Secretory Transport of Methylprednisolone Possibly Mediated by P-Glycoprotein in Caco-2 Cells

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We recently reported that P-glycoprotein (MDR1) is capable of interfering with the absorption of methylprednisolone in the rat small intestine. This study was undertaken to examine the interaction between methylprednisolone and MDR1 using Caco-2 cells. The permeation of various steroid hormones (hydrocortisone, prednisolone, progesterone, β-estradiol, and testosterone) was compared. The basolateral-to-apical (secretory) permeation of methylprednisolone was more than 3-fold greater than the apical-to-basolateral (absorptive) permeation. When verapamil (0.1 mM), a potent modulator of MDR1, was added to both apical and basolateral sides of Caco-2 cells, the absorptive permeation of methylprednisolone was increased and its secretory permeation was decreased. As a result, the secretory-oriented manner of methylprednisolone permeation almost completely disappeared. Prednisolone and hydrocortisone exhibited weaker secretory-oriented movement than did methylprednisolone. The secretory-oriented permeation of prednisolone and hydrocortisone was also diminished by the addition of verapamil. There was no significant directionality in progesterone permeation and the permeation of β-estradiol and testosterone tended to be absorptive. These results appear to suggest that methylprednisolone, prednisolone, and hydrocortisone interact with MDR1 as the substrates. In contrast, there was no evidence that MDR1 was capable of potently interfering with the absorption of the sex hormones tested in this study, supporting our previous findings in the rat. It was further found that apically-added verapamil demonstrated a modulating effect on MDR1 function even at 5 μM.

Key words methylprednisolone; P-glycoprotein; Caco-2; secretory-oriented transport; steroid absorption

P-Glycoprotein (MDR1) is localized on the epithelial membrane of various tissues including the intestine,1,2 and its physiological function may be to detoxify or protect the body from various drugs and cytotoxic compounds.3 Several reports have described the interaction of various steroid hormones with MDR1 as substrates or modulators.4-7 In a recent study, we reported that methylprednisolone disappeared from in situ rat intestinal loops in a region-dependent manner; that is, its disappearance was greater in the upper small intestine and relatively smaller in the lower small intestine.8 It was also found that the addition of verapamil and cyclosporin A, potent modulators of MDR1, significantly increased the disappearance of methylprednisolone from the entire small intestine, though more markedly in the lower small intestine.9,10 A likely explanation was that methylprednisolone absorption was lowered through interaction with MDR1, especially in the lower small intestine.11 It is known that MDR1 expression is rich in the lower small intestine.12 On the other hand, it was found that the steroid sex hormones β-estradiol, progesterone, medroxyprogesterone and testosterone were absorbed very rapidly and almost completely from the entire small intestine, implying that MDR1 did not interfere with the absorption of these hormones in this organ.9 These results supported previous reports which described that progesterone and medroxyprogesterone were not transported by MDR1 on multidrug-resistant cancer cells.7,10 Progesterone and progesterone-like compounds are regarded as potent modulators rather than substrates of MDR1.13 However, our previous results from the rat small intestine are not always direct evidence that MDR1 is able to carry methylprednisolone more efficiently compared with other steroid hormones.

A human colorectal carcinoma cell line, Caco-2, has been used to predict the intestinal absorption of various drugs and new chemical entities in humans for nearly a decade.11,12 Recently, Caco-2 has been used increasingly as an experimental model for evaluating the interactions between MDR1 and drugs, because this cell line expresses abundant MDR1 on its apical membrane.13,14 This study was undertaken to elucidate the permeation of methylprednisolone and several steroid hormones across Caco-2 cell monolayers, with special focus on their interaction with MDR1.

MATERIALS AND METHODS

Materials 6α-Methylprednisolone, prednisolone, hydrocortisone, β-estradiol, and testosterone were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Progesterone and verapamil hydrochloride were obtained from Wako Pure Chemical Ind. (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), and non-essential amino acid solution were obtained from Life Technologies (Grand Island, NY, U.S.A.). Other chemicals were of the highest grade available.

Cell Culture Caco-2 cells (passage #40) were purchased from the Riken Cell Bank (Tsukuba, Japan) and were kept frozen in aliquots in liquid nitrogen. At passage #42—44, the cells were seeded on 60-mm plastic culture dishes coated with rat tail collagen type I (Becton Dickinson, Bedford, MD, U.S.A.) at a density of 1×10⁶ cells/cm² in 5 ml of DMEM with 5% FCS. They were grown at 37 °C in an atmosphere of 5% CO₂ in air and 90% relative humidity. The culturing medium was replaced every 3—4 d after inoculation. Subculturing was carried out every 6 d using 0.02% EDTA and

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0.25% trypsin. Caco-2 cells (passage #45—55) were seeded at a density of 1 × 10^4 cells/cm² on polycarbonate filter inserts (Becton Dickinson) with a surface area of 4.2 cm². For permeation experiments, Caco-2 cell monolayers, with transepithelial electrical resistance over 400 Ω·cm², were used on 20—22 d (mostly on 21 d) after seeding.

**Transport Study across Caco-2 Cell Monolayers** Dulbecco’s phosphate-buffered saline (D’s PBS, 137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, and 5 mM D-glucose, pH 7.4) was used as an experimental medium. The apical-to-basolateral and basolateral-to-apical permeation of test compounds across Caco-2 cell monolayers was determined at 37°C. Caco-2 cells were rinsed three times with 2 ml of D’s PBS at pH 7.4. Caco-2 cells were preincubated for 10 min at 37°C. The permeation experiment was initiated by adding D’s PBS including a test compound either to the apical or basolateral side of cell monolayers that were then incubated for 60 min. The medium volume of the apical and basolateral side was 1.5 and 2.2 ml, respectively. At stated times, aliquots of the receiver medium were taken to measure the amount that had permeated Caco-2 cell monolayers. The cumulative amount of test compounds permeated in both directions was calculated and plotted vs. time. The slope calculated was corrected for membrane surface area and donor concentration to obtain an apparent permeation coefficient (P<sub>app</sub>).

**Analytical Methods** Determination of steroid hormones tested was carried out by HPLC as described previously.8,9

**Protein Assay** A Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) was used to determine the protein contents of the cell monolayers, which were dissolved in 1 M NaOH, with bovine serum albumin used as a standard.

**Statistical Analysis** Statistical significance was evaluated by Tukey’s multiple comparison procedure (ANOVA) and Student’s t-test. The level of significance was p<0.05.

**RESULTS AND DISCUSSION**

**Permeation of Methylprednisolone across Caco-2 Cell Monolayers** Figure 1 shows the apical-to-basolateral (absorptive) and basolateral-to-apical (secretory) permeation of methylprednisolone (50 μM) across Caco-2 cell monolayers in the presence or absence of verapamil. In the absence of verapamil, methylprednisolone was much more permeable in the basolateral-to-apical direction than in the apical-to-basolateral direction, demonstrating a typical secretory-oriented transport in Caco-2 cells. The apparent permeation coefficients (P<sub>app</sub>) in both directions are listed in Table 1. In this study, P<sub>app</sub> was not corrected for the paracellular permeation. The ratio of secretory to absorptive permeability (B to A/A to B) was 3.4 at 50 μM. When verapamil was added to both apical and basolateral sides of Caco-2 cells at a concentration of 0.1 mM, the apical-to-basolateral permeation of 50 μM methylprednisolone significantly increased at each sampling time (Fig. 1). The amount permeated at 60 min was more than 200% of the control (i.e., in the absence of verapamil). On the other hand, the basolateral-to-apical permeation of methylprednisolone decreased to ca. 70% of the control at 60 min in the presence of verapamil. As a result, methylprednisolone permeation became almost equal in both directions, losing its directionality in Caco-2 cell permeation. It is well known that verapamil is capable of modulating MDR1 function. Therefore, it is very likely that this glycoprotein played significant role in the secretory permeation of methylprednisolone, supporting our previous findings that it significantly interfered with methylprednisolone absorption in the rat small intestine.8,9 However, since several recent reports have demonstrated that verapamil is also able to interfere with the function of other efflux transporters like multidrug resistance-associated proteins (MRPs),15,16 which is also present on the Caco-2 cell membrane,17 the possibility that non-MDR1 type transporters are involved in the secretory permeation of methylprednisolone cannot be excluded.

As shown in Table 1, when methylprednisolone permeation was examined at 100 μM, P<sub>app</sub> in the absorptive direction was increased and that in the opposite direction was decreased; the ratio of secretory to absorptive permeability thus
shifted to 1.6. It is probable that this result may relate to a concentration dependence of methylprednisolone transport.

For comparison, we evaluated the transport of vinblastine, a well-known MDR1 substrate, across Caco-2 cell monolayers in this study. The secretory permeation of vinblastine was 5.5-fold greater than its absorptive permeation (Table 1). We did not confirm the degree of MDR1 expression on the apical membrane of Caco-2 cells used in this study, for example, using a technique like immunohistochemical detection. However, the highly secretory-oriented transport of vinblastine could confirm that the present Caco-2 cells retained enough MDR1 to evaluate the transport it mediated. According to these observations, it is likely that methylprednisolone is transported by MDR1 to a lesser extent than vinblastine.

**Permeation of Various Steroid Hormones across Caco-2 Cell Monolayers** The permeability of prednisolone, hydrocortisone, progesterone, β-estradiol, and testosterone across Caco-2 cells is also presented in Table 1. Permeation experiments were done at a concentration of 50 μM, except β-estradiol which was evaluated at 25 μM due to its limited solubility in D’s PBS. Prednisolone and hydrocortisone exhibited greater basolateral-to-apical permeation than apical-to-basolateral permeation, consistent with the results of methylprednisolone (Table 1). The apical-to-basolateral permeation of prednisolone and hydrocortisone was almost identical to methylprednisolone, whereas their basolateral-to-apical permeation was relatively smaller. Although the directionality in their permeation was almost gone in the presence of verapamil, the modulator of MDR1 mainly affected the secretory permeation of the two hormones but not their absorptive permeation. These results might imply that the secretory-oriented transport of prednisolone and hydrocortisone was also related to MDR1, but their interaction with MDR1 might be weaker than methylprednisolone. The order of the ratio of secretory to absorptive permeability was methylprednisolone > prednisolone > hydrocortisone. Earlier, we demonstrated that the absorption of these three steroid hormones from the rat intestine was in the order of hydrocortisone > prednisolone > methylprednisolone. Therefore, it can be said that their absorption is significantly dependent on the extent of their interaction with MDR1. Barnes et al. previously reported that hydrophilic steroid hormones were more suitable substrates for MDR1 in multidrug-resistant cancer cells. We compared the log partition coefficients of methylprednisolone, prednisolone and hydrocortisone between n-octanol and a pH 7.4 buffer and found them to be 2.09, 1.64 and 1.55, respectively. Accordingly, our present results may be inconsistent with those of Barnes et al. Further information on various steroid hormones is required to clarify the discrepancy.

The basolateral-to-apical permeation of progesterone was slightly but not significantly greater than its apical-to-basolateral permeation. In the case of β-estradiol and testosterone, on the other hand, apical-to-basolateral permeation was significantly greater than basolateral-to-apical permeation, suggesting that the transepithelial movements of β-estradiol and testosterone were absorptive-oriented. It seems that these results are associated with earlier reports showing that the effect of MDR1 on the absorption of these three steroid hormones was small. However, it is still possible that MDR1-mediated secretory transport is fully covered by a specialized absorption mechanism for β-estradiol and testosterone; further studies are now being conducted.

**Concentration Dependence of Apically-added Verapamil Effect on Absorptive Permeation of Methylprednisolone** To date, information on the relationship between verapamil concentration and its efficacy to inhibit the function of transporters is inadequate. Table 2 shows the effects of verapamil, which was added to the apical side of cell monolayers over a concentration range between 5 and 100 μM, on the apical-to-basolateral permeation of methylprednisolone. The P_{app} gradually increased with increase in verapamil concentration to 40 μM and then remained constant. For comparison, the effect of 5 μM of apically-added verapamil was examined on the basolateral-to-apical permeation of methylprednisolone, and it was found that secretory permeation of methylprednisolone was decreased to ca. 60% of that in the absence of verapamil (data not shown). The site and mechanism of verapamil action have not been fully characterized. Very recently, we reported that the presence of a substrate on the apical surface of Caco-2 cell membrane was necessary to initiate MDR1 transport of the same substrate in the basolateral-to-apical direction and that 5 μM of verapamil completely inhibited this process. The present results implied that apically-added verapamil is capable of fully displaying the effect at relatively low concentration.

In conclusion, it has been suggested that methylprednisolone is possibly a more potent substrate of MDR1 than either prednisolone or hydrocortisone. The magnitude of the interaction with MDR1 is closely related to the extent of the absorption in the small intestine. Poor response to the methylprednisolone therapy, possibly due to incomplete absorption of the glucocorticoid, has been reported clinically. Our present results propose that methylprednisolone is less absorbable in a patient who has over-expression of MDR1 in the gastrointestinal tract.

**REFERENCES**