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Inhibition effect of flavonoids on monocarboxylate transporter 1 (MCT1) in Caco-2 cells

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Abstract

This study aimed to investigate the inhibition effect of flavonoids on monocarboxylate transporter 1 (MCT1) in Caco-2 cells. The cellular uptake of benzoic acid was examined in the presence and the absence of naringin, naringenin, morin, silybin and quercetin in Caco-2 cells. All the tested flavonoids except naringin significantly inhibited ($P < 0.05$) the cellular uptake of [^{14}C]-benzoic acid. Particularly, naringenin and silybin exhibited strong inhibition effects with IC50 values of 23.4 and 30.2 μM, respectively. Kinetic analysis indicated that the inhibition mode of naringenin and silybin on MCT1 activity was competitive with a Ki of 15~20 μM. The effect of flavonoids on the gene expression of MCT1 was also examined by using RT-PCR and western blot analysis. Results indicated that the expression level of MCT1 was not affected by the treatment with naringenin or silybin. The cellular accumulation of naringenin in Caco-2 cells was not changed in the presence of benzoic acid or L-lactic acid, implying that naringenin might not be a substrate of MCT1. In conclusion, some flavonoids appeared to be competitive inhibitors of MCT1, suggesting the potential for diet–drug interactions between flavonoids and MCT1 substrates.

Introduction

Monocarboxylate transporters (MCTs), proton-linked membrane carriers, play a critical role in the transport of monocarboxylates across the cellular membrane and are important for energy metabolism, homoeostasis and pH control in various tissues (Poole & Halestrap 1993; Halestrap & Price 1999; Enerson & Drewes 2003). Among MCT isoforms, MCT1 has been most extensively characterized as a proton-linked monocarboxylate transporter (Halestrap & Meredith 2004). MCT1 is located in the brush-border membranes of both upper and lower intestines and is involved in the intestinal absorption of pharmacologically active compounds, such as β-lactam antibiotics, atorvastatin and pravastatin (Kang et al 1990; Tamai et al 1995; Li et al 1999; Wu et al 2000). Recently, Choi et al (2006) have reported that the concurrent use of benzoic acid, a substrate of MCT1, could significantly alter the oral exposure of ketoprofen via competition for the common transport pathway during intestinal absorption. Wu et al (2000) also reported that the cellular uptake of atorvastatin was significantly inhibited by benzoic acid in Caco-2 cells. Therefore, inhibition of MCT1 may lead to potential drug interactions in combination therapy.

Flavonoids are polyphenolic compounds widely present in fruits, vegetables, plant-derived foods and beverages (Dixon & Steele 1999). Owing to a variety of biological actions, including anti-oxidation, anti-ulcer, anti-allergic and anti-cancer activity (Kuhnau 1976; Surh 2003), there is an increasing number of patients taking herbal dietary supplements with primary prescription drugs (Richardson & Straus 2002; Cassileth & Deng 2004). A number of studies have reported that flavonoids can interplay with transporter proteins, such as P-gp, MRP1 and ABCG2, suggesting the potential for diet–drug interactions via the inhibition of those efflux transporters (Zhang & Morris 2003; van Zanden et al 2005; Morris & Zhang 2006). However, the interactions between flavonoids and uptake transporters have not been well characterized yet. Recently, Wang & Morris (2007) reported that the uptake of γ-hydroxybutyrate in rat MCT1-gene-transfected MDA-MB231 cells decreased significantly in the presence of some flavonoids via the inhibition of rat MCT1. However, the interaction mechanism of flavonoids against the human MCT1, particularly their effect

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on the human MCT1 gene expression, has not clearly been defined yet. Therefore, this study aimed to investigate the modulation of MCT1 activity by the naturally occurring flavonoids. Quercetin, morin, naringin, naringenin and silybin are widely present in our daily diet and their physiological concentrations achievable from dietary intake are available in the literature (Hertog et al 1993a, b; Ofer et al 2005). This study selected these five flavonoids and investigated their interaction characteristics with MCT1 in human colonic cancer cells (Caco-2 cells).

Materials and Methods

Materials

Naringenin, naringin, silybin, morin, quercetin, benzoic acid, [^{14}C]-benzoic acid (13.1 mCi mmol^{-1}) and BCA protein assay kit were purchased from Sigma Chemical Co. (St Louis, MO). The anti-hMCT1 antibody was supplied from Chemicon (Temecula, CA). Alkaline phosphatase donkey anti-mouse IgG and horseradish peroxidase-conjugated donkey anti-rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA). Anti-actin antibody and the other reagents in the molecular studies were obtained from Sigma Chemical Co. (St Louis, MO). Caco-2 cells were obtained from Korean Cell Line Bank (Seoul, Korea). All other chemicals were reagent grade and all solvents were HPLC grade.

Cell cultures

Caco-2 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1% non-essential amino acids, 1 mM sodium pyruvate, 1% L-glutamine and penicillin (100 U mL^{-1})/streptomycin (100 mg mL^{-1}). All cells were maintained in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

Inhibition studies in Caco-2 cells

Cells were seeded in 12-well culture plates at a density of 10^5 cells/cm^2. At 7 days post-seeding, the cells were washed twice with pH 6.0 uptake buffer containing (in mM) 1 $CaCl_2$, 1 $MgCl_2$, 150 NaCl, 3 KCl, 1 NaH_2PO_4, 5 D-glucose and 10 MES. Drug solution containing [^{14}C]-benzoic acid (10 μM, 0.1 μCi mL^{-1}) with/without each flavonoid (0.1~400 μM) was added to each well and incubated for 15 min. At the end of incubation, drug solution was removed and the cells were washed three times with ice-cold phosphate-buffered saline (PBS) buffer. One milliliter of 1.5% ice-cold Triton X solution was added to each well. After 15 min incubation, cells were harvested and the radioactivity in each sample was determined by a scintillation counter. The IC50 (drug concentration showing 50% inhibition on the uptake of benzoic acid) was determined from the nonlinear regression of a dose–response curve by using the SigmaPlot 9.0 (Systat Software Inc., Point Richmond, CA). To investigate the inhibition mode of flavonoids against MCT1 activity, the concentration-dependent uptake of [^{14}C]-benzoic acid was also determined in the presence and the absence of naringenin or silybin (50 μM) in Caco-2 cells. Based on the uptake of [^{14}C]-benzoic acid under each condition, double reciprocal plot analyses were performed. The slopes and intercepts were compared by using an unpaired *t*-test.

Cellular uptake of naringenin in Caco-2 cells

Cells were seeded in 6-well culture plates at a density of 105 cells/cm^2. At 7 days post-seeding, cells were incubated with a drug solution containing 40 μM naringenin in the absence and the presence of benzoic acid (500 μM) or L-lactic acid (500 μM). At the end of 15 min incubation, drug solution was removed and the cells were washed three times with ice-cold PBS. After the cell lysis, cells were harvested and sonicated for 1–2 min. Acetonitrile (1 mL) was added to the cell lysate, vortexed rigorously, and centrifuged for 5 min at 3000 rev min^{-1}. After filtration of the supernatant through a membrane filter (0.45 μm), samples were analysed by HPLC. The amount of protein in each sample was determined with BCA protein assay kit following the manufacturer's instructions (Sigma Chemical Co., St Louis, MO).

Reverse transcription–polymerase chain reaction (RT-PCR)

After the 6-h incubation of Caco-2 cells in the presence or absence of flavonoids (30 μM), the total RNA was isolated from the cells using total RNA isolation kit (RNAgents, Promega, Madison, WI). The total RNA (1.0 μg) obtained from the cells was reverse-transcribed using an oligo(dT) 18mer as a primer and M-MLV reverse transcriptase (Bioneer, Eumsung, Korea) to produce the cDNAs. PCR was performed using the selective primers for the human MCT1 (sense: 5′-CCATTGTGGAATGCTGTCCT-3′, antisense: 5′-CCTACTTCTTTCCCCCATCC-3′) and S16 ribosomal protein (sense: 5′-TCCAAGGGTCCGCTGCAGTC-3′, antisense: 5′-CGTTCACCTTGATGAGCCCATT-3′) genes. The PCRs were carried out for 35 cycles using the following conditions: denaturation at 98°C for 10 s, annealing at 51°C for 0.5 min, and elongation at 72°C for 1 min. The band intensities of the amplified DNAs were compared after visualization on a UV transilluminator.

Immunoblot analysis

MCT1 expression in Caco-2 cells was determined by western blot analysis as described previously (Kang et al 2006). Briefly, Caco-2 cells were incubated for 6 h or 24 h in the presence or absence of flavonoids (30 μM). At the end of incubation, cells were washed with sterile PBS and were lysed in buffer containing 20 mM Tris·Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride and 1 μg·mL^{-1} leupeptin. Cell lysates were centrifuged at 3000 rev·min^{-1} for 10 min to remove debris, and proteins were fractionated using a 10% separating gel. Fractionated proteins were then electrophoretically transferred to nitrocellulose paper, and proteins were immunoblotted with specific

antibodies. Nitrocellulose papers were developed using an ECL chemiluminescence system.

HPLC analysis

The chromatographic system consisted of a pump (LC-10AD), an automatic injector (SIL-10A) and a UV detector (SPD-10A) (Shimadzu Scientific Instruments, Tokyo, Japan). An octadecylsilane column (Gemini C18, 4.6×250 mm, 5 μm; Phenomenex, Torrance, CA) was eluted with a mobile phase consisting of methanol–water–acetic acid (40:58:2, v/v/v %). The flow rate was 1.0 mL min^{-1} with the detection wavelength set at 280 nm. The calibration curve from the standard samples was linear over the concentration range 0.01–5 μg mL^{-1}.

Statistical analysis

All the means were presented with their standard deviation. Differences between the means were analysed by a one-way analysis of variance followed by a-posteriori testing with the use of the Dunnett correction, or by using an unpaired *t*-test. $P < 0.05$ was considered statistically significant.

Results

Cellular uptake studies in Caco-2 cells

Among MCT isoforms, MCT1 is the most abundant isoform in Caco-2 cells (Hadjiagapiou et al 2000) and the expression of MCT1 protein in Caco-2 cells reached a peak at 7 days post-seeding (data not shown). Therefore, in this study, Caco-2 cells were cultivated for 7 days to elevate the expression of MCT1 and then five flavonoids—morin, naringin, naringenin, silybin and quercetin—were compared in Caco-2 cells with respect to their inhibitory effects on the cellular uptake of benzoic acid, a substrate of MCT1 (Poole & Halestrap 1993). All the tested flavonoids except naringin inhibited significantly ($P < 0.05$) the cellular uptake of [^{14}C]-benzoic acid (Figure 1). Particularly, naringenin and silybin exhibited a strong inhibition effect on the cellular uptake of [^{14}C]-benzoic acid, with IC50 values of 23.4 and 30.2 μM, respectively (Figure 2). Kinetic analysis using Lineweaver–Burk plots indicated that both naringenin and silybin inhibited the cellular uptake of benzoic acid competitively with Ki values of 15.3 μM and 19.7 μM, respectively (Figure 3). On the other hand, the cellular accumulation of naringenin was not significantly affected by the presence of benzoic acid or L-lactic acid (Table 1).

Effect of flavonoids on the gene expression of MCT1

To examine whether flavonoids modulate the expression of MCT1 at both mRNA and protein levels, RT-PCR and western blot analysis were performed using the cell lysates obtained after the incubation of Caco-2 cells with vehicle alone or naringenin (30 μM) or silybin (30 μM). The incubation of cells with naringenin or silybin for 6 h did not affect the mRNA expression of MCT1 (Figure 4A). In western blot analysis, the relative densitometric ratios to the controls

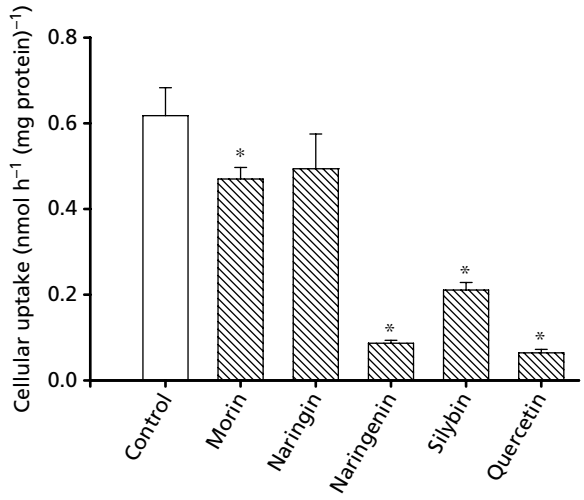

Figure 1 Inhibition effects of flavonoids on the cellular uptake of [^{14}C]-benzoic acid in Caco-2 cells (mean ± s.d., n = 6). *$P < 0.05$ vs control.

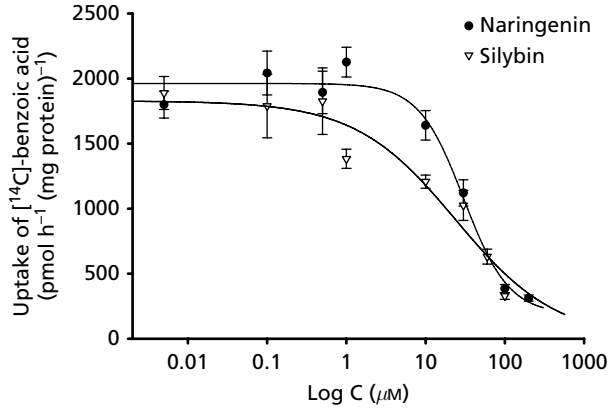

Figure 2 Inhibition effects of naringenin and silybin on the cellular uptake of [^{14}C]-benzoic acid in Caco-2 cells (mean ± s.d., n = 6).

treated with vehicle alone were 1.2 and 1.1 after 24 h incubations with naringenin or silybin, respectively (Figure 4B).

Discussion

Given that numerous xenobiotics contain a carboxyl group, making those compounds potential substrates for MCT1, the modulation of MCT1 activity may cause the pharmacokinetic interactions in combination therapy. Recently, Wang & Morris (2007) reported the inhibition effects of some flavonoids on rat MCT1, while Wenzel et al (2005) have reported that the flavonoid flavone could enhance the uptake of lactate into mitochondria, most likely by an allosteric activation of MCT1. Considering that previous reports on the modulation of MCT1 activity by flavonoids are contradictory in part and

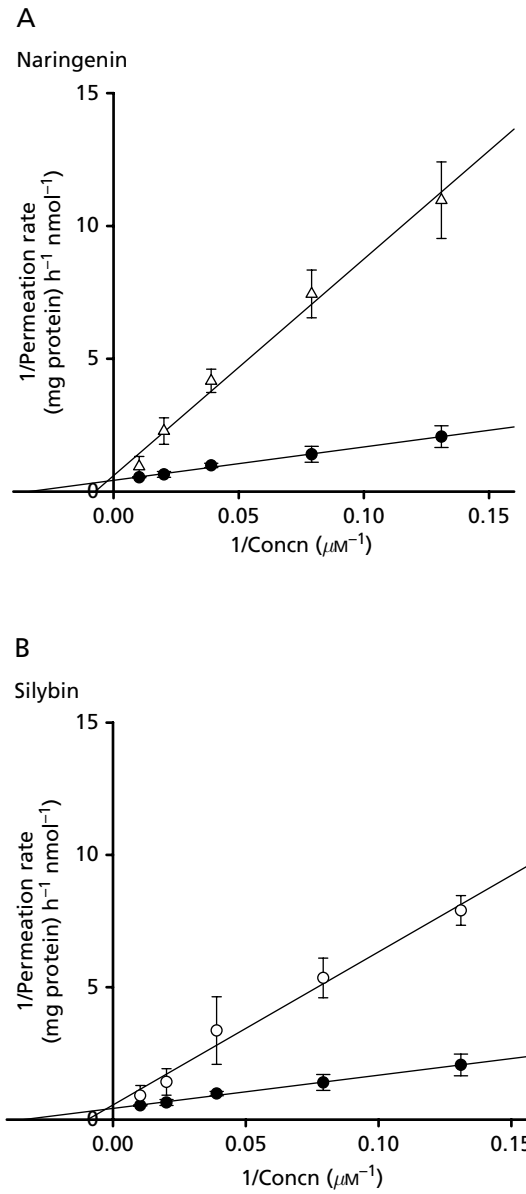

Figure 3 Lineweaver–Burk plots for the cellular uptake of benzoic acid across Caco-2 cell monolayer (mean = s.d., n = 6). The cellular uptake was measured in the absence (●) and the presence of 50 μM naringenin (△) or 50 μM silybin (○).

Table 1 Cellular accumulation of naringenin in Caco-2 cells

	Cellular accumulation (nmol h^{-1} (mg protein)$^{-1}$)
Naringenin 40 μM	8.51 ± 0.44
Naringenin 40 μM + benzoic acid 500 μM	8.53 ± 0.23
Naringenin 40 μM + L-lactic acid 500 μM	7.73 ± 1.66

Data are expressed as mean ± s.d., n = 6.

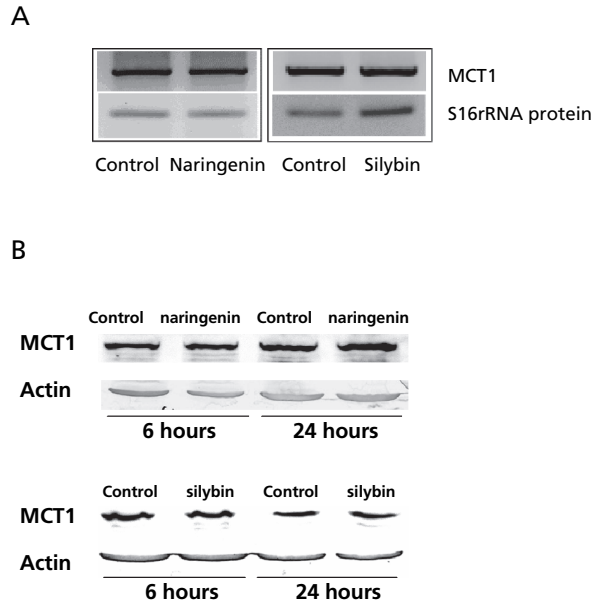

Figure 4 The effect of flavonoids on MCT1 expression in Caco-2 cells. A. mRNA level of MCT1: RT-PCR analysis was performed to evaluate the mRNA expression of MCT1 after the incubation of cells for 6 h in the presence and the absence of naringenin (30 μM) or silybin (30 μM). The mRNA expression of S16r protein was comparable among the samples. B. Protein level of MCT1. Western blot analysis was performed after the incubation of cells for 6 h or 24 h in the presence and the absence of naringenin (30 μM) or silybin (30 μM).

there should be a species difference in the transporter activity, interaction characteristics of flavonoids with human MCT1 are not clearly defined yet. Therefore, this study investigated the modulation of MCT1 activity by the naturally occurring flavonoids in human colonic cancer cells.

As illustrated in Figure 2, naringenin and silybin exhibited a strong inhibition effect on the cellular uptake of [^{14}C]-benzoic acid with IC50 values of 23~30 μM. Given that diclofenac, diflunisal, ketoprofen and naproxen exhibited the inhibition effect on the cellular uptake of [^{14}C]-benzoic acid with IC50 values of 50 μM to 440 μM in Caco-2 cells (Choi et al 2005), the inhibitory potency of flavonoids on MCT1 appeared to be even greater than those of nonsteroidal anti-inflammatory drugs. Furthermore, the inhibitory concentrations of tested flavonoids are in the range of physiologically relevant concentrations that should be achievable from the dietary intake (Hertog et al 1993a, b; Ofer et al 2005). For example, the relevant concentrations of flavonoids achievable from dietary intake are approximately 20 μM for quercetin, 300 μM for naringenin and 1000 μM for naringin (Hertog et al 1993a, b; Ofer et al 2005). Therefore, the results from this study suggest that strong inhibition effects of flavonoids on MCT1 are expected at the concentrations achievable from daily nutrition. Given that MCT1 could play an important role in the cellular uptake of pharmacologically active drugs, including β-lactam antibiotics and statins (Tamai et al 1995; Li et al 1999; Wu et al 2000; Enerson & Drewes 2003), this study raises the awareness of the potential diet–drug interactions via MCT1 inhibition.

Furthermore, considering that certain tumour cells, including hepatocarcinoma and high-grade glial neoplasms, may up-regulate the expression of MCT1 as a pH regulator to prevent the apoptosis caused by cellular acidosis (Froberg et al 2001; Kang et al 2006), flavonoids may be useful to improve the effectiveness of anti-cancer chemotherapy by promoting cellular acidosis via MCT1 inhibition.

Kinetic analysis indicated that the inhibition of MCT1 by naringenin and silybin was competitive in nature (Figure 3), which is also comparable with the observation by Wang & Morris (2007) with rat MCT1. A competitive type of inhibition against MCT1 suggested that flavonoids could presumably bind into the substrate-binding site. However, interestingly, the cellular accumulation of naringenin was not affected by the presence of MCT1 substrates, such as benzoic acid and L-lactic acid, implying that naringenin might not be translocated by MCT1. Konishi et al (2003) reported a similar observation with tea polyphenols in Caco-2 cells. Besides the direct inhibition on the substrate binding sites, flavonoids may modulate the gene expression of transporter proteins. Therefore, mRNA and protein level of MCT1 were determined in the presence of naringenin or silybin. As shown in Figure 4, RT-PCR and western blot analysis indicated that there were no changes in the expression level of either MCT1 mRNA or protein in the presence of naringenin or silybin. Therefore, the inhibition effect of flavonoids on MCT1 activity is unlikely to be due to the modulation of MCT1 gene expression. Taken all together, some flavonoids could interact with MCT1 as blockers of its transport function rather than as its transportable substrates. Further studies are necessary to reveal the inhibition mechanism of MCT1 by flavonoids.

Conclusion

The flavonoids morin, naringenin, silybin and quercetin appeared to be competitive inhibitors of MCT1, suggesting the potential for diet–drug interactions between flavonoids and MCT1 substrates.

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