5	6.6±3.4
	M/W	2.7±2.0*	1.2±0.5*

Figures indicate the rate of Pi liberation in $\mu\text{g}/\text{mg}$ protein/min determined in 10 minutes' incubation.

* Mean±s.d. was calculated from each M/W value.

M, membrane; W, whole tissue.

Fig. 5. $\text{Na}^+\text{-K}^+$ ATPase activity of isolated trophoblastic membranes from normal and SFD placentas. ●—●, normal ($n=11$); ○—○, SFD ($n=10$).

SFD placenta. The corresponding ratio in G-6-Pase was 1.6 and 1.2, respectively. These values were not compatible with those reported in other organs (10~15 in ATPase and 0.5~1 in G-6-Pase). These discrepancies may suggest that our membrane specimens were not completely pure but rather crude containing some organelle components. And also, our results may be attributable to the characteristic structure of the placenta that the epithelium is mainly composed of inseparable syncytiotrophoblasts.

The differences between the normal and the SFD placenta in the activity feature of Na-K ATPase in the trophoblastic membrane are shown in Fig. 5. The activity increased gradually with time in like manner in both placentas. But the level of activity in SFD was only half as high as that in the normal placenta at each scheduled time of measurement, with a difference invariably significant ($p < 0.05$).

DISCUSSION

In our previous study, we found a lower f/m ratio of blood amino acids in SFD pregnancy with no maternal diseases, and suggested a probability that the active transport of amino acids is depressed in the SFD placenta (Matsuda et al. 1977). In an attempt to substantiate this probability, we conducted experiments of tissue slice incubation. Although this method has a difficulty in indicating the direction of transport of substances, we adopted it in this study according to the opinion of Miller and Berndt (1977) that the amino acid uptake in the placental slice is somehow indicative of the transport more inclining from mother to fetus.

The features of placental uptakes of serine and lysine were different. Christensen et al. (1973), experimenting with Ehrlich ascites tumor cells, stated that neutral amino acid serine is transported by the Na⁺-dependent system, while diamino acid lysine is transported by an unknown system, probably with the aid of neutral amino acid. This may suggest that the transport processes of serine and lysine are different also in the placenta. Enders et al. (1976) showed no difference in the uptake of amino acids between the placenta and other organs.

Regarding the total amount of amino acids absorbed in the placenta, Schneider and Dancis (1974), experimenting with placental slices, observed that after 90 min of incubation, the intracellular (ICS) concentrations of serine and lysine are about 20 times and 2 times as high as the corresponding values of the extracellular spaces (ECS). Higher distinctions between ICS and ECS were found in our data, i.e. 35 times for serine and 13 times for lysine. The differences between the results of Schneider and Dancis and the present study are perhaps due to different experimental methods. Smith et al. (1973) and Gussek et al. (1975) reported that the longer the pre-incubation period of the placental slice, the intracellular uptakes of amino acids are the greater.

Whether do our data indicate an example of active transport of amino acids in the placenta or not? Miller and Berndt (1974), using α -amino-isobutyric acid (AIB), carried out experiments under varying conditions of pH, temperature,

aerobic atmosphere, and with specimen groups added ouabain or 2,4-dinitrophenol (DNP). They showed that amino acid uptake in the placenta represents active transport depending on Mg^{++} ion and Na-K ATPase. Similar results were shown by other authors; Litonjua et al. (1967) in their experiments under aerobic and anaerobic atmosphere, Dancis et al. (1968) through experiments with metabolic inhibitors such as DNP and cyanide with the use of AIB, and Gussek et al. (1975) with protein synthesis inhibitors. Our experiment with ouabain seems to present an instance of active transport of serine, showing a 31% decrease at 90-min. A greater decrease of 58% in the corresponding figure was shown by Sybulski and Tremblay (1967) in their experiment with glycine. The difference may be due to the dissimilarity in the concentration of ouabain used (theirs 0.07 mM) as well as in the marker amino acid (glycine against serine). On the other hand, ouabain addition less affected lysine uptake, showing only an insignificant decrease. This may account for the difference in transport mode as mentioned above.

It is very suggestive that in the SFD placenta, the uptake of serine and lysine became significantly reduced at 30 min and later. It is notable that the figures are close to those observed in the $10^{-3}M$ ouabain-added normal placenta. This tends to strengthen our previous presumption that the low f/m ratio of blood amino acids in SFD pregnancy would account for a depressed active transport of amino acids in the SFD placenta, and emphasizes the possibility that such depression is attributable to the depressed activity of membrane-bound Na-K ATPase.

It is important to discuss in what manner amino acids once taken up in the placental tissues are released into the medium. The presumption prior to the experiment was that: (1) the release of amino acids into the medium would be greater from the normal placenta than from the SFD placenta, and (2) the amount of release would be greater for lysine than for serine inasmuch as the previous clinical data showed the f/m ratio higher for lysine than for serine, and the present data indicated intracellular uptakes two- to three-fold greater for serine than for lysine. Contrary to the presumption, the actual results disclosed that the amino acid release into the medium was slightly greater from the SFD than from the normal placenta, and also there was no significant difference in the release between serine and lysine. Considering no positive correlation observed between the f/m ratio and the amino acid uptake in the placental slice, the actual results seem to point out technical problems in experiments rather than indicating the absence of idiosyncrasy in extracellular release mechanism of amino acids.

In the test to substantiate the probability that placental uptake of amino acids is done through the active transport, it is necessary to analyse the activity of membrane-bound Na-K ATPase, which is known to play a key role in amino acid transport. So far the fairly successful removal of the membrane has been reported for the erythrocytes, liver cells and free culture cells, but there are no reports available for the trophoblastic membrane perhaps because of difficulties in its isolation. In our present experiment, the trophoblastic membrane could be

removed using Emmelot's method although it was not fairly pure. But the data are enough to suggest that the depressed activity of transport ATPase in the trophoblastic membrane had probably caused a decrease in uptake of amino acids in the SFD placenta.

Among reports on transport ATPase in the placenta available at present, Ishii et al. (1971) stated that the enzyme's activity is less in the placenta than in the erythrocytes, kidney and brain, and has no distinct difference between the early and term placentas. Performing more detailed analysis of the enzyme, Miller and Berndt (1973) stated that the placenta has at least two varieties of ATPase; one is Ca^{++} -ATPase that concerns the transport of Ca^{++} , and other is Mg^{++} -dependent Na-K ATPase which acts in the transport of amino acids and other organic compounds. Some of other authors' reports tend to support our review: Marton and Spearing (1972) in their electrophysiological research in the frog's skin, found a decrease in membrane potential with addition of pre-eclamptic serum; Lister (1966), in his electron-microscopic study on the placenta, suggested the phosphatase activity decreased in hypertensive pregnancy. Contrarily, Kuhnert et al. (1977), citing no change in ATPase activity in the pre-eclamptic placenta, suspected that the depressed amino acid transport in the pre-eclamptic placenta is likely to account for choline acetyl transferase activity.

For identifying the mechanism of amino acid transport in the placenta, including that of SFD, it seems necessary to pursue the role of transport ATPase, choline acetyl transferase, γ -glutamyl cycle, adenyl cyclase-cyclic AMP system, and their interrelationships.

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