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Carrier-mediated transport system for cephalexin in human placental brush-border membrane vesicles

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The uptake of cephalosporin antibiotics, cephalexin, was studied with brush-border microvillous plasma membrane vesicles prepared and purified from human full-term placental syncytiotrophoblasts. The uptake of cephalexin by the membrane vesicles was not stimulated in the presence of an Na^+ gradient from the outside to the inside of the vesicles, whereas α-(methylamino)isobutyrate uptake into the vesicles of the same preparation was stimulated by an Na^+ gradient. The equilibrium level of cephalexin uptake decreased with increasing osmolarity of the medium, which indicates that cephalexin is transported into the membrane vesicles. When cephalexin concentrations were varied, the initial rate of uptake obeyed Michaelis-Menten kinetics with K_m and V_{max} values of 2.29 mM and 2.98 nmol/mg of protein per 60 s, respectively. The uptake of cephalexin was inhibited by structural analogues and sulfhydryl modifying reagents. These results indicate the existence of a carrier-mediated transport system for cephalexin in the human placental brush-border membranes.

Introduction

Transplacental passage of cephalosporin antibiotics, commonly used for suspected intrauterine infection, has been investigated by administrating them to mothers with subsequent estimations of the levels obtained in maternal serum and umbilical cord blood at time of delivery [1–3]. However, it is difficult to obtain further information on the mechanisms of transport of cephalosporin antibiotics through the brush-border membranes of placental syncytiotrophoblasts by this method. In other tissues including small intestine [4–6] and renal cortex [7–9], transport of cephalosporin antibiotics has been extensively studied using brush-border membrane vesicles; the results suggest the existence of a carrier-mediated transport system for these compounds. In placenta, although brush-border membrane vesicles have been recently used to study transport of amino acids [10–13], dipeptides [14], glucose [15] and ions [16], information is not yet available regarding transport of cephalosporin antibiotics by membrane vesciles. These backgrounds prompted us to investigate the mechanisms of placental transport of cephalexin, one of the most widely used cephalosporin antibiotics, using brush-border membrane vesicle prepared and purified from human full-term placental syncytiotrophoblasts. The results suggest that cephalexin is taken up across the placental brush-border membranes by a carrier-mediated system.

Materials and Methods

Preparation of membrane vesicles. Brush-border membrane vesicles were prepared from freshly obtained human full-term placenta by a modified procedure of the method of Smith et al. [17]; this procedure has been described previously [13]. Membrane vesicles were finally suspended in 2 mM Tris-Hepes buffer (pH 7.5) containing 300 mM D-mannitol and 0.1 mM $MgSO_4$, to give a final protein concentration of approx. 4 to 6 mg/ml. This membrane vesicle preparation showed a degree of alkaline phosphatase (EC 3.1.3.1) enrichment 33-times greater than that of the starting homogenate,

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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while acid phosphatase (EC 3.1.3.2) showed a one-fifth decrease in its specific activity. The purity of this membrane vesicle preparation was higher than that of those described previously [18,19]. The transport acitivty was observed to be intact even after the membrane vesicles had been stored for 8 weeks at −70°C.

Uptake studies. The uptake of cephalexin was carried out according to the procedure as described previously [13]. In the regular assay, cephalexin uptake was initiated by adding 50 μl of the membrane suspension to 50 μl of an incubation medium composed of 5 mM cephalexin, 50 mM D-mannitol, 0.1 mM $MgSO_4$, 20 mM Tris-Hepes buffer (pH 7.5) and either 200 mM NaCl or 200 mM KCl. Other additions are described in the figure legends. In the case of α-(methylamino)isobutyrate transport, a double-isotope medium containing α-[1-^{14}C](methylamino)isobutyrate (25 μCi/ml) and L-[^{3}H]glucose (75 μCi/ml) was used for determining the specific transport of α-(methylamino)isobutyrate. Corrections for simple diffusion and non-specific trapping of substrate were made by subtracting the amount of L-glucose associated with each sample. α-(Methylamino)isobutyrate uptake was initiated by adding 50 μl of the membrane suspension to 60 μl of an incubation medium composed of α-[1 − ^{14}C](methylamino)isobutyrate, L-[^{3}H]glucose, 50 mM D-mannitol, 0.1 mM $MgSO_4$, 20 mM Tris-Hepes buffer (pH 7.5) and either 220 mM NaCl or 220 mM KCl. Both the membrane suspension and the incubation medium were preincubated independently at 37°C for 5 min before mixing, followed by further incubation at 37°C. The uptake of the substrate was terminated by diluting the sample with a 40-fold excess of an ice-cold buffer composed of 150 mM NaCl, 50 mM $MgCl_2$, 30 mM D-mannitol and 10 mM Tris-Hepes buffer (pH 7.5). The diluted sample was immediately filtered through a Millipore filter (HAWP, 0.45 μm, 2.5 cm diameter) and washed once with 3 ml of the same ice-cold buffer. Cephalexin trapped on the filter was extracted with 300

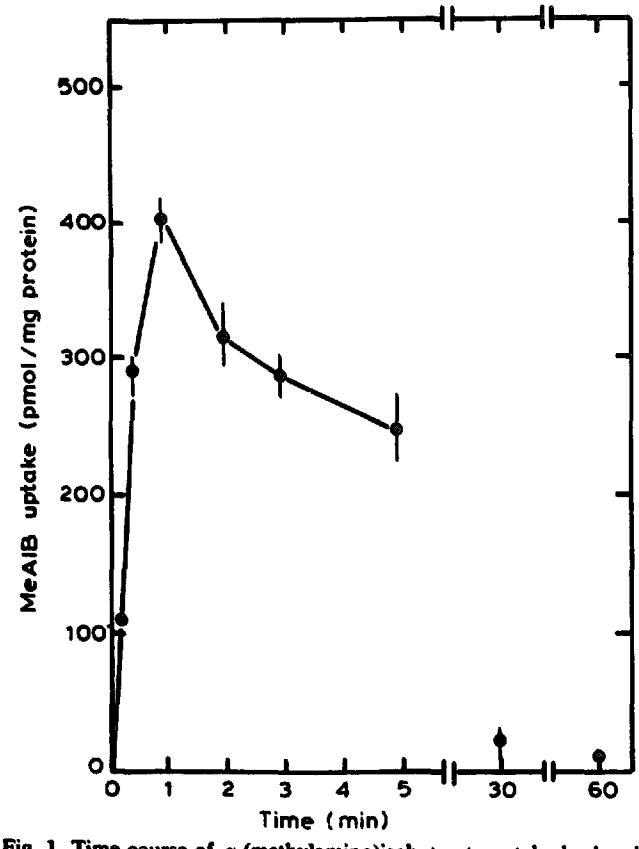

Fig. 1. Time course of α-(methylamino)isobutyrate uptake by brush-border membrane vesicles of human placenta in the presence of an Na^+ gradient (out > in). Membrane vesicles were suspended in a medium containing 300 mM D-mannitol, 0.1 mM $MgSO_4$ and 2 mM Tris-Hepes (pH 7.5). α-(Methylamino)isobutyrate (MeAIB) uptake was initiated by adding 50 μl of the membrane suspension (4–6 mg protein/ml) to 60 μl of an incubation medium composed of 36.7 μM α-[1 − ^{14}C](methylamino)isobutyrate, 36.7 μM L-[^{3}H]glucose, 50 mM D-mannitol, 0.1 mM $MgSO_4$, 20 mM Tris-Hepes (pH 7.5) and 220 mM NaCl. Both the membrane suspension and the incubation medium were preincubated independently at 37°C for 5 min before mixing, followed by further incubation at 37°C. Each point represents the mean ± S.D. for four experiments.

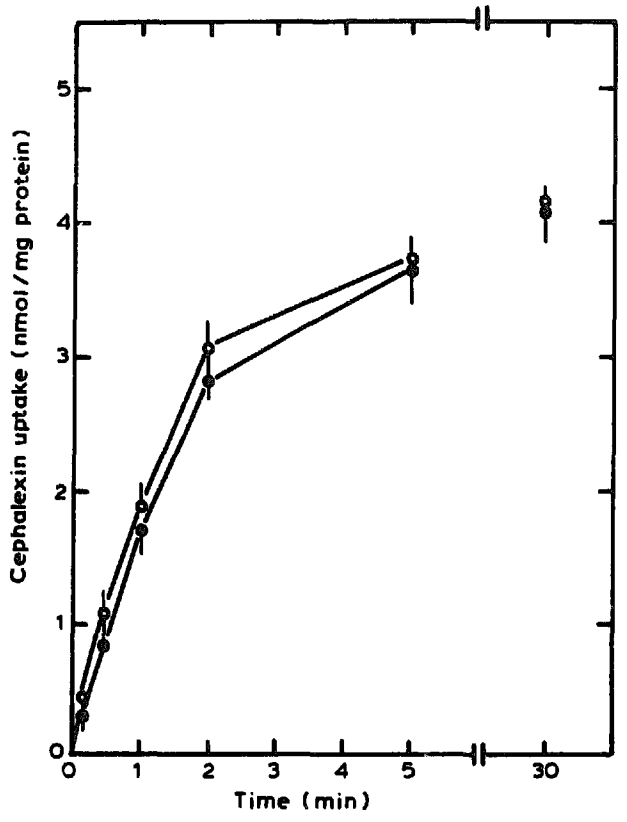

Fig. 2. Time course of cephalexin uptake by brush-border membrane vesicles. Membrane vesicles were suspended in a medium containing 300 mM D-mannitol, 0.1 mM $MgSO_4$ and 2 mM Tris-Hepes (pH 7.5). Cephalexin uptake was initiated by adding 50 μl of the membrane suspension (4–6 mg protein/ml) to 50 μl of an incubation medium composed of 5 mM cephalexin, 50 mM D-mannitol, 0.1 mM $MgSO_4$, 20 mM Tris-Hepes (pH 7.5) and either 200 mM NaCl or 200 mM KCl. Both the membrane suspension and the incubation medium were preincubated independently at 37°C for 5 min before mixing, followed by further incubation at 37°C. NaCl (●); KCl (○). Each point represents the mean ± S.D. for four experiments.

μl of distilled water and used for the determination by high-performance liquid chromatography [7]. The recovery of cephalexin from the filter was more than 95%. In separate experiments, non-specific trapping of substrate was determined by the addition of 50 μl of incubation medium to 3 ml of ice-cold stop solution containing 50 μl of membrane suspension. This value was substracted from the uptake data. The radioactivity of labeled substrate retained on the filter was counted by liquid scintillation. α-(Methylamino)isobutyrate uptake was proportional to membrane vesicle concentration up to a protein concentration of 9 mg/ml.

Analysis of cephalexin. Cephalexin taken up by the membrane vesicles was analyzed by a high-performance liquid chromatography using LC-6A system (Shimadzu, Kyoto, Japan) with the NOVA-PAK$^{TM}C_{18}$ column, 15 cm × 3.9 mm (Waters, MA, U.S.A.). The mobile phase consisted of 30 mM sodium phosphate buffer (pH 7.0) and 20% methanol, which were used after filtration through a 0.45 μm membrane filter and degassed by a vacuum aspirator. A 50-μl volume of the extract was injected on the column, chromatographed and detected at 260 nm. The minimum amount of cephalexin reproducible detected was 10 pmol and the calibration graph was linear to 1 nmol of cephalexin.

Protein estimation. Protein concentration of the vesicle preparation was determined by the method of Lowry et al. [20] using bovine serum albumin as a standard.

Chemicals. Cephalexin, cephalothin, cephaloridine, cefamandole, latamoxef (Shionogi Co. & Ltd., Osaka, Japan) and cephradine (Sankyo Co., Tokyo, Japan) were kindly supplied. α-[1 −^{14}C](Methylamino)isobutyrate (48.4 mCi/mmol) and L-[^{3}H]glucose (10.7 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were of the highest purity commercially available.

Results

Time course of α-(methylamino)isobutyrate uptake

In order to confirm the presence of the amino acid transport system in the membrane vesicles as shown in a previous paper [13], the time course of α-(methylamino)isobutyrate uptake by the membrane vesicles was examined. As shown in Fig. 1, the presence of an Na^+ gradient toward the inside from the outside of the membrane vesicles stimulated α-(methylamino)isobutyrate uptake and showed a typical 'overshoot' phenomenon of transport. In the absence of Na^+ gradient, α-(methylamino)isobutyrate uptake was below 2 pmol/mg of protein at any period (data not shown), indicating that Na^+ gradient-independent uptake was negligible. These results indicate the presence of a secondary active transport system for α-(methylamino)isobutyrate in brush-border membrane vesicles prepared from human full-term placental syncytiotrophoblasts and suggest that this membrane vesicle preparation is useful in uptake studies of cephalosporin antibiotics.

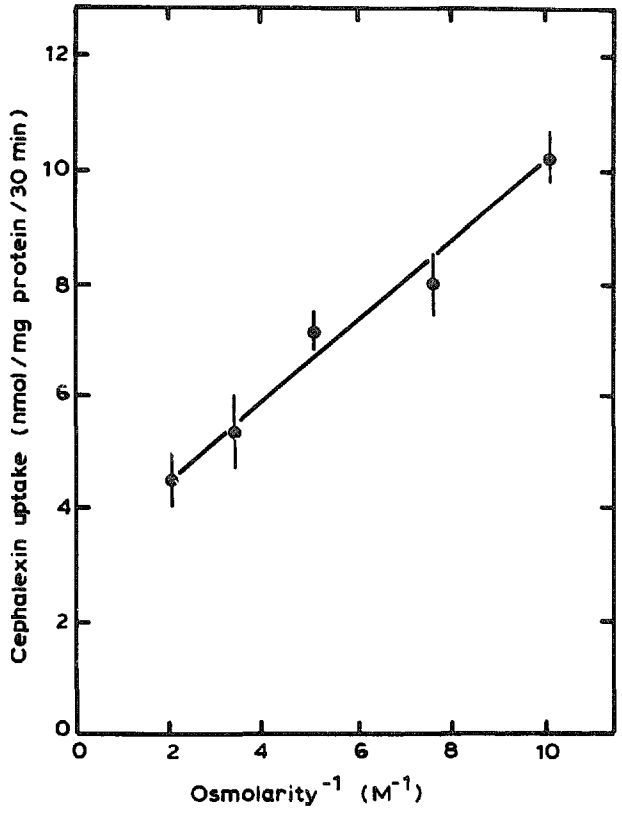

Fig. 3. Effect of medium osmolarity on cephalexin uptake by brush-border membrane vesicles. Cephalexin uptake was measured 30 min after incubation in a medium containing 2.5 mM cephalexin, 0.1 mM $MgSO_4$, 10 mM Tris-Hepes (pH 7.5) and 20 mM NaCl at final concentrations and D-mannitol was added to the medium to give the indicated osmolarities. Each point represents the mean ±S.D. for four experiments.

Time course of cephalexin uptake

The uptake of cephalexin by brush-border membrane vesicles as a function of incubation time is shown in Fig. 2. There was no significant difference between the cephalexin uptake in the presence of an Na^+ gradient and a K^+ gradient. A typical overshoot phenomenon observed in α-(methylamino)isobutyrate uptake was not observed. These results suggest that cephalexin uptake by the membrane vesicles is not dependent on an Na^+ gradient toward the inside from the outside of the vesicles.

Effect of medium osmolarity on cephalexin uptake

To ascertain that the uptake of cephalexin by the membrane vesicles represented the transport into a vesicular space rather than membrane binding, the relationship between the medium osmolarity and cephalexin uptake was studied (Fig. 3). When the intravesicluar space was decreased by increasing the medium osmolarity with D-mannitol, there was a linear relationship

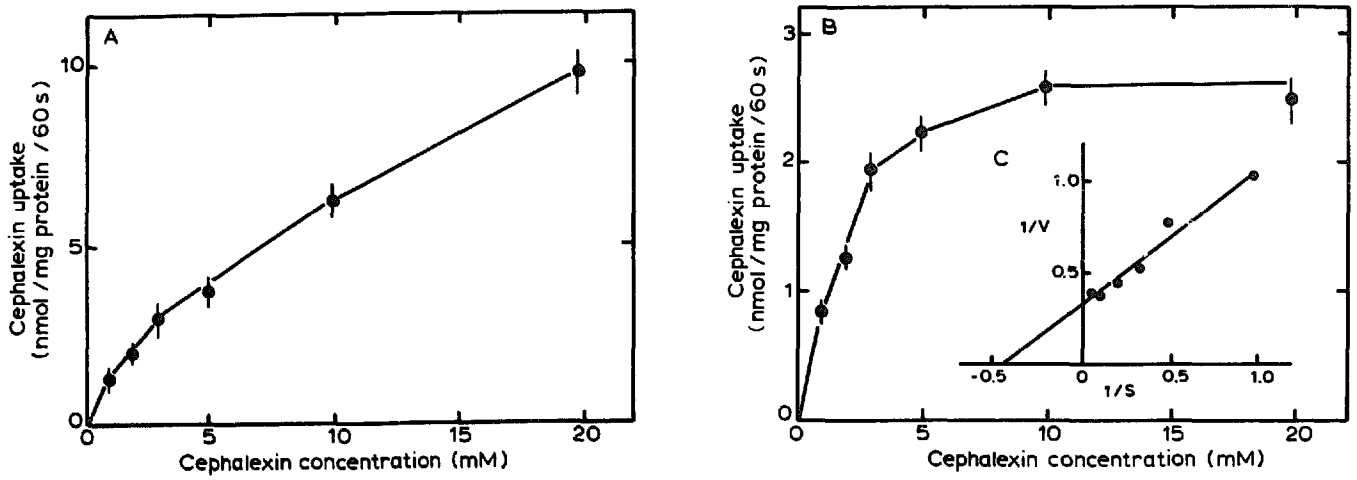

Fig. 4. Effect of cephalexin concentration on the initial rate of cephalexin uptake by brush-border membrane vesicles. (A) The uptake over a 60-s period was measured in medium containing cephalexin at the indicated concentrations, 175 mM D-mannitol, 0.1 mM $MgSO_4$, 10 mM Tris-Hepes (pH 7.5) and 100 mM NaCl at final concentrations. (B) Initial rate of uptake was obtained by correcting for the non-saturable component as described in the text. (C) A Lineweaver-Burk plots of the data; the line was determined by least squares linear regression analysis. Each point represents the mean ±S.D. for four experiments.

between cephalexin uptake and the reciprocal of the medium osmolarity. This indicates that cephalexin is transported through the brush-border membrane into the intravesicular space.

Effect of cephalexin concentrations on the initial rate of uptake

The effect of different concentrations of cephalexin over a range of 1 to 20 mM on the initial rate of uptake is illustrated in Fig. 4. There was a non-linear relationship between the concentration and the initial rate of uptake, providing evidence for saturability (Fig. 4A). In order to analyze the saturation of cephalexin uptake, the amount of the non-saturable component was subtracted from the total uptake at each concentration. The amount of the non-saturable uptake could be determined by employing the straight-line equation generated at higher cephalexin concentrations. As shown in Fig. 4B, the uptake rate showed a saturable hyperbolic curve that obeyed Michaelis-Menten kinetics after the correction for the non-saturable component. A Lineweaver-Burk plots (Fig. 4C) corresponding to the initial rate of uptake over a concentration range of 1 to 20 mM showed a straight line. The calculated values of K_m and V_{max} for the cephalexin uptake were 2.29 mM and 2.98 nmol/mg of protein per 60 s, respectively.

Effect of other cephalosporin antibiotics on cephalexin uptake

The initial rate of cephalexin uptake at 1.25 mM was measured in the presence of a structural analogue at a concentration of 10 mM. As shown in Table I, the uptake of cephalexin was significantly inhibited by any of the inhibitors tested. The results indicate that a carrier-mediated system is involved in cephalexin uptake by brush-border membrane vesicles isolated from human full-term placental syncytiotrophoblasts.

Effect of sulfhydryl modifying reagents on cephalexin uptake

In order to further confirm the existence of a carrier-mediated transport system for cephalexin in placental brush-border membranes, the effect of sulfhydryl modifiying reagents, *N*-ethylmaleimide or $HgCl_2$, on the initial rate of uptake was determined (Fig. 5). Both reagents inactivated cephalexin uptake in a concentration-dependent manner. The half-maximal inhibition of cephalexin uptake was observed at about 1.8 mM and approximately 96% inhibition at 5 mM $HgCl_2$. However, as high as 10 mM *N*-ethylmaleimide was needed to produce 52% inhibition of cephalexin uptake. These findings support the view described above that a

TABLE I

Effect of other cephalosporin antibiotics on cephalexin uptake by brush-border membrane vesicles

Cephalexin uptake over a 60-s period was measured in medium containing 1.25 mM cephalexin, 150 mM D-mannitol, 0.1 mM $MgSO_4$, 10 mM Tris-Hepes (pH 7.5) and 100 mM NaCl in the presence of 10 mM other cephalosporin antibiotics at final concentrations. The osmolarity of the medium was adjusted to a constant value by the addition D-mannitol. Values are mean ±S.D. for four experiments.

Inhibitor	Cephalexin uptake	
	nmol/mg protein per 60 s	%
None	2.95±0.46	100
Cephradine	1.35±0.03	46
Cefamandole	1.43±0.15	49
Cephalothin	1.33±0.22	45
Cephaloridine	1.79±0.30	61
Latamoxef	1.71±0.26	58

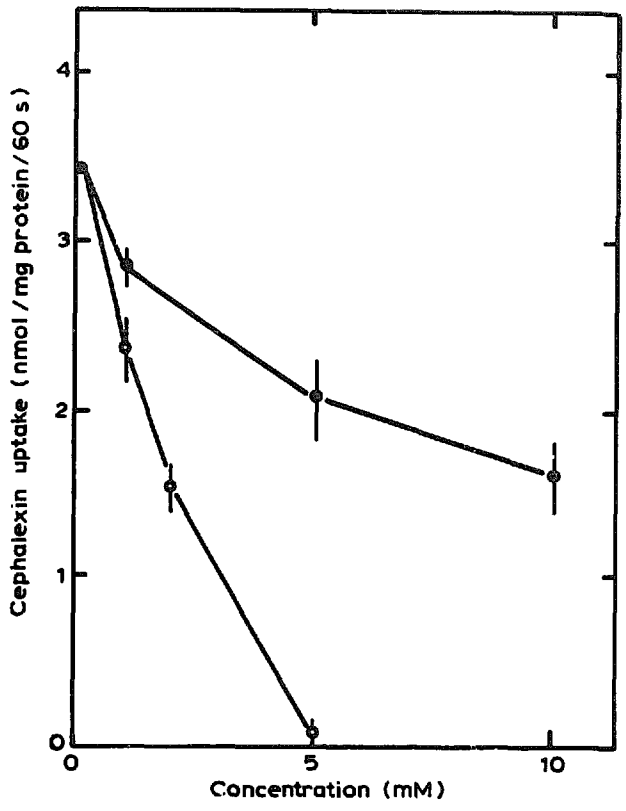

Fig. 5. Effect of sulfhydryl modifying reagents on cephalexin uptake by brush-border membrane vesicles. Membranes vesicles were pre-cincubated with the indicated concentrations of either N-ethylmaleimide or $HgCl_2$ for 10 min at 25°C prior to starting the uptake. Cephalexin uptake over a 60-s period was measured in medium containing 2.5 mM cephalexin, 175 mM D-mannitol, 0.1 mM $MgSO_4$, 10 mM Tris-Hepes (pH 7.5) and 100 mM NaCl in the presence of the indicated concentrations of sulfhydryl modifying reagents. *N*-Ethylmaleimide (●); $HgCl_2$ (○). Each point represents the mean ± S.D. for four experiments.

carrier-mediated system is involved in cephalexin uptake by human placental brush-border membrane vesicles and suggest that sulfhydryl groups are essential for this system.

Discussion

The placenta is the primary site of solutes exchange between the mother and the fetus, and it occurs across the brush-border membrane of the syncytiotrophoblasts. This paper describes the properties of cephalexin, one of the most widely used cephalosporin antibiotics, uptake by the brush-border membrane vesicles prepared from human full-term placental syncytiotrophoblasts, which has not been reported previously. The brush-border membrane vesicles used in the present experiments show a higher degree of purity than those described previously [18,19] and contain both Na^+-dependent and Na^+-independent transport systems for various amino acids [13]. These indicate this membrane preparation is suitable for analysis of the uptake properties of cephalexin across the human placental brush-border membranes.

The results obtained indicate the existence of a carrier-mediated transport system for cephalexin in the human full-term placental brush-border membranes. The uptake of cephalexin is saturable and inhibited by structural analogues and sulfhydryl modifying reagents such as *N*-ethylmaleide and $HgCl_2$. The presence of an Na^+ gradient from the outside to the inside of the vesicles did not stimulate cephalexin uptake, whereas α-(methylamino)isobutyrate uptake was accelerated by an Na^+ gradient under the same condition.

In previous investigations of cephalosporin antibiotics transport using brush-border membrane vesicles prepared from small intestine [4–6] and renal cortex [7–9], it was reported that cephalosporin antibiotics such as cephalexin and cephradine were taken up by an Na^+-independent carrier-mediated system, and that the uptake of them was stimulated by an H^+ gradient. Furthermore, photoaffinity labeling studies have demonstrated that cephalosporin antibiotics and dipeptides interact with an identical membrane protein in the brush-border membranes of the small intestine [6]. The existence of a transport system for dipeptides has also been demonstrated in human placental brush-border membrane vesicles [14]. This dipeptide transport system may be available for the transport of cephalosporin antibiotics in placental membranes as it is in renal and intestinal membranes.

In the first series of experiments in cephalosporin antibiotics transport by placental brush-border membrane vesicles, we investigated the uptake properties of cephalexin. The results obtained provide strong evidence for the existence of a carrier-mediated transport system for cephalexin in human placental brush-border membrane vesicles. Since therapeutic concentrations of cephalosporin antibiotics in plasma are several orders of magnitude lower than those of used in this paper, we cannot clearly define the pharmacological significance of our observation. However, fetal plasma concentrations of cephalosporin antibiotics often exceed maternal plasma concentrations during therapy and this transport system may play an important role in placental transfer of these compounds. In order to further explore the mechanisms of cephalosporin antibiotic transport in brush-border membrane vesicles of human placenta, we are currently examining the energetics and specificity of this transport system.

Acknowledgements

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