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Kinetic and time-dependent features of sustained inhibitory effect of myricetin on folate transport by proton-coupled folate transporter

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ABSTRACT

Myricetin is a flavonoid that has recently been suggested to induce sustained inhibition of proton-coupled folate transporter (PCFT/SLC46A1), which operates for intestinal folate uptake. The present study was conducted to characterize the inhibitory effect in more detail, using human PCFT stably expressed in Madin–Darby canine kidney II cells, to gain information to cope with problems potentially arising from that. The kinetics of saturable folate transport was first assessed in the absence of myricetin in the cells pretreated with the flavonoid for 60 min. The pretreatment induced PCFT inhibition in a manner dependent on the concentration of myricetin, where the maximum transport rate was reduced by 35.5% and 83.1%, respectively, at its concentrations of 20 μM and 50 μM. The inhibitory effect was, however, less extensive at lower folate concentrations, because the Michaelis constant was also reduced similarly in a manner dependent on myricetin concentration. The inhibition was induced depending on the time of pretreatment and, after removal of myricetin (50 μM) upon the manifestation of an extensive inhibition at 60 min, reversed almost completely in 90 min. This rather short time required for recovery may suggest that the sustained inhibition of PCFT is of a reversible type.

1. Introduction

Flavonoids have been of increasing concern for their inhibitory effects on transporters and enzymes involved in the disposition of drugs and nutrients [1–6]. Myricetin, which is present in various fruits and vegetables, is one of such flavonoids. This flavonoid has recently been found to inhibit proton-coupled folate transporter (PCFT/SLC46A1) [7–9], which is responsible for the intestinal uptake of folate (vitamin B_9) and antifolate drugs analogous to the vitamin [10–14]. This myricetin-induced inhibition of PCFT, which could lead to the malabsorption of folate, has been suspected to be involved in some cases in the increased risk of folate deficiency in those who consume alcoholic beverages excessively, since this flavonoid is contained abundantly in wine as one of such beverages [15–17]. It might also cause a decrease in the absorption of antifolate drugs, such as methotrexate, as a potential case of drug interaction.

Myricetin has been indicated to inhibit PCFT not only in a transient manner, in which inhibition is induced instantaneously upon the addition of an inhibitor and manifested only during its presence, but also in a sustained manner, in which inhibition is induced typically in a time-dependent manner in the presence of an inhibitor and persistent after its removal. The transient types of inhibition are typically caused by the action of an inhibitor on a transporter or an enzyme in a competitive manner or in a noncompetitive manner, and the myricetin-induced inhibition of PCFT was found to be of the noncompetitive type in our previous study [8]. On the other hand, the sustained types of inhibition are in general more complicated in their mechanisms and manifestations. In our previous study conducted using a single test condition, in which Madin–Darby canine kidney II (MDCKII) cells stably expressing human PCFT tagged with green fluorescent protein (GFP) were pretreated with 100 μM of myricetin for 60 min, it was found that the pretreatment induces an extensive reduction in the maximum transport rate (V_{max}) for folate, although there were no major alterations in the expression and localization of PCFT, which were examined by western blot analysis probing for GFP and

Abbreviations: CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GFP, green fluorescent protein; IBMX, 3-isobutyl-1-methylxanthine; MDCK, Madin–Darby canine kidney; OATP, organic anion transporting polypeptide; PMA, phorbol 12-myristate 13-acetate; PI3K, phosphoinositide 3-kinase; PCFT, proton-coupled folate transporter.

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observation of fluorescence from GFP [9]. Together with the reduction in V_{max}, the Michaelis constant (K_m) was also found to be reduced. It was, therefore, assumed to be likely that myricetin induces some kind of modulation of PCFT that makes it less functional for the translocation of substrates across the plasma membrane, while enhancing its affinity for substrates. However, several important issues, such as the effect of the concentration of myricetin on the inhibition and time-dependent changes in the inhibition in the phases of induction and recovery, have not been clarified yet. It should be of interest and needed to clarify them to elucidate the mechanism of the inhibition and to cope with clinical problems potentially arising from that. We, therefore, addressed those issues in the present study as an extension of our previous study. We also intended to confirm the sustained inhibitory effect of myricetin on native human PCFT without any artificial modulation, since GFP-tagged one was used in our previous study for the visualized assessment of its expression and localization [8].

2. Materials and methods

2.1. Materials

[^{3}H]Folate (19.4 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA), unlabeled folate was from Wako Pure Chemical Industries (Osaka, Japan), and myricetin was from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were of analytical grade and commercially obtained.

2.2. Cell culture

MDCKII cells were obtained from RIKEN BioResource Center (Ibaraki, Japan). The 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 (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin.

2.3. Preparation of MDCKII cells stably expressing PCFT

MDCKII cells were transfected with the plasmid carrying the cDNA of human PCFT, which had been prepared using pCI-neo vector (Promega, Madison, WI, USA) in our previous study [11], by using Lipofectamine 2000 (Invitrogen) as a transfection reagent, according to the manufacturer's instructions, and cultured in DMEM supplemented with 10% FBS and 400 μg/mL G418 for 2–3 weeks. Antibiotic-resistant clones were selected and tested for the transport of [^{3}H]folate as a probe substrate.

2.4. 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, and 25 mM D-glucose) modified by supplementation with 10 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 5.5). MDCKII cells stably expressing PCFT were seeded at the density of 1.5×10^5 cells/mL (1 mL/well) on 24-well plates and grown for 2 days to confluence. The cells in each well were preincubated for 60 min, unless otherwise indicated, in 1 mL of the modified Hanks' solution with or without myricetin and subsequently, after replacing the solution with fresh one without myricetin, preincubated for 5 min, unless otherwise indicated, 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. The concentrations of folate were 0.005, 0.1, 0.5, 1, 2, 5, 10 and 20 μM in experiments to assess its concentration-dependent uptake. In experiments to examine the effect of protein kinase modulators, the cells were preincubated for 60 min in the presence of a modulator to be tested. All the procedures were conducted at 37 °C. The 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 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 BCA method (BCA Protein Assay Reagent Kit; Thermo Fisher Scientific Waltham, MA, USA), using bovine serum albumin as the standard. Mock cells, which were transfected with empty pCI-neo vector, were used to estimate nonspecific uptake. The specific uptake of folate by PCFT was estimated by subtracting its uptake in mock cells from that in PCFT-expressing cells.

2.5. Data analysis

The uptake rate (v) was estimated for the initial 2-min uptake period, in which uptake was almost in proportion to time. The saturable transport of folate as the substrate was analyzed by assuming Michaelis–Menten type carrier-mediated transport represented by the following equation: $v = V_{max} \times s/(K_m + s)$. The parameters of maximum transport rate (V_{max}) and the Michaelis constant (K_m) were estimated by fitting this equation to the experimental profile of v *versus* the substrate concentration (s), using a nonlinear least-squares regression analysis program, WinNonlin (Pharsight, Mountain View, CA, USA), and v^{-2} as the weight. A set of parameters was estimated for each 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 standard error (SE), using three sets of parameters.

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

3. Results and discussion

3.1. Effect of the concentration of myricetin on its induced inhibition of PCFT

The saturable transport of folate by PCFT was first assessed after preincubation for 60 min in the presence of myricetin and subsequently for 5 min in its absence for washout. The uptake assays for the evaluation of transport was conducted at pH 5.5, where the H^+-dependent folate transporter can operate efficiently [10,11]. As shown in Fig. 1, the rate of PCFT-specific folate uptake was reduced by the pretreatment with myricetin, indicating its induced inhibition of PCFT. The inhibitory effect was greater in extent at the higher myricetin concentration of 50 μM than at 20 μM, indicating the inhibitory response depending on myricetin concentration, and was also greater in extent at the higher concentrations of folate at each myricetin concentration. As summarized in Table 1, kinetic analyses indicated that a reduction in V_{max} was responsible for the inhibition, where V_{max} was reduced by 35.5% and 83.1%, respectively, at the myricetin concentrations of 20 μM and 50 μM. It was also indicated that K_m was reduced at the same time by the extent comparable to that for V_{max} at each myricetin concentration, resulting in insignificant alterations in V_{max}/K_m. This implies that an enhancement in the affinity of PCFT for folate, as indicated by the reduction in K_m, counteracted the reduction in V_{max} and, as a consequence, the inhibitory effect was less extensive at lower folate concentrations. This was most evident at the lowest folate