Drug Metabolism and Pharmacokinetics 30 (2015) 154–159

Contents lists available at ScienceDirect

Drug Metabolism and Pharmacokinetics

journal homepage: http://www.journals.elsevier.com/drug-metabolism-and-pharmacokinetics

Regular article

Sustained inhibition of proton-coupled folate transporter by myricetin

Mai Furumiya [a, b], Takahiro Yamashiro [a], Katsuhisa Inoue [c], Chihiro Nishijima [a], Kinya Ohta [a], Yayoi Hayashi [b], Hiroaki Yuasa [a, *]

[a] Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan
[b] Department of Biopharmaceutics, College of Pharmacy, Kinjo Gakuin University, Nagoya, Japan
[c] Department of Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan

ARTICLE INFO

Article history:
Received 10 August 2014
Received in revised form
14 October 2014
Accepted 18 November 2014
Available online 25 November 2014

Keywords:
Proton-coupled folate transporter
Caco-2 cells
Folate
Methotrexate
Myricetin
Flavonoid
Sustained inhibition
Drug interaction

SUMMARY

Myricetin is a flavonoid that has recently been suggested to interfere with the intestinal folate transport system. To examine that possibility, focusing on its sustained inhibitory effect on proton-coupled folate transporter (PCFT), the uptake of folate was examined in Caco-2 cells, in which PCFT is known to be in operation, in the absence of myricetin in the medium during uptake period after preincubation of the cells with the flavonoid (100 μM) for 1 h. This pretreatment induced an extensive and sustained reduction in the carrier-mediated component of folate uptake, which was attributable to a reduction in the maximum transport rate (V_{max}). Although the affinity of the transporter for folate was increased at the same time as indicated by a reduction in the Michaelis constant (K_m), the change in K_m was overwhelmed in extent by that in V_{max}. Consistent with the finding, folate transport by human PCFT stably expressed in Madin–Darby canine kidney II cells was reduced in a similar manner with simultaneous reductions in V_{max} and K_m by myricetin pretreatment. Attention may need to be given for a possibility that such a sustained inhibition of PCFT could potentially be a cause of the malabsorption of folate and also antifolate drugs.

1. Introduction

Malfunction of the intestinal folate transport system has been suspected as a possible mechanism that could be involved in the increased risk of folate deficiency brought about by continuous ingestion of alcoholic beverages [1–3]. To address this issue, studies have been conducted initially with the focus on the effect of ethanol as a potentially hazardous constituent of such beverages. However, the role of ethanol in the problem still remains inconclusive. On the other hand, it has recently been suggested that flavonoids, rather than ethanol, might be alternatively involved in the problem [4,5]. This indication would now raise an additional concern that the malabsorption of folate and analogs that share the same transport system for absorption might be also caused potentially by ingestion of some other types of beverages and food rich with some specific flavonoids. In drug therapy using antifolate drugs that are analogous to folate and transported by the folate transport system, this could be a case of drug interaction caused by flavonoids, which have been of increasing concern for their effects on enzymes and transporters involved in drug disposition [6–11].

Myricetin, a flavonoid contained abundantly in wines as a component originating from grapes, is one of those that have recently been suggested to interfere with the intestinal folate transport system in the Caco-2 cell as an intestinal epithelial cell model [4,5]. Indeed this flavonoid has more recently been found to inhibit proton-coupled folate transporter (PCFT/SLC46A1), which is responsible for intestinal folate uptake [12–16], in a noncompetitive manner [17]. In addition to such an instantaneous and transient effect caused in the presence of myricetin, this flavonoid has also been suggested to induce a different mode of inhibitory effect on the folate transport system, which is sustained in its absence after preincubation of Caco-2 cells with it [5]. Such a sustained effect could be more harmful to the absorption of folate than the instantaneous and transient one, due to the nature that it lasts for a certain period even after the disappearance of the flavonoid. However, the detailed characteristics and mechanism of the sustained effect have little been clarified yet. We, therefore, examined that aspect of the effect of myricetin on intestinal folate uptake,

* Corresponding author. Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan. Tel./fax: +81 52 836 3423.
E-mail address: yuasa@phar.nagoya-cu.ac.jp (H. Yuasa).

http://dx.doi.org/10.1016/j.dmpk.2014.11.001
1347-4367/

focusing on the functional modulation of PCFT, to further clarify the mechanism that could be potentially involved in the malabsorption of folate.

2. Materials and methods

2.1. Chemicals

[^{3}H]Folate (35 Ci/mmol) and [^{3}H]methotrexate (50.8 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA), unlabeled folate and methotrexate were from Sigma–Aldrich (St. Louis, MO), and myricetin and naringenin were from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of analytical grade and commercially obtained.

2.2. Cell culture

Caco-2 cells were obtained from RIKEN BioResource Center (Ibaraki, Japan) and Madin–Darby canine kidney II (MDCKII) cells were from Cell Resource Center for Biomedical Research, Tohoku University. Caco-2 cells and MDCKII cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin.

2.3. Uptake study

Solutions for experiments were prepared using Hanks' solution (136.7 mM NaCl, 5.36 mM KCl, 0.952 mM $CaCl_2$, 0.812 mM $MgSO_4$, 0.441 mM KH_2PO_4, 0.385 mM Na_2HPO_4, 25 mM D-glucose) modified by supplementation with 10 mM MES (pH 5.5). Caco-2 cells were seeded at the density of 1.5×10^5 cells/mL (1 mL/well) on 24-well plates coated with poly-L-lysine and grown for 4 days to confluence. The cells in each well were preincubated for 1 h in 1 mL of the modified Hanks' solution with or without myricetin (100 μM) as a test flavonoid and, after replacing the solution with fresh one without myricetin, preincubated for 5 min for washout. Then the solution was replaced with 0.25 mL of the modified Hanks' solution added with [^{3}H]folate as a substrate to start uptake assays in the absence of myricetin in the medium. All the procedures were conducted at 37 °C. Assays were stopped by addition of ice-cold modified Hanks' solution (2 mL), and the cells were washed two times with 2 mL of the same solution. The cells were solubilized in 0.5 mL of 0.2 M NaOH solution containing 0.5% sodium dodecyl sulfate (SDS) at room temperature for 1 h, and the associated radioactivity was determined by liquid scintillation counting, using 3 mL of Clear-sol I (Nacalai Tesque, Kyoto, Japan) as a scintillation fluid, for the evaluation of uptake. Cellular protein content was determined by the method of Lowry et al. [18], using bovine serum albumin as the standard. In experiments to assess the PCFT function at pH 7.4 for comparison with that at pH 5.5 as the regular condition, 10 mM MES in the modified Hanks' solution was replaced with 10 mM HEPES to adjust pH.

Similarly, MDCKII cells stably expressing human PCFT tagged with green fluorescent protein (GFP), which had been prepared as described previously [13], were grown on 24-well plates (1.5×10^5 cells/mL and 1 mL/well) for 2 days to confluence and, after pretreatment with or without myricetin (100 μM), uptake assays were conducted using [^{3}H]folate as a substrate. Mock cells, which were transfected with empty vector, were used to estimate nonspecific uptake. Experiments were also conducted using [^{3}H]methotrexate as another substrate and naringenin (100 μM) as another test flavonoid. The GFP-tagged PCFT was used in this study for inspection of its expression by observation of GFP-derived fluorescence and by western blot analysis probing for GFP.

2.4. Western blot analysis

MDCKII cells stably expressing GFP-tagged human PCFT were grown on 24-well plates and then pretreated with or without myricetin (100 μM), as described above. Thereafter, the cells in each well were washed twice with 2 mL of ice-cold phosphate-buffered saline (pH 7.4) and solubilized for 30 min on ice in 0.1 mL of lysis buffer, which consisted of 1% SDS, 4 M urea, 1 mM EDTA, 150 mM NaCl and 50 mM Tris (pH 8.0), and was supplemented with protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA), according to the manufacturer's instructions. The cell lysate was homogenized by sonication and then centrifuged at 15,000 × *g* for 20 min at 4 °C to obtain the supernatant as the sample of crude membrane fraction. The sample (20 μg protein) was separated on the 10% SDS-polyacrylamide gel by electrophoresis and transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 2% skim milk in Tris-buffered saline (pH 7.6)/0.1% Tween 20 and then probed with the primary antibody of mouse anti-GFP (mFX75; Wako Pure Chemical Industries) at a dilution of 1:1000 overnight at 4 °C. After washing three times with Tris-buffered saline (pH 7.6)/Tween 20, the membrane was incubated with the secondary antibody of goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma–Aldrich) at a dilution of 1:10,000 for 1 h at room temperature. Finally, the expression level of PCFT was determined by enhanced chemiluminescence using ECL reagent (Millipore, Billerica, MA), thus probing for GFP tagged to it. For the detection of β-actin, mouse anti-β-actin antibody (Sigma–Aldrich) was used as the primary antibody at a dilution of 1:1000 and, thereafter, the obtained sample was processed by the same procedures as those for the detection of PCFT.

2.5. Data analysis

In Caco-2 cells, the uptake rate (v) was estimated for the initial 10-min uptake period, in which uptake was almost in proportion to time. In kinetic analysis, the v was assumed to consist of a carrier-mediated component represented by a single Michaelis–Menten equation and a nonsaturable component as follows: $v = V_{max} \times s/(K_m + s) + CL_{ns} \times s$. The apparent parameters of maximum transport rate (V_{max}), the Michaelis constant (K_m) and the clearance of nonsaturable transport (CL_{ns}) were estimated by fitting this equation to the experimental profile of v *versus* s, using a nonlinear least-squares regression analysis program, WinNonlin (Pharsight, Mountain View, CA), and v^{-2} as the weight. A set of parameters was estimated for a profile, in which v was estimated as the mean of duplicate determinations at each s, and then the mean of each parameter was estimated with its SE using three sets of parameters.

In PCFT-transfected MDCKII cells, the initial rate of specific uptake by PCFT was estimated for a shorter period of 2 min and by subtracting the uptake rate in mock cells from the total rate of uptake in the PCFT-transfected cells. It was designated to be v_c as the one equivalent to the carrier-mediated component of v and assumed to consist of the single Michaelis–Menten term. The v_c was kinetically analyzed as described above for the analysis of v in Caco-2 cells.

Experimental data are presented as means ± SE. Statistical analysis was performed by using Student's t-test or, when multiple comparisons were needed, ANOVA followed by Dunnett's test, with $p < 0.05$ considered significant.