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TRANSPORT OF CEFADROXIL, AN AMINOCEPHALOSPORIN ANTIBIOTIC, ACROSS THE SMALL INTESTINAL BRUSH BORDER MEMBRANE

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Abstract—The transport characteristics of cefadroxil, an aminocephalosporin antibiotic, across the brush border membrane of rat small intestine were investigated by a rapid filtration technique. The uptake of cefadroxil was not affected by Na^+ gradient, suggesting the absence of a cotransport system between cefadroxil and Na^+ in the brush border membrane. The uptake was slightly inhibited by $HgCl_2$ pretreatment and stimulated by the countertransport effect, where cyclacillin played a role as an elicitor. These results suggest the existence of a carrier-mediated transport system for cefadroxil in the brush border membrane, which is shared with cyclacillin. Papain treatment increased the specific transport activities for the antibiotic. This may be the first step of purification of the cefadroxil transport carrier.

Our previous report demonstrated carrier-mediated transport systems for aminocephalosporins in rat small intestine [1]. In *in situ* recirculation experiments through the whole small intestine, cefadroxil showed the highest absorption among the tested antibiotics, in spite of its lowest lipophilicity, and its absorption was inhibited by the simultaneous perfusion of other amino-β-lactam antibiotics. So, the carrier system for cefadroxil seems to be shared, at least in part, with the others. The mucosal-to-serosal uphill transport for cefadroxil was also demonstrated by the *in vitro* technique.

In all these complex preparations, net transport represents the sum of numerous processes, both in series and parallel. The present study was designed to further characterize the carrier system for cefadroxil absorption by using the small intestinal brush border membrane vesicles.

MATERIALS AND METHODS

Materials. Cefadroxil (Bristol Meyers Co., Tokyo) and cyclacillin (Takeda Chemical Industries, Osaka) were used as supplied. Papain and D-[^{14}C]glucose were purchased from E. Merck, Darmstadt, West Germany, and the New England Nuclear Corp., Boston, MA, U.S.A., respectively. All other reagents used in these experiments were of reagent grade and were used without further purification.

Preparation of brush border membrane vesicles. Male Wistar rats weighing 180–250 g were used. Brush border membrane vesicles were prepared by the method of Kessler *et al.* [2]. Briefly, scraped intestinal mucosa was suspended in a buffer containing 50 mM mannitol and 2 mM Tris–HCl (pH 7.1), and vigorously homogenized in a Waring blender for 2 min. After the addition of $CaCl_2$ (final concentration, 10 mM), the suspension was centrifuged at 3000 *g* for 15 min. The brush border membrane was collected from the supernatant fraction at 27,000 *g* for 30 min, and washed once by centrifugation.

For transport studies, the final pellet was resuspended in a buffer containing 300 mM mannitol and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–Tris (pH 6.5) (buffer A). The specific activity of a typical brush border enzyme, alkaline phosphatase, was enriched 9.3-fold compared to those found in the homogenate, as described elsewhere [3].

Transport experiments. The composition of the incubation medium was 300 mM mannitol, 10 mM HEPES–Tris (pH 6.5), 200 mM NaSCN (or KSCN), and as a drug either 0.42 mM D-[^{14}C]glucose (2 μCi/ml) or 2.5 mM cefadroxil in the final concentration. The uptake of substrates was determined by a rapid filtration technique [4]. Transport studies were initiated by the addition of 20 μl of the medium to 20 μl of the vesicle suspension (5–10 mg protein/ml) at room temperature. At the desired times, 2 ml of ice-cold stop solution (250 mM NaCl and 1 mM Tris–HCl, pH 6.5) was added to the mixture. The resulting mixtures were immediately filtered through pre-wetted 0.45 μm filters (Fuji Photo Film, Tokyo). The filters were quickly rinsed with 5 ml of the ice-cold stop solution twice and transferred into a counting vial for the determination. Background value or nonspecific adsorption to the filter was determined by using buffer A without brush border membrane vesicles. This value was subtracted from the uptake data.

Pretreatment of vesicles with $HgCl_2$. Brush border membrane vesicles were treated with 25 μM $HgCl_2$ for 5 min at 0° according to the method of Klip *et al.* [5]. The reaction was stopped by 7- to 10-fold dilution with buffer A either in the presence or absence of 5 mM dithiothreitol (DTT). After standing for

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10 min at 0°, the suspensions were centrifuged at 6000 *g* for 30 min. The pelleted membranes were resuspended in buffer A for transport studies.

Papain digestion of vesicles. Papain digestion was carried out according to the modified procedure of Louvard *et al.* [6]. Briefly, the commercial papain was activated before use by incubating it in buffer A containing 5 mM cysteine at 0°. Vesicle suspensions were incubated with 0.5 mg/ml activated papain at room temperature for 5 min. After the incubation, the mixture was diluted 10-fold with ice-cold buffer A and centrifuged at 27,000 *g* for 30 min. The pelleted membranes were resuspended in buffer A for transport studies.

Analytical methods. For D-glucose uptake, radioactivities were measured by a Beckman LS-232 liquid scintillation counter.

For cefadroxil uptake, a high pressure liquid chromatography (HPLC) method was used. The cephalosporin trapped on the filter was extracted with 300 μl of distilled water as described by Inui *et al.* [7], and aliquots were injected into HPLC. A high pressure liquid chromatograph TRI ROTAR (Japan Spectroscopic Co., Tokyo) was equipped with an ultraviolet detector (UVIDEC 100-III, Japan Spectroscopic Co.) and a Cosmosil 5C18 column (15 cm × 4.6 mm i.d., Nakarai Chemical Co., Kyoto). The mobile phase of methanol–water containing 0.02 M, pH 7.5, sodium phosphate buffer and 5 mM tetra-*n*-butylammonium bromide was 18:82 (v/v). The flow rate was maintained at 0.8 ml/min, and the wavelength of the detector was 262 nm. The drug concentration was calculated from the peak height using the calibration curve.

Protein was assayed by the method of Lowry *et al.* [8] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

D-Glucose transport across the brush border membrane at pH 7.5 showed an "overshoot" phenomenon, that is, a transient 2.3-fold accumulation above the equilibrium level by the addition of NaSCN. This agrees well with the data reported previously [3, 4], and prepared vesicles proved to be suited for transport studies.

Since the physiological pH of the small intestinal lumen seems to be 6.5 [9], it may be reasonable to examine the transport characteristics at pH 6.5. Similar overshoot phenomenon in the presence of a 100 mM NaSCN gradient (outside to inside) was observed, suggesting that the Na^+-dependent D-glucose transport system is operative at this pH. The transient accumulation above the equilibrium level was 1.7-fold at this condition.

Figure 1 shows cefadroxil uptake into brush border membrane vesicles. Contrary to the case of D-glucose, no "overshoot" was observed, and the time course curves were almost similar in the presence of a 100 mM NaSCN gradient or a KSCN gradient. This agrees well with the uptake characteristics of other aminocephalosporins, cephalexin and cephradine, in rat renal brush border membrane [7]. This non-Na^+ dependency will be discussed later.

To distinguish between binding of cefadroxil to the brush border membrane and transport of this antibiotic into the intravesicular space, the uptake was measured by raising the osmolarity of the outer medium with mannitol. As shown in Fig. 2, the amount of the antibiotic taken up was dependent on the medium osmolarity, suggesting that the drug entered an intravesicular space. However, extrapolation to infinite osmolarity (zero space) showed the positive intercept, indicating that considerable binding is also involved. The estimated binding under the incubation conditions normally used was approximately 38%. All the uptake data shown below were not corrected for the binding.

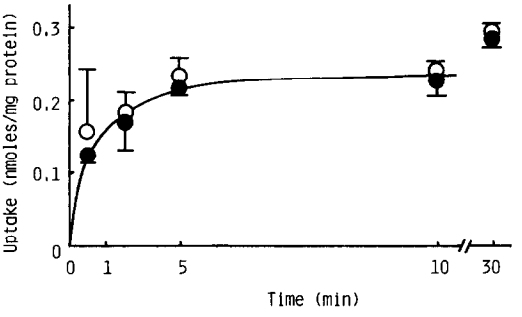

Fig. 1. Time course of cefadroxil uptake by intestinal brush border membrane vesicles. Cefadroxil concentration was 2.5 mM. Two different media prepared in 10 mM HEPES–Tris buffer (pH 6.5) containing 300 mM mannitol and either 200 mM NaSCN (●) or 200 mM KSCN (○) were used. Each point represents the mean ± range of two experiments.

To clarify the presence of the transport carrier for cefadroxil, the effect of $HgCl_2$ pretreatment of the brush border membrane on cefadroxil uptake was examined first. Results were compared among the ratios of the initial uptake at 1 min to the equilibrium uptake (mean ± S.E.). Initial cefadroxil uptake tended to be inhibited by the $HgCl_2$ pretreatment

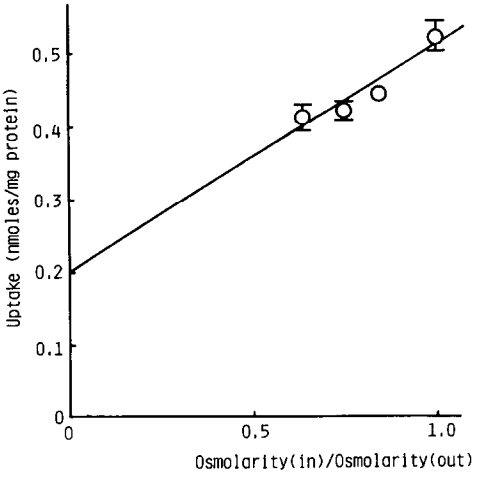

Fig. 2. Cefadroxil uptake as a function of the osmolarity of the extravesicular medium. Cefadroxil uptake is shown after 10 min. The osmolarity was varied by the addition of mannitol. Each point represents the mean ± S.E. of three experiments.

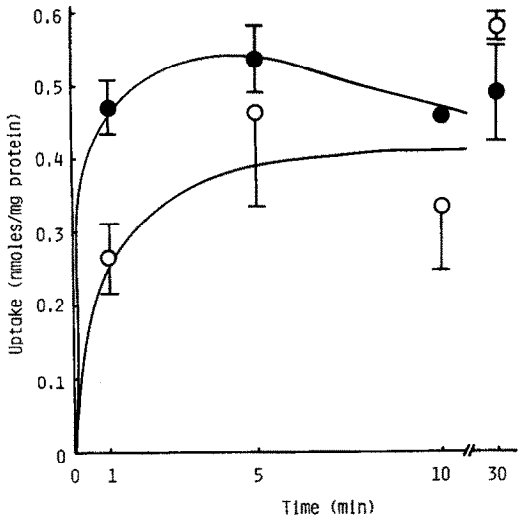

Fig. 3. Countertransport effect on cefadroxil uptake by intestinal brush border membrane vesicles. The vesicles were preincubated with 10 mM HEPES–Tris buffer (pH 6.5) containing 300 mM mannitol in either the presence (●) or absence (○) of 25 mM cyclacillin. Each point represents the mean ± S.E. of two or three experiments.

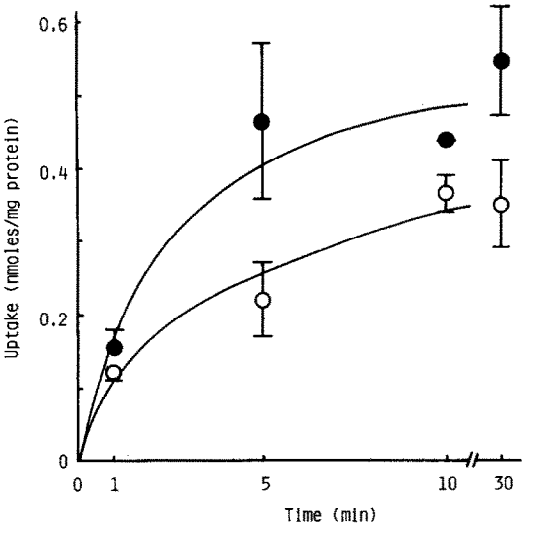

Fig. 4. Time course of cefadroxil uptake by normal (○) and papain-treated (●) brush border membrane vesicles. Drug concentration was 2.5 mM. The medium contained 10 mM HEPES–Tris (pH 6.5), 100 mM NaSCN, and 300 mM mannitol. Each point represents the mean ± S.E. of three experiments.

(0.527 ± 0.063 to 0.443 ± 0.031), although the differences were not statistically significant. This inhibition was reversed by 5 mM DTT (0.634 ± 0.037, $P < 0.05$). This suggests that sulfhydryl groups within the brush border membrane participate in cefadroxil uptake, which is consistent with the observations in the intact tissue [1].

Figure 3 shows the result of countertransport experiments for cefadroxil uptake. For preloading, the pellet of brush border membranes was resuspended with buffer A containing 25 mM cyclacillin, whose carrier system might be common, at least in part, to cefadroxil [1]. At the start of incubation, the vesciles were diluted 10-fold. As is evident from the figure, vesicles containing cyclacillin showed higher cefadroxil uptake than control vesicles, except at equilibrium, which indicates that cyclacillin played a role as an elicitor for the countertransport. This result suggests that cefadroxil is transported across the brush border membrane by a carrier-mediated process and shares a common carrier system in the brush border membrane with cyclacillin.

As shown in Fig. 1, this carrier system for cefadroxil transport in the brush border membrane was non-Na^+ dependent, i.e. cotransport system between cefadroxil and Na^+ was absent in the brush border membrane. This is puzzling in view of the results of our previous study which showed the Na^+ dependency of the mucosal-to-serosal flux of this drug in the everted intestinal sacs [1]. The dependency of the aminocephalosporin transport on Na^+ observed in the intact tissue preparation could possibly be due at least in part to solvent drag [10] driven by the Na^+-dependent bulk flow of fluid [11], which is absent in the brush border preparation. In addition, metabolic energy converting reaction for uphill transport of cefadroxil observed in everted sacs [1] seems not to be located in the brush border membrane, since accumulation of the antibiotic against the concentration gradient did not take place in the vesicle preparation (estimated by comparing equilibrium values of uptake for D-glucose and the cephalosporin).

The enzyme proteins of the brush border membrane are mainly hydrophilic molecules that reside in the membrane with a hydrophobic tailpiece. The linkage between the hydrophobic tail and the main body of some of these enzymes is sensitive to proteolytic cleavage by papain. As an approach to the purification of the carrier protein, the effect of papain treatment of brush border membrane vesicles on cefadroxil transport into the vesicles was examined. As shown in Fig. 4, the treatment of brush border membrane vesicles with soluble papain led to an increase in the specific activity of cefadroxil uptake. This indicates that some brush border membrane proteins were removed by papain treatment without loss of the transport activity. Similarly, Berteloot *et al.* [12] reported that papain-treated vesicles exhibited increased specific activities of leucine and glucose uptake. This can be the first step of purification of the cefadroxil transport carrier in the brush border membrane.

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REFERENCES

1. T. Kimura, T. Yamamoto, M. Mizuno, Y. Suga, S. Kitade and H. Sezaki, *J. Pharmacobio-Dynamics* **6**, 246 (1983).

2. M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Müller and G. Semenza, *Biochim. biophys. Acta* **506**, 136 (1978).
3. M. Yasuhara, T. Kimura and H. Sezaki, *J. Pharmacobio-Dynamics* **6**, 888 (1983).
4. U. Hopfer, K. Nelson, J. Perrotto and K. J. Isselbacher, *J. biol. Chem.* **248**, 25 (1973).
5. A. Klip, S. Grinstein, J. Biber and G. Semenza, *Biochim. biophys. Acta* **598**, 100 (1980).
6. D. Louvard, S. Maroux, Ch. Vannier and P. Desnuelle, *Biochim. biophys. Acta* **375**, 236 (1975).
7. K. Inui, T. Okano, M. Takano, S. Kitazawa and R. Hori, *Biochem. Pharmac.* **32**, 621 (1983).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
9. T. Koizumi, T. Arita and K. Kakemi, *Chem. pharm. Bull., Tokyo* **12**, 421 (1964).
10. A. Karino, M. Hayashi, S. Awazu and M. Hanano, *J. Pharmacobio-Dynamics* **5**, 670 (1982).
11. S. G. Schultz, in *Physiology of the Gastrointestinal Tract* (Eds. L. R. Johnson, J. Christensen, M. I. Grossman, E. D. Jacobson and S. G. Schultz), p. 983. Raven Press, New York (1981).
12. A. Berteloot, R. W. Bennetts and K. Ramaswamy, *Biochim. biophys. Acta* **601**, 592 (1980).