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Title page.

Functional expression of stereoselective metabolism of cephalexin by exogenous transfection of oligopeptide transporter PEPT1

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Running title page.

Running title:

Transfection of PEPT1 confers stereoselective metabolism of CEX

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Abbreviations:

PEPT1, H^+/oligopeptide transporter 1; D-CEX, D-stereoisomer of cephalexin, (6R,7R)-7-[[(2R)-2-amino-2-phenyl-acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid; L-CEX, L-stereoisomer of cephalexin, (6R,7R)-7-[[(2S)-2-amino-2-phenyl-acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid; 7-ADCA, 7-aminodesacetoxycephalosporanic acid, 7-amino-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride

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Abstract

Gastrointestinal absorption of the ß-lactam antibiotic cephalexin (CEX) is highly stereoselective: L- and D-CEX are both taken up by intestinal epithelial cells through the brush-border membrane, most likely via oligopeptide transporter PEPT1, but L-CEX is not found in serum or urine after oral administration due to its rapid intestinal metabolism, whereas D-CEX is well absorbed in the unchanged form. We examined the contribution of PEPT1 to the stereoselective uptake and metabolism of CEX. We observed stereoselective metabolism of CEX after exogenous transfection of PEPT1 alone into mammalian cell lines: L-CEX, but not D-CEX, was metabolized to 7-aminodesacetoxycephalosporanic acid (7-ADCA) in HeLa and HEK293 cells stably and transiently expressing human PEPT1, respectively, whereas such metabolism was minor in cells expressing the vector alone. The formation rate of 7-ADCA depended on the amount of PEPT1 cDNA transfected. L-CEX metabolism was rapid, since only 7-ADCA was found inside and outside the cells during incubation with L-CEX. The characteristics of PEPT1-mediated metabolism of L-CEX were similar, but not identical, to those of PEPT1-mediated transport. PEPT1-mediated metabolism was also observed in permeabilized cells expressing PEPT1, in which PEPT1-mediated intracellular substrate accumulation was negligible, suggesting that the increase in L-CEX metabolism by PEPT1 transfection cannot be fully explained by an increase in uptake and subsequent exposure to intracellular hydrolases. The present findings demonstrate that stereoselectivity in CEX absorption can be fully explained in terms of PEPT1, implying that the L-CEX hydrolase is PEPT1 itself, or is induced by PEPT1.

Introduction

Cephalexin (CEX), an orally effective first-generation α-amino-ß-lactam antibiotic, exists as two diastereoisomers, L-CEX and D-CEX (Fig. 1), but as with all other commercially available ß-lactam antibiotics, only the D-isomer is used as an ethical drug. L-CEX is not used as a medicine, because it is rapidly metabolized after oral administration (Tamai et al., 1988) and it lacks antibacterial activity (Snyder et al., 1997). D-CEX is a highly hydrophilic compound, and it mainly exists in ionized form in to the vicinity of the brush border membrane of intestinal epithelial cells, where the microclimate pH is 6.5 - 7.5 in the rat jejunum (Iwatsubo et al., 1986), since the pKa values of D-CEX are 2.56 and 6.88 (Yamana and Tsuji 1976). Nevertheless, permeability of D-CEX through the small intestine is good, and the oral bioavailability is more than 90% (Hardman and Limbird (eds.) 2001). Many studies have been performed to understand the mechanism(s) of the efficient intestinal absorption, and it has been suggested that the transport of D-CEX through the intestinal membrane is governed by a carrier-mediated mechanism (Tsuji et al., 1981a; 1981b; Nakashima et al., 1984; Dantzig and Bergin 1990; Kramer et al., 1990), which also recognizes di- and tripeptides.

The transport system(s) responsible for D-CEX uptake in small intestine is proton-dependent, but sodium-independent, and is stereoselective, i.e., L-CEX was not found in either serum or urine as an unchanged form, though 7-ADCA (Fig. 1), a metabolite of L-CEX generated by hydrolysis at the peptide bond, was detected. On the other hand, D-CEX was detected as the unchanged form in serum and urine after oral administration in rats (Tamai et al., 1988). It was initially suggested that L-CEX is not transported by the oligopeptide transporter, since L-CEX was not detected in rabbit intestinal brush-border membrane vesicles (BBMV) or proteoliposomes prepared from rabbit intestinal BBMV (Kramer et al., 1992). However, it was demonstrated that L-CEX binds to the peptide

transporter with high affinity, and L-CEX is transported, by assessing substrate-induced intracellular acidification caused by H^+ co-transported with the substrate in Caco-2 cells (Wenzel et al., 1995). As regards affinity of CEX to the H^+-coupled peptide transporter, L-CEX competitively inhibited D-CEX uptake more strongly than did D-CEX itself in a rat intestinal everted sac, a human intestinal model cell line Caco-2 cells (Tamai et al., 1988; Wenzel et al., 1995; Snyder et al., 1997). Thus, although L-CEX seems to be transported via the same carrier system as D-CEX, and the affinity of the transporter for L-CEX is higher than that for D-CEX, L-CEX is not detected inside the cells because it is metabolized very rapidly.

The oligopeptide transporter PEPT1 (SLC15A1) (Fei et al., 1994; Liang et al., 1995; Saito et al., 1995), which belongs to the proton/oligopeptide cotransporter (POT) family, has been shown to contribute to the uptake from the apical side of CEX and other ß-lactam antibiotics with peptide-type structures (Ganapathy et al., 1995; Tsuji and Tamai 1996; Tamai et al., 1997) in the small intestine. PEPT1 is expressed on the apical membranes of epithelial cells in the small intestine (Sai et al., 1996), and its expression level correlates with absorptive transport of ß-lactam antibiotics, as well as a dipeptide model compound, glycylsarcosine (Gly-Sar), in rats (Shiraga et al., 1999; Naruhashi et al., 2002). PEPT1 also mediates uptake of various other drugs, including certain angiotensin-converting enzyme inhibitors, an anticancer agent (ubenimex) and an antiviral agent (valacyclovir), as well as di- and tripeptides derived from nutrients (Balimane et al., 1998; Tsuji, 2002; Rubio-Aliaga and Daniel, 2002; Terada and Inui 2004; Daniel, 2004).

Although the pharmacokinetic properties of CEX have been well investigated, the molecular mechanism(s) responsible for the rapid metabolism of L-CEX has not yet been clarified. We hypothesized that PEPT1 would play a major role in the molecular mechanism of the stereoselective metabolism, as well as the membrane transport of CEX.

Therefore, we examined the contribution of PEPT1 to the stereoselective uptake and metabolism of CEX using *in vitro* PEPT1-expressing cell lines.

Methods

Materials

L-CEX was obtained from Nippon Bulk Yakuhin Co., Ltd. (Osaka, Japan). 7-ADCA and all other reagents of analytical grade were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Nacalai tesque, Inc. (Kyoto, Japan) and used without further purification. HEK293 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HeLa cells stably expressing human PEPT1 (HeLa/PEPT1) or vector alone (Mock) were previously established in our laboratory (Nakanishi et al., 2000). The protein assay kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

Cell culture

HeLa/PEPT1 and Mock cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; from Cansera International, Ontario, Canada), 2 mM L-glutamine, and 1 mg/mL geneticin (G418), as described previously (Nakanishi et al., 2000). For the uptake and metabolism assay, each cell line was seeded on 6-well plates (Nunc, Naperville, IL) and cultured for 3 days. HEK293 cells were grown in DMEM supplemented with 10% FBS, 1 mg/mL sodium pyruvate, 100 µg/mL streptomycin and 100 unit/mL benzylpenicillin.

In Vitro Gene Transfer to HEK293 Cells

HEK293 cells were plated onto 6-well plates for 24 hours before cDNA transfection. Transfection was performed after the cells had reached 40 to 60% confluence, by means of the calcium phosphate precipitation method (Watanabe et al.,

2005). The uptake and metabolism assays were performed 40 to 48 hours after transfection.

Uptake and Metabolism Experiments

The experiments that were conducted with HeLa cells were performed at 37°C in Hanks' balanced salt solution (HBSS) (136.7 mM NaCl, 5.36 mM KCl, 25 mM D-glucose, 0.952 mM $CaCl_2$, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 0.385 mM Na_2HPO_4 and 10 mM MES or HEPES) adjusted to pH 5.5 to 8.0. The osmolality of the HBSS was 305 ± 5 mOsm/kg. The experiments that were conducted with HEK293 cells were performed in MES buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM MES) adjusted to pH 6.0. The osmolality of the MES buffer was 290 ± 5 mOsm/kg. Cultured cells were washed and pre-incubated in the buffer without test compounds for 20 min at 37°C and pH 7.4. Then the reaction was initiated by replacing the medium with fresh buffer (2 mL) containing L- or D-CEX (28.8 μM). After incubation for desired times at 37°C, the experiment was terminated by removing the medium, followed by washing three times with ice-cold buffer.

To prepare digitonin-permeabilized cells, HeLa cells were treated in HBSS (pH 6.0) containing 125 μg/mL of digitonin for 10 min at room temperature. HBSS was replaced with hypotonic buffer (10 mM MES, 25 mM D-glucose, adjusted to pH 6.0) for hypotonic treatment of the cells. Cellular protein was determined using a protein assay kit with bovine serum albumin as a standard.

Metabolism Experiments in Cellular Homogenates

Cells were collected with a cell scraper (Asahi Techno Glass Corporation Co. Ltd., Chiba, Japan) and suspended in 10 mM MES/NaOH (pH 6.0) or 10 mM Tris/HCl (pH 7.4). Cell suspensions were then homogenized on ice with 100 strokes of a 1 mL tapered tissue grinder (Potter-Elvehjem Teflon homogenizer; 358133, Wheaton Instruments, Millville, NJ), and centrifuged at 1500 rpm and 4°C for 5 min (Hitachi Himac CF15R; Tokyo, Japan). The 180 µL of supernatant was used for assay. The reaction was initiated by adding the medium (20 µL) containing L-CEX (final concentration, 288 µM). After incubation for 60 min at 25°C, the experiment was terminated by adding 800 µL of ice-cold methanol.

HPLC Analysis

The medium sample was first centrifuged at 15000 rpm and 4°C for 15 min. The cellular samples were collected by adding 500 µL of a mixture of mobile phase : methanol = 1 : 4 and using a cell scraper, then sonicated for 30 min and gently shaken at 4°C for 1 h for deproteinization with a petite rotor (Model 2210, Wakenyaku Co. Ltd., Kyoto, Japan), followed by centrifugation at 15000 rpm and 4°C for 15 min. Quantities of L-CEX, D-CEX and 7-ADCA were determined by HPLC as described previously (Tamai et al., 1988). The HPLC system consisted of a constant-flow pump (JASCO PU-2080 *Plus*), a UV detector (JASCO UV-2075 *Plus*), an automatic sample injector (JASCO AS-2057 *Plus*) (JASCO International Co., Ltd., Tokyo, Japan) and an integrator (Chromatopac C-R7A; Shimadzu Corporation, Kyoto, Japan). The UV detector was set at 260 nm. Reversed-phase columns, J'sphere ODS-M80 (4.6 x 150 mm; YMC Co., Ltd., Kyoto Japan) for L-CEX and 7-ADCA, and COSMOSIL $5C_{18}$-MS-II (4.6 x 150 mm; Nacalai tesque, Inc., Kyoto, Japan) for D-CEX, were maintained at 35°C in a column oven (JASCO CO-2065 *Plus*; JASCO International Co., Ltd., Tokyo, Japan). The mobile

phase was 10 mM sodium phosphate buffer (pH 3.0) containing 10 mM ammonium acetate and 10 mM pentanesulphonic acid-methanol (10/90, v/v) for L-CEX, 10 mM ammonium acetate-methanol (22/78, v/v) for D-CEX and 10 mM ammonium acetate and 10 mM tetra-n-butylammonium bromide-methanol (8/92, v/v) for 7-ADCA. The flow rate was 1.0 mL/min.

Measurement of Lactate Dehydrogenase (LDH) Activity

LDH activity in the medium was determined using an LDH-Cytotoxicity Test (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's protocol. Cells treated with PBS(-) containing 0.2% Tween or PBS(-) alone were used as the positive and negative controls, respectively. The results were expressed as LDH-cytotoxicity ratio and corrected LDH-cytotoxicity ratio based on equation (1) and (2), respectively.

$$\text{LDH-Cytotoxicity Ratio} = (S - N) / (P - N) \times 100\ (\%) \quad (1)$$

$$\text{Corrected LDH-Cytotoxicity Ratio}\ (\%) = 100 - \text{LDH-Cytotoxicity Ratio} \quad (2)$$

where S, N and P represents LDH activity observed under the tested condition, and those of negative and positive controls, respectively.

Data Analysis

Metabolism of L-CEX was represented as generation of 7-ADCA during incubation with L-CEX. All data were expressed as mean ± S.E.M. Statistical analysis was performed with Student's *t* test. The criterion of significance was taken to be $p < 0.05$.

Results

Uptake and Metabolism of L- and D-CEX in HeLa/PEPT1 cells

To analyze metabolism and transport after exogenous transfection of PEPT1, we first used the cell line HeLa/PEPT1 which has already been demonstrated to exhibit functional expression of PEPT1 (Nakanishi et al., 2000). The HeLa/PEPT1 cells exhibited a marked increase in formation of 7-ADCA, compared with Mock cells (Fig. 2A). Disappearance of L-CEX from the medium of HeLa/PEPT1 cells was faster than from that of Mock cells (Fig. 2B). Thus, exogenous transfection of PEPT1 gene resulted in an increase of L-CEX metabolism. Accumulation of 7-ADCA in HeLa/PEPT1 cells was significantly higher than in Mock cells, whereas L-CEX was under the quantification limit (0.1 nmol/mg protein/180 min) in both cell lines (Fig. 2C). On the other hand, D-CEX was minimally hydrolyzed to 7-ADCA in both HeLa/PEPT1 and Mock cells (Figs. 2D and 2E). D-CEX was taken up as the unchanged form by HeLa/PEPT1 cells, whereas such uptake was extremely small in Mock cells (Fig. 2F).

To check the stability in the incubation medium, L- and D-CEX was incubated at pH 6.0 and 7.4 in the assay buffer without cells at 37°C. The decrease in L- and D-CEX concentration in the buffer after 180 min was less than 0.4% of initial dose, suggesting that both compounds are stable in the buffer.

To confirm such an increase in L-CEX metabolism by PEPT1 transfection, we next performed similar experiments in different host cells, HEK293 cells, which were transiently transfected with PEPT1 (HEK293/PEPT1). This is because HeLa/PEPT1 was a stable clone, and L-CEX metabolism observed in the HeLa/PEPT1 might represent any experimental artifact that may happen by selecting such specific clone of HeLa cells. For example, stable expression of PEPT1 may up-regulates endogenous peptidase, resulting in significant L-CEX metabolism. In addition, L-CEX metabolism in HeLa/PEPT1 might

be HeLa cell-specific phenomenon. Consequently, to exclude these possibilities, we also performed metabolism studies using another cell line HEK293 which was transiently transfected with PEPT1. Very much higher metabolism of L-CEX was observed in HEK293/PEPT1 cells than in Mock cells (Figs. 3A and 3C). A similar increase in L-CEX metabolism was also observed after transient transfection of PEPT2, another POT family member (Figs. 3A and 3C). Disappearance of L-CEX was much faster in HEK293/PEPT1 and HEK293/PEPT2 cells than in Mock cells (Fig. 3B). Accumulation of L-CEX was not observed in HEK293/PEPT1 or HEK293/PEPT2 cells (Fig. 3C).

cDNA-dependent Activity of L-CEX Metabolism

To confirm PEPT1-mediated L-CEX metabolism, various amounts (0.25 - 4.0 μg/well) of plasmid cDNA encoding PEPT1 or PEPT2 were transiently transfected into HEK293 cells, and L-CEX metabolism was examined. Since the amounts of 7-ADCA inside HeLa/PEPT1 and HEK293/PEPT1 cells at 180 min (~7 and 18 nmol/mg protein, respectively) were much lower than those in the medium (~64 and 87 nmol/mg protein, respectively) (Figs. 2 and 3), appearance of 7-ADCA was measured only in the medium in the following studies to analyze metabolism of L-CEX. The L-CEX metabolic activity increased almost linearly in proportion to the amount of cDNA encoding PEPT1 or PEPT2 up to 2 μg/well (Figs. 4A and 4B).

Comparison of pH-dependence between PEPT1-mediated Metabolism and Uptake

To compare the characteristics of PEPT1-mediated metabolism and uptake, both metabolism of L-CEX and uptake of D-CEX were determined at various values of pH. A strong dependency on extracellular pH was observed for both metabolism and uptake in HeLa/PEPT1, the optimal pH being 6 - 6.5, whereas minimal pH dependence

was observed in Mock cells (Fig. 5). Thus, the pH dependence of PEPT1-mediated metabolism was similar to that of PEPT1-mediated uptake.

Recognition specificity of PEPT1-mediated Metabolism and Uptake

To examine the substrate specificity of the PEPT1 activity involved in L-CEX metabolism, the inhibitory effects of various compounds on L-CEX metabolism were examined in HeLa/PEPT1 cells and compared with those on PEPT1-mediated D-CEX uptake activity. L-CEX metabolism in HeLa/PEPT1 was significantly inhibited by EDTA and substrates of PEPT1 e.g., D-CEX, L-Ala-L-Ala-L-Ala, glycylsarcosine, valacyclovir, enalapril and ubenimex (Table I). A similar inhibition profile was observed for D-CEX uptake in HeLa/PEPT1 cells (Table I). All the compounds that inhibited D-CEX uptake also decreased the metabolism of L-CEX in HeLa/PEPT1 cells (Table I). Among the compounds examined, the inhibition of L-CEX metabolism by L-Ala-L-Ala-L-Ala, enalapril, ubenimex and EDTA was most marked, and the inhibitory effects of these compounds on L-CEX metabolism were greater than those on D-CEX uptake (Table I). In Table I, metabolism of L-CEX was measured for 180 min, since formation of 7-ADCA increased in time-dependent manner until this time (Fig. 2A). On the other hand, it should be noted that substantial degree (30~50%) of medium concentration of L-CEX was reduced until 180 min (Fig. 2B), suggesting that the substrate was partially depleted. Therefore, further studies are required for more strict analysis of kinetics of L-CEX metabolism.

L-CEX Metabolism Does Not Solely Depend on Uptake Activity of PEPT1 in PEPT1-expressing Cells after Membrane-permeabilization

To examine whether the increase in L-CEX metabolism following PEPT1 transfection can be simply explained by the increase in PEPT1-mediated uptake and consequent exposure of L-CEX to intracellular hydrolases, the plasma membranes of HeLa/PEPT1 cells were first perforated by exposure to hypotonic solution or digitonin-containing solution, and both metabolism and uptake activity were determined (Table II). As an index of increased membrane permeability, the corrected LDH-cytotoxicity ratio was determined and was found to be 7 - 26% after the treatment with hypotonic or digitonin solution, indicating that the plasma membranes had been successfully permeabilized (Table II). The concentration of digitonin was set at 125 μg/mL, since, in our preliminary analysis, digitonin at this concentration exhibited the lowest value of the corrected LDH-cytotoxicity ratio (data not shown). In intact cells, exposure to L-CEX inside the cells could be much higher in PEPT1-transfected cells, compared with that in Mock cells, because of PEPT1-mediated transport activity. On the other hand, it was considered that such permeabilization of the cells may increase the exposure to L-CEX in intracellular space of both cells, resulting in almost comparable exposure to L-CEX inside the cells. This was confirmed by the fact that PEPT1-mediated uptake of D-CEX was almost completely reduced in hypotonic (~10% of control) and digitonin-treated (~8% of control) conditions (Table II). Even in such condition, L-CEX metabolism was higher in HeLa/PEPT1 than that in Mock cells, as indicated by PEPT1-mediated L-CEX metabolism which was maintained at 30-50% of control level both in hypotonic and digitonin-treated conditions (Table II). This suggests that L-CEX metabolism enhanced in HeLa/PEPT1 cells (Fig. 2) does not solely results from the increase in exposure to L-CEX inside the cells by PEPT1-mediated transport in HeLa/PEPT1 cells.

L-CEX Metabolism in Homogenates of PEPT1-expressing Cell Lines

Metabolism of L-CEX was also examined in cellular homogenates prepared from HeLa/PEPT1 and Mock cells. The generation of 7-ADCA at pH 6.0 in HeLa/PEPT1 and Mock cells was 415 ± 20 and 135 ± 11 nmol/mg protein/60 min, respectively (mean ± S.E.M., n = 3-6), whereas that at pH 7.4 in HeLa/PEPT1 and Mock cells was 305 ± 5 and 245 ± 3 nmol/mg protein/60 min, respectively (mean ± S.E.M., n = 3-6). PEPT1-mediated L-CEX metabolism, assessed by subtracting the formation of 7-ADCA in Mock cells from that in HeLa/PEPT1 cells, was 281 ± 21 and 60.3 ± 5.1 nmol/mg protein/60 min at pH 6.0 and 7.4, respectively (mean ± S.E.M., n = 3-6). Thus, L-CEX metabolism observed in cellular homogenates was also more obvious at pH 6.0 than that in pH 7.4, as in case of that found in intact cell lines (Fig. 5).

Discussion

While D-CEX is orally well absorbed, L-CEX is quite rapidly hydrolyzed in the small intestine to form 7-ADCA, and little L-CE is detected in circulating plasma *in vivo* or brush border membrane vesicles *in vitro* (Tamai et al., 1988; Kramer et al., 1992). On the other hand, Wenzel et al. (1995) demonstrated that L-CEX is a good substrate of oligopeptide transporter. Thus, it is likely that transport and metabolism of L-CEX occur almost simultaneously *in vivo*. We therefore hypothesized that oligopeptide transporter plays a fundamental role not only in transport of L-CEX, but also its metabolism. Since PEPT1 is thought to be a major oligopeptide transporter in small intestine, we examined whether PEPT1 could account for the stereoselective uptake and metabolism of CEX in the present study.

L-CEX hydrolysis was indeed dramatically stimulated by exogenous transfection of PEPT1 (Figs. 2 and 3). This phenomenon is independent of the type of host cell line or the transfection method, since such enhancement of L-CEX metabolism by PEPT1 was observed in both HeLa and HEK293 cells (Figs. 2 and 3), and after both stable and transient transfection of the PEPT1 gene (Figs. 2 and 3). Activity of L-CEX metabolism depended on the amount of PEPT1 cDNA transfected (Fig. 4A). On the other hand, D-CEX was not metabolized even after transfection of PEPT1, although its uptake into the cells was enhanced by expression of PEPT1 (Figs. 2D and 2F). These results suggest that PEPT1 alone is responsible for the stereoselective uptake and metabolism of CEX. It is noteworthy that the PEPT1-mediated stereoselective disposition of CEX observed in *in vitro* is quite similar to the stereoselective disposition observed in the small intestine after oral administration (Tamai et al., 1988), implying a predominant role of this transporter not only in transport, but also in metabolism of L-CEX.

To characterize such PEPT1-mediated metabolism and uptake of CEX, we determined the pH-dependence and substrate specificity of the two processes (Fig. 5, Table II). Considering that PEPT1 exhibits proton-coupled transport of substrates, it is reasonable that uptake of CEX was greater at lower pH values (Fig. 5B). The optimal pH for L-CEX metabolism, on the other hand, was 6.0 - 6.5 (Fig. 5A), which is slightly higher than that for D-CEX uptake (Fig. 5B). As for the substrate specificity of the L-CEX metabolic activity, substrates of PEPT1 inhibited both L-CEX metabolism and D-CEX uptake (Table I), whereas several other compounds, including L-Ala-L-Ala-L-Ala, enalapril, ubenimex and EDTA, inhibited L-CEX metabolism much more strongly than D-CEX uptake (Table I). Inhibition of D-CEX uptake by EDTA (Table I) may imply possible involvement of metal ion(s) in PEPT1-mediated transport, and further studies are required for analyzing detailed mechanism(s). Thus, PEPT1-mediated metabolism and uptake have similar, though not identical, pH-dependence and substrate recognition specificity.

The metabolism of L-CEX observed after PEPT1 transfection in *in vitro* transfectant systems (Figs. 2, 3 and 4) can simply be explained, if we hypothesize that the PEPT1-mediated uptake process of L-CEX is the rate-limiting step in its overall hydrolysis. It is possible that transfection of PEPT1 gene increases the membrane permeation of L-CEX, resulting in a higher exposure of L-CEX to intracellular peptidases. To examine whether uptake is the rate-limiting step or not, PEPT1-mediated metabolism of L-CEX was also examined after plasma membranes had been perforated using hypotonic or digitonin-containing solution (Table II). While PEPT1-mediated D-CEX uptake was greatly reduced under such highly permeabilized conditions, the decrease in L-CEX metabolism was less marked (Table I), indicating that PEPT1-mediated metabolism cannot be fully explained by the hypothesis that the membrane permeation

process is rate-limiting. This is consistent with our present finding that PEPT1-mediated metabolism also occurs in homogenates prepared from HeLa/PEPT1 cells (see Results). If we assume that exogenous transfection of PEPT1 gene only increases PEPT1 protein, these results suggest that PEPT1 itself is directly involved in the hydrolysis of L-CEX. Up to now, this protein has been recognized only as a transporter for various substrates including di- and tripeptides, and ß-lactams, and no information has been available on its potential for hydrolytic activity. Alternatively, it might be possible that exogenous transfection of PEPT1 gene increases the expression of some endogenous hydrolytic enzyme(s) and/or activates it. Since mammalian cell lines stably transfected with PEPT1 gene also express various types of undetermined endogenous proteins, it is difficult to finally conclude that PEPT1 itself is a metabolizing enzyme. A purified *in vitro* system may be useful to clarify whether PEPT1 itself exhibits hydrolytic activity for L-CEX. Several studies have aimed at construction of such *in vitro* system in which only PEPT1 protein purified from animal small intestine is present. Kramer et al. (1992) performed purification and reconstitution of the 127-kDa binding protein for ß-lactam antibiotics and oligopeptides, expressed in brush border membrane of rabbit small intestinal enterocytes. This liposome contained the binding protein which exhibited stereospecific transport activity, but did not ensure a purity of the transporter, i.e., contamination of other proteins might be present. Iseki et al. (1998) also reported purification of oligopeptide transporter(s) from rat small intestinal brush-border, although identification of this protein has not yet been fully characterized. Therefore, for the final demonstration of the hypothesis that PEPT1 may act as a metabolic enzyme, we need a novel experimental system that exclusively express PEPT1 protein.

Our results indicate a fundamental role of PEPT1 not only in transport, but also in metabolism of oligopeptides. In humans, more than 80% of orally ingested protein was

digested to small peptides (Adibi and Mercer, 1973), and the absorption rates of peptides mediated by the oligopeptide transport systems, including PEPT1, are greater than those of free amino acids (Adibi, 1971). Therefore, it seems reasonable that the uptake and subsequent hydrolysis processes would be tightly coupled, leading to efficient absorption of the nutrients.

7-ADCA is generated as a result of hydrolytic cleavage of the peptide bond of L-CEX (Tamai et al., 1988). Chemical degradation of the peptide bond of D-CEX was stimulated at higher pH, whereas the bond is quite stable at lower pH (Yamana and Tsuji, 1976). Considering that L-CEX is a diastereomer of D-CEX, it could also be stable under lower pH conditions. Since 7-ADCA generation was marked at pH 6 - 6.5 (Fig. 5A), hydrolysis of L-CEX is thought to be more likely to be mediated by metabolic enzyme(s) than by chemical degradation.

In the present study, exogenous transfection of PEPT2, which has 50% amino acid homology with PEPT1 (Meredith and Boyd, 2000; Smith et al., 2004; Daniel and Kottra, 2004) also increases the metabolism of L-CEX (Fig. 4B). PEPT2 is expressed at apical membranes of renal proximal tubules and epithelial cells of choroid plexus, and is thought to play a role in (re)absorption of peptides and other substrates (Rubio-Aliaga et al., 2003; Shen et al., 2003). Considering the similar physiological roles of the two homologous transporters, it is reasonable that both proteins could be involved in the hydrolysis of L-CEX. Human peptide transporter 1 (HPT1, also named CDH17) is another peptide transporter that accepts cephalexin, ubenimex (Dantzig et al., 1994) and valacyclovir (Landowski et al., 2003) as substrates. In preliminary studies, we transfected human HPT1 gene (2 μg/well) into HEK293 cells, but found no increase in 7-ADCA formation (10.9 ± 0.3 and 9.6 ± 0.8 pmol/min/mg protein in HPT1- and Mock-

transfected cells, respectively), suggesting that HPT1 is unlikely to be involved in CEX metabolism.

In the present study, the profiles of 7-ADCA formation in HeLa/PEPT1 cells (Fig. 2A) may exhibit unusual one, the slope being faster after a certain lag-time (~30 min). The reason could be that metabolism of L-CEX may occur after it is taken up by the cells. In this case, such a lag-time would be necessary for L-CEX molecules to permeate the plasma membranes and subsequently be exposed to the catalytic site of metabolizing enzymes inside the cells. A similar profile of the 7-ADCA formation was also observed in HEK293 cells expressing PEPT1 (Fig. 3A). If there is a lag-time, after which 7-ADCA formation becomes much faster, and this is caused by the uptake and/or exposure processes of the parent compound in the cells, it would be difficult to perform Michaelis-Menten type analysis of the 7-ADCA formation. Therefore, the experimental systems other than the intact cell lines are necessary for detailed analysis of the 7-ADCA formation.

In conclusion, PEPT1-mediated L-CEX metabolism has similar, though not identical, characteristics to those of PEPT1-mediated L-CEX transport, in terms of pH-dependence and substrate specificity, and can take place even when the PEPT1-mediated transport process is hindered, implying an intrinsic involvement of PEPT1 itself and/or PEPT1-associated peptidases in L-CEX metabolism in the small intestine.

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References.

Adibi SA (1971) Intestinal transport of dipeptides in man: relative importance of hydrolysis and intact absorption. *J Clin Invest* **50**:2266-2275.

Adibi SA and Mercer DW (1973) Protein digestion in human intestine as reflected in luminal, mucosal, and plasma amino acid concentrations after meals. *J Clin Invest* **52**:1586-1594.

Balimane PV, Tamai I, Guo A, Nakanishi T, Kitada H, Leibach FH, Tsuji A and Sinko PJ (1998) Direct evidence for peptide transporter (PepT1)-mediated uptake of a nonpeptide prodrug, valacyclovir. *Biochem Biophys Res Commun* 250:246-251.

Daniel H (2004) Molecular and integrative physiology of intestinal peptide transport. *Annu Rev Physiol* **66**:361-384.

Daniel H and Kottra G (2004) The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. *Pflugers Arch* **447**:610-618.

Dantzig AH and Bergin L (1990) Uptake of the cephalosporin, cephalexin, by a dipeptide transport carrier in the human intestinal cell line, Caco-2. *Biochim Biophys Acta* **1027**:211-217.

Dantzig AH, Hoskins JA, Tabas LB, Bright S, Shepard RL, Jenkins IL, Duckworth DC, Sportsman JR, Mackensen D, Rosteck PR Jr and Skatrud PL (1994) Association of intestinal peptide transport with a protein related to the cadherin superfamily. *Science* **264**:430-433.

Fei YJ, Kanai Y, Nussberger S, Ganapathy V, Leibach FH, Romero MF, Singh SK, Boron WF and Hediger MA (1994) Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature* **368**:563-566.

Ganapathy ME, Brandsch M, Prasad PD, Ganapathy V and Leibach FH (1995) Differential recognition of ß-lactam antibiotics by intestinal and renal peptide transporters, PEPT 1 and PEPT 2. *J Biol Chem* **270**:25672-25677.

Hardman JG and Limbird LE eds (2001) *Goodman & Gilman's: The Pharmacological Basis of Therapeutics* 10th Ed., McGraw-Hill Companies, Inc., NY

Iseki K, Yonemura K, Kikuchi T, Naasani I, Sugawara M, Kobayashi M, Kohri N, Miyazaki K (1998) Purification by ceftibuten-affinity chromatography and the functional reconstitution of oligopeptide transporter(s) in rat intestinal brush-border membrane. *Biochim Biophys Acta* **1370**: 161-168

Iwatsubo T, Miyamoto Y, Sugiyama Y, Yuasa H, Iga T and Hanano M (1986) Effects of potential damaging agents on the microclimate-pH in the rat jejunum. *J Pharm Sci* **75**:1162-1165.

Kramer W, Gutjahr U, Girbig F and Leipe I (1990) Intestinal absorption of dipeptides and ß-lactam antibiotics. II. Purification of the binding protein for dipeptides and ß-lactam antibiotics from rabbit small intestinal brush border membranes. *Biochim Biophys Acta* **1030**:50-59.

Kramer W, Girbig F, Gutjahr U, Kowalewski S, Adam F and Schiebler W (1992) Intestinal absorption of ß-lactam antibiotics and oligopeptides. Functional and stereospecific reconstitution of the oligopeptide transport system from rabbit small intestine. *Eur J Biochem* **204**:923-930.

Landowski CP, Sun D, Foster DR, Menon SS, Barnett JL, Welage LS, Ramachandran C and Amidon GL (2003) Gene expression in the human intestine and correlation with oral valacyclovir pharmacokinetic parameters. *J Pharmacol Exp Ther* **306**:778-786.

Liang R, Fei YJ, Prasad PD, Ramamoorthy S, Han H, Yang-Feng TL, Hediger MA, Ganapathy V and Leibach FH (1995) Human intestinal H^+/peptide cotransporter.

Cloning, functional expression, and chromosomal localization. *J Biol Chem* **270**:6456-6463.

Meredith D and Boyd CA (2000) Structure and function of eukaryotic peptide transporters. *Cell Mol Life Sci* **57**:754-778.

Nakanishi T, Tamai I, Takaki A and Tsuji A (2000) Cancer cell-targeted drug delivery utilizing oligopeptide transport activity. *Int J Cancer* **88**:274-280.

Nakashima E, Tsuji A, Kagatani S and Yamana T (1984) Intestinal absorption mechanism of amino-ß-lactam antibiotics. III. Kinetics of carrier-mediated transport across the rat small intestine *in situ*. *J Pharmacobiodyn* **7**:452-464.

Naruhashi K, Sai Y, Tamai I, Suzuki N and Tsuji A (2002) PepT1 mRNA expression is induced by starvation and its level correlates with absorptive transport of cefadroxil longitudinally in the rat intestine. *Pharm Res* **19**:1417-1423.

Rubio-Aliaga I and Daniel H (2002) Mammalian peptide transporters as targets for drug delivery. *Trends Pharmacol Sci* **23**:434-440.

Rubio-Aliaga I, Frey I, Boll M, Groneberg DA, Eichinger HM, Balling R and Daniel H (2003) Targeted disruption of the peptide transporter Pept2 gene in mice defines its physiological role in the kidney. *Mol Cell Biol* **23**:3247-3252.

Sai Y, Tamai I, Sumikawa H, Hayashi K, Nakanishi T, Amano O, Numata M, Iseki S and Tsuji A (1996) Immunolocalization and pharmacological relevance of oligopeptide transporter PepT1 in intestinal absorption of ß-lactam antibiotics. *FEBS Lett* **392**:25-29.

Saito H, Okuda M, Terada T, Sasaki S and Inui K (1995) Cloning and characterization of a rat H^+/peptide cotransporter mediating absorption of ß-lactam antibiotics in the intestine and kidney. *J Pharmacol Exp Ther* **275**:1631-1637.

Shen H, Smith DE, Keep RF, Xiang J and Brosius FC 3rd (2003) Targeted disruption of the PEPT2 gene markedly reduces dipeptide uptake in choroid plexus. *J Biol Chem* **278**:4786-4791.

Shiraga T, Miyamoto K, Tanaka H, Yamamoto H, Taketani Y, Morita K, Tamai I, Tsuji A and Takeda E (1999) Cellular and molecular mechanisms of dietary regulation on rat intestinal H^+/peptide transporter PepT1. *Gastroenterology* **116**:354-362.

Smith DE, Johanson CE and Keep RF (2004) Peptide and peptide analog transport systems at the blood-CSF barrier. *Adv Drug Deliv Rev* **56**:1765-1791.

Snyder NJ, Tabas LB, Berry DM, Duckworth DC, Spry DO and Dantzig AH (1997) Structure-activity relationship of carbacephalosporins and cephalosporins: antibacterial activity and interaction with the intestinal proton-dependent dipeptide transport carrier of Caco-2 cells. *Antimicrob Agents Chemother* **41**:1649-1657.

Tamai I, Ling HY, Timbul SM, Nishikido J and Tsuji A. (1988) Stereospecific absorption and degradation of cephalexin. *J Pharm Pharmacol* **40**:320-324.

Tamai I, Nakanishi T, Hayashi K, Terao T, Sai Y, Shiraga T, Miyamoto K, Takeda E, Higashida H and Tsuji A (1997) The predominant contribution of oligopeptide transporter PepT1 to intestinal absorption of ß-lactam antibiotics in the rat small intestine. *J Pharm Pharmacol* **49**:796-801.

Terada T and Inui K (2004) Peptide transporters: structure, function, regulation and application for drug delivery. *Curr Drug Metab* 5:85-94.

Tsuji A, Nakashima E, Kagami I and Yamana T (1981a) Intestinal absorption mechanism of amphoteric ß-lactam antibiotics I: Comparative absorption and evidence for saturable transport of amino-ß-lactam antibiotics by *in situ* rat small intestine. *J Pharm Sci* **70**:768-772.

Tsuji A, Nakashima E, Kagami I and Yamana T (1981b) Intestinal absorption mechanism of amphoteric ß-lactam antibiotics II: Michaelis-Menten kinetics of cyclacillin absorption and its pharmacokinetic analysis in rats. *J Pharm Sci* **70**:772-777.

Tsuji A and Tamai I (1996) Carrier-mediated intestinal transport of drugs. *Pharm Res* **13**:963-977.

Tsuji A (2002) Transporter-mediated Drug Interactions. *Drug Metab Pharmacokinet* **17**:253-74.

Watanabe C, Kato Y, Ito S, Kubo Y, Sai Y and Tsuji A. (2005) Na^+/H^+ exchanger 3 affects transport property of H^+/oligopeptide transporter 1. *Drug Metab Pharmacokinet* **20**:443-451.

Wenzel U, Thwaites DT and Daniel H (1995) Stereoselective uptake of ß-lactam antibiotics by the intestinal peptide transporter. *Br J Pharmacol* **116**:3021-3027.

Yamana T and Tsuji A (1976) Comparative stability of cephalosporins in aqueous solution: kinetics and mechanisms of degradation. *J Pharm Sci* **65**:1563-1574.

Foot Notes

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Legends for figures.

Figure 1

The chemical structures of L-CEX, D-CEX and 7-ADCA

Figure 2

Stereoselective metabolism of cephalexin in HeLa cells stably expressing PEPT1

HeLa/PEPT1 (closed circles) or Mock (open circles) cells were incubated for 180 min at 37°C and pH 6.0 in assay buffer containing L-CEX (A, B, C) or D-CEX (D, E, F). Amounts of 7-ADCA (A, D), L-CEX (B) and D-CEX (E) in the incubation medium (supernatant) were determined at the designated times. After the incubation period, accumulation of each compound in HeLa/PEPT1 (closed columns) or Mock (open columns) cells was also determined (C, F). Each value represents the mean ± S.E.M. (n = 9 - 18).

*$p < 0.05$, compared with the Mock cells (Student's *t* test).

‡, below the quantification limit.

Figure 3

Stereoselective metabolism of cephalexin in HEK293 cells transiently expressing PEPT1 or PEPT2

HEK293/PEPT1 (closed circles), HEK293/PEPT2 (closed triangles) or Mock (open circles) cells were incubated over 180 min at 37°C and pH 6.0 in assay buffer containing L-CEX (28.8 μM). Amounts of 7-ADCA (A) and L-CEX (B) in the incubation medium (supernatant) were determined at designated times. After the incubation period, accumulation of 7-ADCA in HEK293/PEPT1 (closed column), HEK293/PEPT2 (hatched

column) or Mock (open column) was also determined (C). Each value represents the mean ± S.E.M. (n = 3).

*$p < 0.05$, compared with the Mock cells (Student's *t* test).

Figure 4

Metabolism of L-CEX in HEK293 cells transiently transfected with various amounts of cDNA encoding PEPT1 (A) or PEPT2 (B)

HEK293 cells were transiently transfected with various amounts of cDNA encoding PEPT1 (A, circles) or PEPT2 (B, triangles). Cells were then incubated over 180 min at 37°C and pH 6.0 in assay buffer containing L-CEX (28.8 μM), and the amount of 7-ADCA in the medium was determined. Each value was normalized with respect to maximal activity and represents the mean ± S.E.M. (n = 3).

Figure 5

Effect of extracellular pH on metabolic (A) and transporting (B) activities of PEPT1 in HeLa cells stably expressing PEPT1

Metabolism of L-CEX (28.8 μM, A) and uptake of D-CEX (28.8 μM, B) over 180 min and 10 min, respectively, was measured under various pH conditions in HeLa/PEPT1 (closed symbols) or Mock (open symbols) cells, respectively. Each value was normalized with respect to maximal activity and represents the mean ± S.E.M. (n = 6 or 3).

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Tables.

Table I

Inhibitory effects of various compounds on L-CEX metabolism or D-CEX uptake in HeLa/PEPT1 cells [a)]

Test Compound	Concentration		L-CEX Metabolism [b)] (pmol/min/mg proterin)	D-CEX Uptake [c)] (pmol/min/mg proterin)
Control			473 ± 14	95.4 ± 12.9
D-CEX	10	mM	**172 ± 9	N.T.
L-CEX	10	mM	N.T.	*37.8 ± 7.7
7-ADCA	10	mM	N.T.	92.1 ± 4.3
L-Ala	10	mM	*394 ± 7	86.4 ± 9.0
D-Ala-D-Ala	10	mM	**330 ± 7	76.0 ± 10.8
L-Ala-L-Ala-L-Ala	10	mM	**44.0 ± 0.6	**36.5 ± 2.0
Gly-Sar	10	mM	**231 ± 2	**45.3 ± 6.1
Valacyclovir	10	mM	**109 ± 6	**33.8 ± 3.8
Enalapril	10	mM	**16.1 ± 5.0	*36.3 ± 7.6
Ubenimex	10	mM	**0.572 ± 0.755	*39.5 ± 6.0
PMSF	1	mM	*382 ± 12	94.9 ± 7.1
Aprotinin	2	μg/mL	444 ± 11	81.6 ± 10.7
Leupeptin	100	μM	*417 ± 12	75.3 ± 5.4
EDTA	5	mM	**103 ± 2	67.6 ± 6.5
Pepstatin-A	1	μM	*381 ± 20	79.4 ± 9.9
Mock			**145 ± 5	**40.3 ± 4.3

[a] Each value was shown as mean ± S.E.M. (n = 3).

[b] Metabolism of L-CEX (28.8 μM) was measured for 180 min at pH 6.0.

[c] Uptake of D-CEX (28.8 μM) was measured for 10 min at pH 6.0.

*$p < 0.05$; **$p < 0.01$ (compared with the control).

N.T.: not tested

Table II

PEPT1-mediated metabolism of L-CEX in permeabilized HeLa cells[a)]

Treatment [b)]	Relative Activity (% of Control) L-CEX Metabolism [c)]	D-CEX Uptake [d)]	Corrected LDH-Cytotoxicity Ratio [e)] (%)
Control	100 ± 11.7	100 ± 6.0	99.6 ± 3.8
Hypotonic	27.7 ± 9.2	9.5 ± 2.6	25.9 ± 2.7
Digitonin	49.4 ± 12.2	8.4 ± 18.1	6.9 ± 4.9

[a] Each value represents the mean ± S.E.M. (n = 3 - 10).

[b] Cells were pretreated with hypotonic or digitonin (125 μg/mL) solution.

[c] Data were obtained by subtracting the metabolism of L-CEX (28.8 μM) for 180 min at pH 6.0 in Mock from that in HeLa/PEPT1 cells.

[d] Data were obtained by subtracting the uptake of D-CEX (28.8 μM) for 180 min at pH 6.0 in Mock from that in HeLa/PEPT1 cells.

[e] LDH activity is shown as corrected LDH-cytotoxicity ratio, obtained by using equation (2).

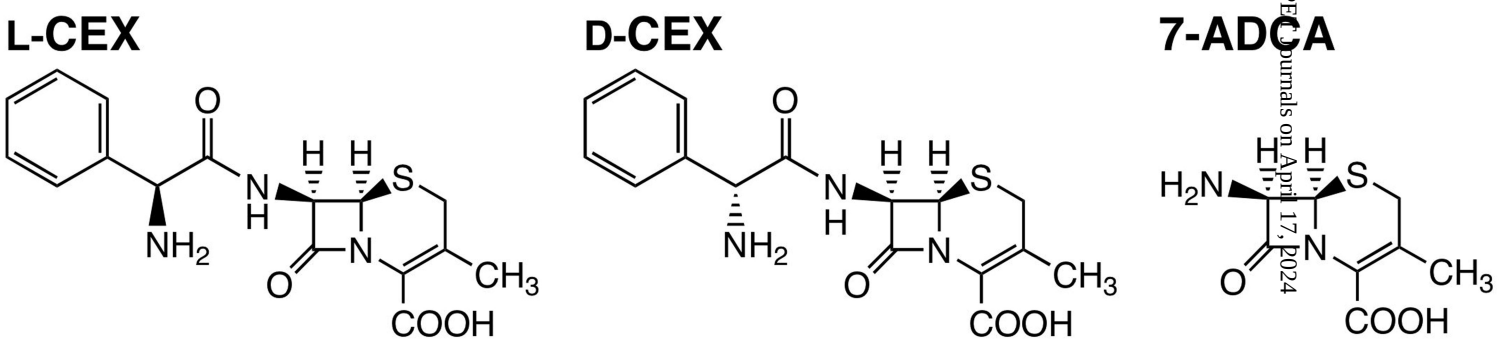

Figure 1

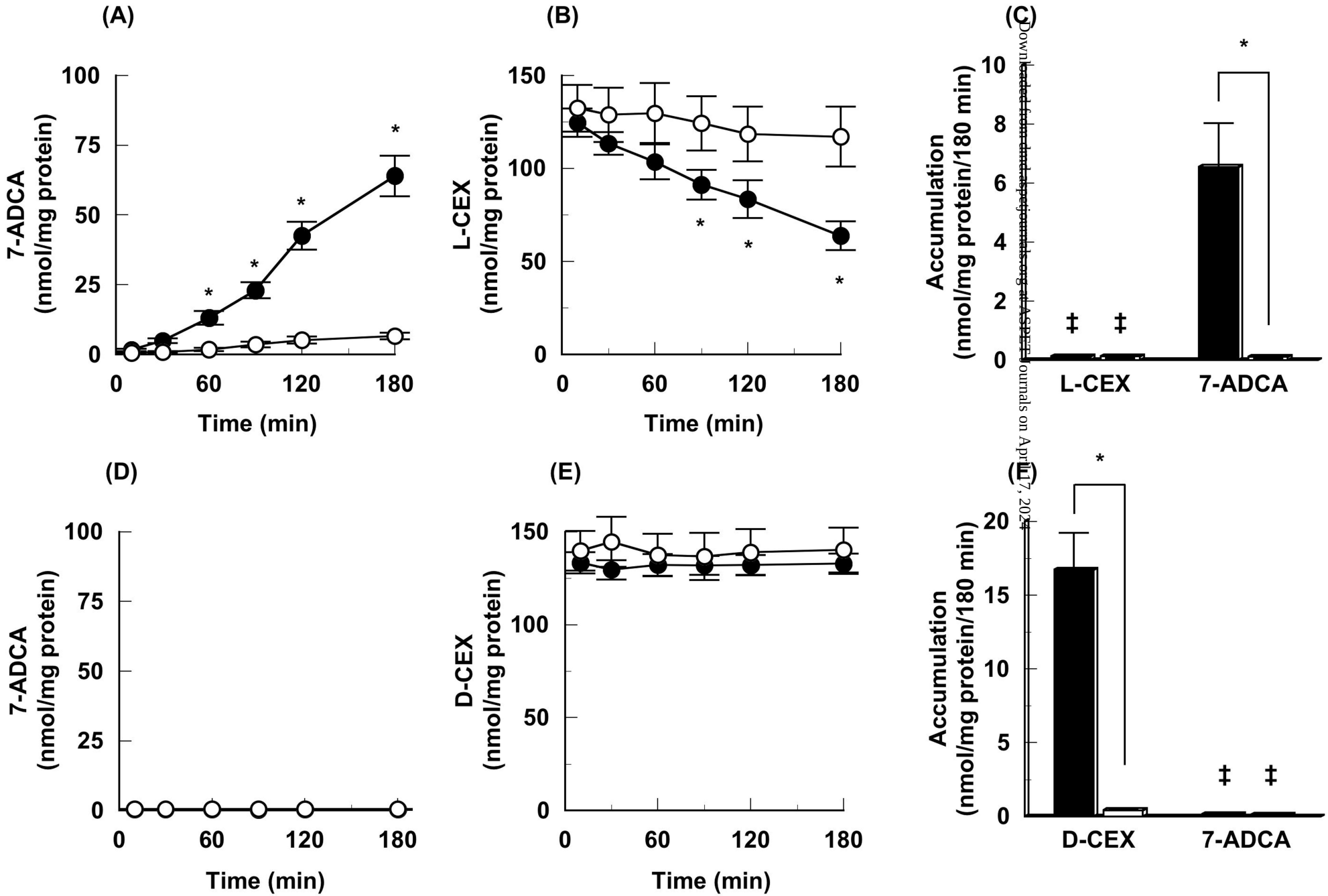

Figure 2

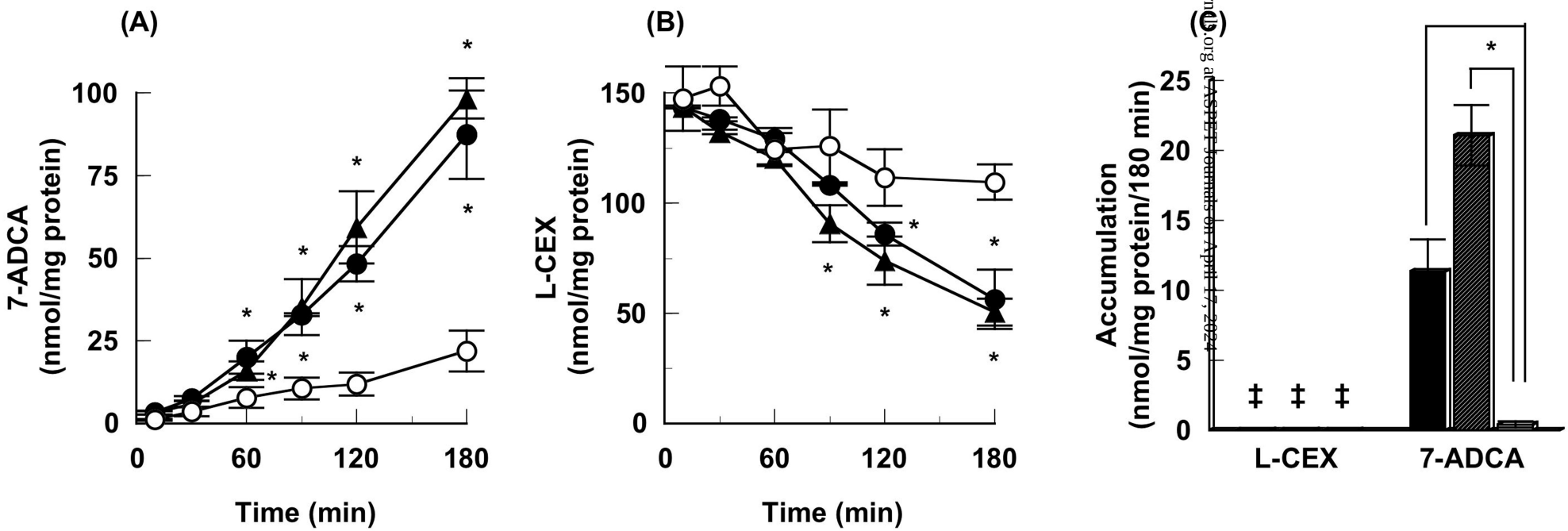

Figure 3

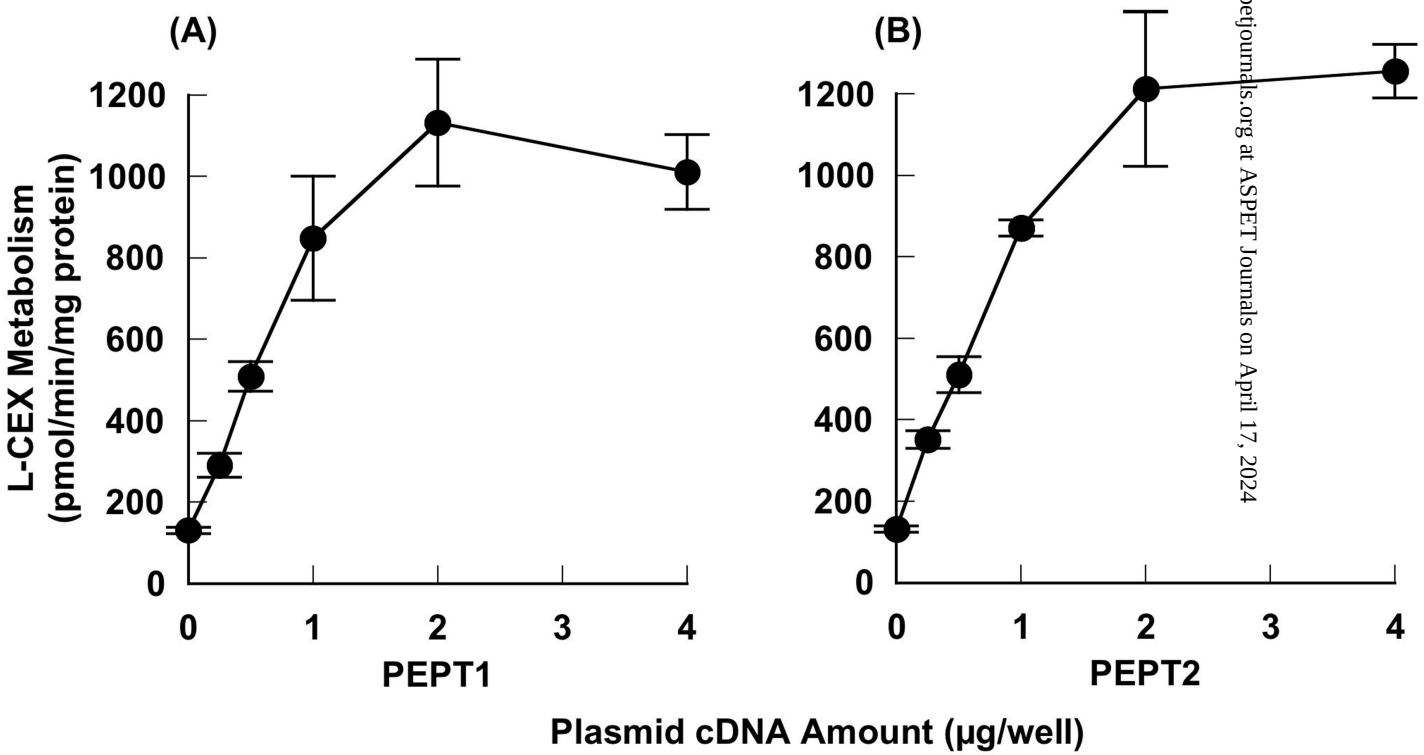

Figure 4

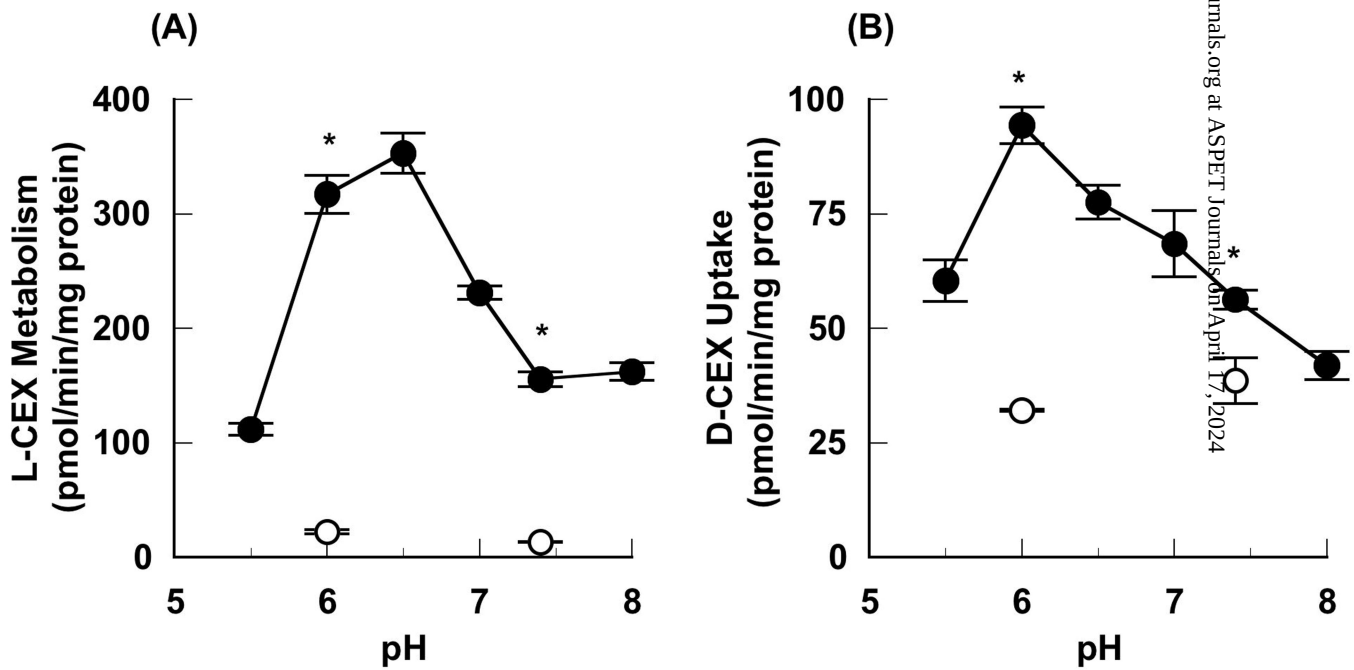

Figure 5