Effects of Progesterone and Norethisterone on Cephalexin Transport and Peptide Transporter PEPT1 Expression in Human Intestinal Cell Line Caco-2

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We investigated the effects of progesterone and norethisterone on the apical-to-basolateral and basolateral-to-apical transports of cephalexin, a typical peptide transporter PEPT1 substrate, and the PEPT1 mRNA and protein expression levels, using the human intestinal cell line, Caco-2. Caco-2 cell monolayers (passages 50 to 60) were cultured on permeable membrane, plastic culture dish and culture tube. The Caco-2 cell monolayers were pretreated with progesterone and norethisterone (3, 10, 30 μM) for 24 h. After the pretreatment, the apical-to-basolateral and basolateral-to-apical transports of cephalexin were measured, and the densities of PEPT1 mRNA and protein expression levels were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot, respectively. The apical-to-basolateral transport of cephalexin was significantly decreased by the progesterone and norethisterone (30 μM each) pretreatments. By contrast, the basolateral-to-apical transport of cephalexin was not altered by the same pretreatments. The densities of PEPT1 mRNA and protein expressions were significantly decreased by progesterone and norethisterone (each at 3 and 10 μM) pretreatments compared with those of the non-treated Caco-2 cells. The results suggest that the transcription of the PEPT1 gene is down-regulated by the progesterone and norethisterone pretreatments. Further studies are needed to clarify whether the inhibition of the PEPT1 gene transcription by progesterone pretreatment proceeds via σ1-receptor or progesterone receptor.

Key words: progesterone; norethisterone; PEPT1; cephalexin; Caco-2 cell

Intestinal drug transporters play an important role in the absorption of orally administered drugs. PEPT1, a peptide transporter located in the apical membrane, and the basolateral peptide transporter cooperate in the efficient transepithelial transport of small peptides and peptide-like drugs from the cells to the circulating blood.

The human intestinal cell line, Caco-2, constitutively expresses such drug transporters as peptide transporter, P-glycoprotein, organic cation transporter and organic anion transporter. Therefore, this cell line is frequently used as an in vitro cell culture model of the gastrointestinal tract to determine drug permeability and metabolism. To this day, some peptide transporters of the apical membrane, such as PEPT1, HPT1, PTR3 and PHT1, have been identified. Of these, many studies have been conducted on the functions of PEPT1 in particular.

Recent information is available on the regulation of PEPT1 activity and mRNA expression; for instance, triiodothyronine and an epidermal growth factor inhibited PEPT1 transport activity and mRNA expression. By contrast, human growth hormone, insulin, and the σ-receptor ligand, pentazocine, enhanced PEPT1 transport activity and protein expression. Although there is no known endogenous σ-receptor ligand to date, Ramamoorthy et al. have shown that progesterone is a potent σ-receptor inhibitor. However, little is known about the relationship between PEPT1 activity and steroid hormones, such as progesterone. In our previous study, the effects of progesterone and its related compounds on the uptake of cephalexin, a typical PEPT1 substrate, in Caco-2 cells, we noted a significant decrease in cephalexin uptake after progesterone and norethisterone pretreatments in the Caco-2 cell monolayers. The effects of progesterone and norethisterone pretreatments on the kinetic parameters of cephalexin uptake were also investigated. The saturable component of cephalexin uptake was markedly inhibited by the progesterone and norethisterone pretreatments. The maximal carrier-mediated uptake rate \( J_{\text{max}} \) of cephalexin uptake with the progesterone and norethisterone pretreatments was significantly decreased compared with the control, whereas the Michaelis constant \( K_m \) and the rate constant for nonsaturable uptake \( K_s \) values were not affected. Therefore, the quantitative decrease in PEPT1 protein expression is considered to be one cause of the decrease in cephalexin uptake by the progesterone and norethisterone pretreatments. The aim of the present study is to investigate whether cephalexin transport (apical-to-basolateral and basolateral-to-apical) and PEPT1 mRNA and protein expressions in Caco-2 cells are regulated by progesterone and norethisterone.

MATERIALS AND METHODS

Materials: Cephalexin, progesterone and norethisterone were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HEPES and MES were purchased from ICN Biomedicals Inc. (Costa Mesa, CA, U.S.A.). Cephradine, fetal bovine serum (FBS) and tissue culture reagents were purchased from Sigma (St. Louis, MO, U.S.A.). Biotinylated SDS-PAGE standard (broad range) and 100 bp DNA ladder were purchased from Bio-Rad (Richmond, CA, U.S.A.) and New England Biolabs (Beverly, MA, U.S.A.), respectively. All other chemicals used in the experiments were of the highest purity commercially available.

Cell Culture: Caco-2 cells at passage 41 were obtained from RIKEN Gene Bank (Tsukuba, Japan) and cultured as described previously. The cells from passages 50 to 60 were used. For the transport study, Caco-2 cells were seeded at a density of \( 3.5 \times 10^4 \) cells/permeable membrane (Cell culture insert, 0.45 \( \mu \) M, 4.3 cm \(^2 \) growth area, Becton Dickinson).

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Transport Studies  The culture medium of both sides was aspirated and the Caco-2 cell monolayers were washed twice with FBS-free culture medium. Then, the monolayers were preincubated for 10 min at 37 °C with 1.5 ml and 2.2 ml of FBS-free culture medium in the apical and basolateral sides, respectively. After the preincubation, the medium was aspirated immediately, and the monolayers were washed twice with FBS-free culture medium. The FBS-free culture medium containing progesterone and northerthisterone was added to either the apical side (1.5 ml) or the basolateral side (2.2 ml), and the monolayers were incubated for 24 h. Progesterone and northerthisterone were dissolved in DMSO, the final concentration of DMSO in the incubation medium being less than 1%. After the incubation, the culture medium of the apical and basolateral sides was aspirated, and the monolayers were washed twice with incubation medium consisting of HEPES buffer (145 mM NaCl, 3 mM KCl, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5 mM D-glucose, 5 mM HEPES (pH 7.4); basolateral side) or MES buffer (145 mM NaCl, 3 mM KCl, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5 mM D-glucose, 5 mM MES (pH 6.0); apical side), respectively. Then, the monolayers were preincubated for 10 min at 37 °C with 1.5 ml (MES buffer) and 2.2 ml (HEPES buffer) of the incubation medium in the apical and basolateral sides, respectively. After the preincubation, the medium was removed immediately and the incubation medium containing cephalexin (1.0 mM) was added to either the apical side (1.5 ml) or the basolateral side (2.2 ml), and the incubation medium (without cephalexin) was added to the opposite side (basolateral, 2.2 ml; apical, 1.5 ml). The monolayers were incubated for the indicated time at 37 °C. Cephalexin was dissolved in MES or HEPES buffer, and the other compounds were dissolved in DMSO or H$_2$O. The final concentration of DMSO in the incubation medium was less than 1%. After the incubation, the medium in the opposite side was transferred to a sample tube and filtered through a Millipore filter (Millex-LG), and the concentration of cephalexin in the filtrate was determined by HPLC, as described below. For the determination of the intracellular accumulation of cephalexin, the membrane filters were detached from the insert well after the incubation, and the cells on the membrane filters were extracted with 0.5 ml of 30 mM phosphate buffer (pH 7.0)/methanol (50/50, v/v) and cephradine (2.5 μg) for 1 h. The extract was centrifuged at 6000 rpm for 20 min, and the supernatant filtered through a Millipore filter. The filtrate was subjected to HPLC.

HPLC  HPLC was carried out with the same machine as that described previously.$^{12}$ For the determination of cephalexin (wavelength: 262 nm), the mobile phase was a mixture of 30 mM phosphate buffer (pH 7.0) and methanol (70/30, v/v), and the flow rate was 0.85 ml/min. Calibration curves were obtained by injecting cephalexin (2—4000 ng) containing a quantitative amount (200 ng) of cephradine as the internal standard into the HPLC system. For the determination of progesterone (wavelength: 240 nm), the mobile phase was a mixture of 30 mM phosphate buffer (pH 7.0) and acetonitrile (40/60, v/v), and the flow rate was 1.00 ml/min. Calibration curves were obtained by injecting progesterone (15—1500 ng) into the HPLC system.

Western Blot and RT-PCR Analyses  For the Western blot and RT-PCR analyses, Caco-2 cells were seeded at a density of 8.0×10$^6$ cells/cm$^2$ in a culture dish or tube. Caco-2 cell monolayers cultured for 14 to 16 d were used in the experiments. The culture medium was aspirated and the Caco-2 cell monolayers were washed twice with FBS-free culture medium. Then, the monolayers were incubated with FBS-free culture medium for 10 min at 37 °C. The medium was aspirated after the incubation and the monolayers were incubated with fresh FBS-free culture medium containing progesterone or northerthisterone for 24 h at 37 °C. The incubation medium was aspirated and the monolayers were washed twice with MES buffer.

The preparation of apical membrane vesicles (AMVs) from Caco-2 cells and PEPT1 polyclonal antibody was performed as described previously.$^{12}$ AMV proteins (150 μg) were suspended in SDS buffer (2% SDS, 62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 0.0625% dithiothreitol). The samples were subjected to 7.5% SDS-PAGE in a Laemmli system.$^{13}$ The resolved proteins were transferred to PVDF membranes (Bio-Rad) and blocked with 5% blocking agent (Amersham, U.K.) in 0.1% Tween–PBS buffer (pH 7.4) for 1 h at room temperature. After washing with 0.1% Tween–PBS buffer, the membranes were incubated with polyclonal antibody (1 : 1000) raised against PEPT1 protein at room temperature for 1 h. Then, the PVDF membranes were washed with 0.1% Tween–PBS buffer and incubated with the second antibody (horseradish peroxidase (HRP) conjugated donkey anti-rabbit IgG, 1 : 25000, Amersham). The PVDF membranes were washed again with 0.1% Tween–PBS buffer, and PEPT1 protein in the AMVs was detected by an enhanced chemiluminescence system (ECL Plus, Amersham). Band density was quantified with Luminous Imager Ver. 2.0 (Aisin Cosmos R&D Co., Aichi, Japan).

The isolation of total RNA from Caco-2 cells and the RT-PCR analysis were performed as described previously.$^{12}$

Statistical Analysis  The results are expressed as means±S.D. Differences between groups were evaluated.
using the Student’s t-test or the Welch’s t-test after initial analysis by the F-test. Three or more groups were evaluated using Bonferroni/Dunn’s multiple comparison test or Scheffe’s multiple comparison test after analysis by the Bartlett test and one-way analysis of variance. p values less than 0.05 were considered significant.

RESULTS

Effects of Progesterone and Norethisterone Pretreatments on Cephalexin Transepithelial Transport After the apical membrane side and/or the basolateral membrane side of Caco-2 cell monolayers were pretreated with progesterone, the effect of progesterone on the apical-to-basolateral transport and the basolateral-to-apical transport of cephalexin was investigated. As shown in Figs. 1A and B, the apical-to-basolateral transport of cephalexin was significantly decreased by progesterone pretreatment for 30—60 min on the apical side, and significantly and markedly decreased by progesterone pretreatment for 15—60 min on the basolateral side and on both sides, compared with that of the control. When progesterone was added to the apical side, intracellular cephalexin accumulation from the apical side in the Caco-2 cell monolayers was not affected; however, progesterone pretreatment of the basolateral side and both sides led to a significant decrease in intracellular cephalexin accumulation compared with that of the control. By contrast, neither the basolateral-to-apical transport of cephalexin nor the intracellular cephalexin accumulation from the basolateral side was altered by progesterone pretreatment of the apical and/or basolateral sides (Figs. 1C, D). The apical-to-basolateral transport of cephalexin and the intracellular cephalexin accumulation from the apical side were decreased markedly by progesterone pretreatment of the basolateral side and the both sides, compared to that of the apical side. To clarify this discrepancy, we determined the intracellular progesterone level after the apical side and/or the basolateral side were pretreated with progesterone for 24 h. As shown in Table 1, the intracellular progesterone levels after progesterone pretreatment of the basolateral side and both sides were significantly enhanced compared with that after the treatment of the apical side.

Next, we investigated the effect on the apical-to-basolateral transport and the basolateral-to-apical transport of cephalexin after the Caco-2 cell monolayers were pretreated with norethisterone. As shown in Fig. 2, the results were similar to those of the progesterone pretreatment.

Effects of Progesterone and Norethisterone Pretreatments on PEPT1 mRNA and Protein Expressions The PEPT1 mRNA expression level in Caco-2 cells with or without progesterone and norethisterone pretreatments is shown in Fig. 3. The PEPT1 mRNA expression level was significantly and dose-dependently decreased by the progesterone and norethisterone pretreatments. We performed Western blot analysis to determine the PEPT1 protein expression level.
on the AMVs in Caco-2 cells. As shown in Fig. 4, the progesterone- and norethisterone-pretreated Caco-2 cells showed a significant decrease in the PEPT1 protein expression level on the apical membrane compared with the non-treated Caco-2 cells.

DISCUSSION

It was about 10 years ago when cDNAs encoding PEPT1 were isolated from human, rabbit, and rat intestine. Subsequently, there has been substantial progress in the identification of the factors controlling the regulation and the mechanisms of PEPT1 activity. Adibi was the first to compile an excellent review of the recent advances in the regulation of PEPT1 expression by physiological factors, pathological conditions and pharmacological agents. However, the relationship between PEPT1 activity and steroid hormones was not described in the review at all.

Fujita et al. have reported that dipeptide glycylsarcosine uptake and PEPT1 mRNA expression level were increased by treatment with (+)-pentazocine, a selective σ1-receptor ligand, in Caco-2 cell monolayers. Recently, we investigated the effect of the female sex hormone, progesterone, on the expression of cephalexin uptake in Caco-2 cells, and found that cephalexin uptake was inhibited by progesterone (30 μM) pretreatment for 12—72 h in the Caco-2 cell monolayers. However, no significant decrease in cephalexin uptake was observed upon progesterone pretreatment...
ment for 0—6 h. Therefore, we proposed that the transcription of the PEPT1 gene is regulated by progesterone treatment. Moreover, progesterone at concentrations of 1—30 µM and the synthetic progesterin, norethisterone, at concentrations of 3—30 µM significantly inhibited only the saturable component of cephalexin uptake. The absorption of cephalexin is mediated by PEPT1\(^{25}\) and its secretion is mediated by other drug transporters. Cephalexin exists as an amphoteric compound under physiological conditions. Therefore, although the secretion of cephalexin from intestinal epithelial cells is mediated by organic anion transporter\(^{26}\) and organic cation/carnitine transporter,\(^{25}\) it is not mediated by P-glycoprotein.\(^{26}\) In the present study, we investigated the effect of progesterone and norethisterone pretreatments at 30 µM on cephalexin transport (apical-to-basolateral and basolateral-to-apical) using Caco-2 cell monolayers.

The apical-to-basolateral transport of cephalexin and the intracellular cephalexin accumulation from the apical side were decreased by the progesterone and norethisterone pretreatments. By contrast, neither the basolateral-to-apical transport of cephalexin nor the intracellular cephalexin accumulation from the basolateral side was altered by the progesterone and norethisterone pretreatments. From these findings, we considered that the decrease in the apical-to-basolateral transport of cephalexin by the progesterone and norethisterone pretreatments originated in the decrease in PEPT1 activity on the apical membrane. Moreover, when the basolateral side was pretreated with progesterone and norethisterone, the apical-to-basolateral transport of cephalexin and the intracellular cephalexin accumulation from the apical side were decreased markedly compared with those of progesterone and norethisterone pretreatments of the apical side. To clarify this discrepancy, the intracellular progesterone levels at the apical and basolateral sides were determined.

The intracellular progesterone level after progesterone pretreatment of the basolateral side was significantly higher than that after the pretreatment of the apical side. Progesterone is mainly transported by simple diffusion and the secretion transport activity is known to be higher than the absorption transport activity\(^{27,28}\) in Caco-2 cells. Our results correspond to the results of those reports. Therefore, it is considered that the high intracellular progesterone level is one cause of the marked decrease in the apical-to-basolateral transport (absorption) and the intracellular accumulation of cephalexin when the basolateral side is pretreated with progesterone and norethisterone. A similar mechanism is also considered in the case of norethisterone pretreatment.

Then, the densities of PEPT1 mRNA and protein expressions on the apical membrane were measured by using progesterone- and norethisterone-pretreated Caco-2 cells, to examine the mechanism of the PEPT1 transport activity decrease. As a result, the decreases in the density of PEPT1 mRNA expression and that of PEPT1 protein expression on the apical membrane were due to progesterone and norethisterone pretreatments. From these findings, the decrease in PEPT1 activity by the progesterone and norethisterone pretreatments was considered to be caused by the decrease in the transcriptional activity of the PEPT1 gene. Recently, Li et al.\(^{29}\) reported that PEPT1 mRNA expression was not altered significantly by progesterone treatment in Caco-2 cells. They investigated Caco-2 cells (passages 30—60) treated with progesterone (10 µM) for 72 h on day 14 after seeding. In the present study, we demonstrated the inhibition of cephalexin uptake by the progesterone and norethisterone treatments for 24 h on days 14—16 after seeding using Caco-2 cells (passages 50—60). This discrepancy may be attributed to differences in the experimental conditions, although the actual reason is not clear.

In conclusion, we have demonstrated that the apical-to-basolateral transport of cephalexin is decreased by progesterone pretreatment, and that the effect of progesterone is due to the inhibition of PEPT1 mRNA and protein expressions. The present results suggest that the transcription of the PEPT1 gene may be downregulated by the progesterone and norethisterone pretreatments. Although the σ1-receptor\(^{30}\) is expressed in Caco-2 cells, the expression of progesterone receptor\(^{11}\) in the same is not known; however, it has been reported that the receptor is expressed in human bowel. Cephalexin uptake was decreased by the treatments of progesterone and its related compounds, however dehydroepiandrosterone and pregnenolone, both of which are neurosteroids and σ1-receptor ligands and the other compounds was not affected cephalexin uptake. From these findings,\(^{15}\) we speculated that the uptake and transport of PEPT1-mediated drugs was not necessarily increased by treatment with a σ1-receptor ligand. However, further studies are needed to clarify whether the inhibition of the transcription of the PEPT1 gene by progesterone proceeds via the σ1-receptor or the progesterone receptor using the agonists and antagonists of these receptors.

REFERENCES