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The *t*ransport mechanism of monocarboxylate transporter on spinosin in Caco-2 cells

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Abstract *Objectives:* The aim of this study was to determine the uptake mechanism of spinosin (SPI) by the monocarboxylic acid transporters (MCTs) in Caco-2 cells. *Methods:* The Caco-2 cells were pretreated with various monocarboxylic acids, and the uptake of spinosin from Caco-2 cells was measured by High Performance Liquid Chromatography (HPLC). *Key findings:* Preloading of various monocarboxylic acids enhanced the uptake of SPI, especially salicylic acid (a substrate of MCTs) had a 23.4 times increase in SPI uptake, indicating that the monocarboxylic acid transporters had an efflux effect on SPI uptake and salicylic acid had a strong inhibition on SPI efflux in Caco-2 cells. At the same time, the uptake of SPI through Caco-2 cells was Na^+- and temperature-dependent, pretreatment without Na^+ significantly increased the uptake of SPI by 1.85 times and incubated at low temperature (4 °C) SPI uptake increased 20% than that of 37 °C. Furthermore, SPI was transported mainly via a carrier-mediated transport: [Vmax = 5.364 μg/mg protein, Km = 657.0 μg/mL]. *Conclusion:* The uptake of spinosin (SPI) in Caco-2 cells was mainly regulated by the monocarboxylic acid transporters along with Salicylic acid.

1. Introduction

Spinosin (2″-b-O-glucopyranosyl swertisin, $C_{28}H_{38}O_{15}$), a C-glycoside flavonoid, is one of the major flavonoids of semen Zizhiphi spinozae (Yuan et al., 1987; Kawashima et al., 1997) Previous studies showed that spinosin played an important role in sedation and hypnosis (Li and Bi, 2006), and exerted anxiolytic-like effects and its mechanism appeared to be modulated by $GABA_A$ and 5-HT_{1A} receptors (Liu et al., 2015). Several pharmacokinetic investigations of spinosin revealed that it had a wide brain regional tissue distribution, particularly in corpus striatum and hippocampus (Zhang et al., 2015). However, the absolute bioavailability of spinosin in rat was only 2.2% (Li et al., 2008). It had demonstrated that efflux pump P-glycoprotein (P-gp) affected the absorption of spinosin by vivo microdialysis (Ma et al., 2012) and situ perfusion method (Huang et al., 2014), which may be one of the reasons of its low bioavailability. But remaining less

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research about the intestinal absorption mechanisms of spinosin was studied.

Transporter-mediated disposition plays an important role in pharmacokinetic changes of many drugs (Feng et al., 2014). Monocarboxylate transporters involve proton dependent monocarboxylate transporters (MCTs; SLC16A) contained 14 members which were identified based on sequence homology (Halestrap and Price, 1999) and sodium coupled monocarboxylate transporters (SMCTs) which contain only two members, SLC5A8 and SLC5A12 (Coady et al., 2004; Gopal et al., 2004; Srinivas et al., 2005). MCT1 is strongly expressed on the basolateral surface of enterocytes, whereas members of the SLC5A8 are expressed primarily on the apical surface (Iwanaga et al., 2006). Monocarboxylate transporters have significant impact on the intestinal absorption of its substrates including some short-fatty acids such as acetic acid, L-lactic acid, butyric acid, salicylic acid, nicotinic acid, succinic acid, citric acid, propionic acid and methanoic acid, and α-cyano-4-hydroxycinnamate (CHC) is a specific competitive inhibitor of MCT1, MCT2, and MCT4 (Halestrap and Wilson, 2012; Halestrap, 2013).

The human colon adenocarcinoma cell line Caco-2 cells are widely used as a valuable transport model system for the small intestinal epithelium when grown on dishes or permeable membranes (Hidalgo et al., 1989). Caco-2 cells express five isoforms of MCTs: MCT1, MCT3, MCT4, MCT5, and MCT6; particularly, MCT1 is most abundant (Hadjiagapiou et al., 2000). Furthermore, Caco-2 cells are often used to test whether a compound is transporter-mediated by MCTs when cultured on dishes or permeable membranes (Martel et al., 2006; Kimura et al., 2014; Kensuke et al., 2014). Shim had reported that the uptake of some flavonoids of naringin, naringenin, morin, silybin and quercetin was affected by MCT1 in Caco-2 cells (Shim et al., 2007). As spinosin which has a relatively low absolute bioavailability is a C-glycoside flavonoid, the aim of the study was to investigate whether the mechanism of SPI uptaked in Caco-2 cells was mediated via MCTs.

2. Materials and methods

2.1. Materials

Caco-2 cells were obtained from Institute of Basic Medical Cell Resource Center of Chinese Academy of Medical Sciences. Spinosin and phlorizin were purchased from Si Chuan Weikeqi Medical Technology Co., Ltd. (Chengdu, China), purity ⩾98%. Acetic acid, L-lactic acid, butyric acid, salicylic acid, nicotinic acid, succinic acid, citric acid, propionic acid, methanoic acid, α-cyano-4-hydroxycinnamate (CHC) and NaN_3 were obtained from Dengke Chemical Industries, Ltd. (Tianjin, China), purity ⩾98%. Thymidine, inosine, and uracil were purchased from Solarbio Science & Technology, Ltd. (Beijing, China), purity ⩾98%. Dulbecco's Modified Eagle's Medium F-12 (DMEM F-12) and fetal bovine serum (FBS) were purchased from Hyclone Life Technologies (Beijing, China). Acetonitrile of HPLC grade was from Tedia Company, Inc. (Fairfield, OH, USA). Dimethyl sulfoxide (DMSO) was purchased from MP Biomedicals, LLC (Illkirch, France). The highest grade reagents were purchased in this experiment.

2.2. Cell culture

The Caco-2 cells were grown in the 100 ∗ 20 mm culture dishes at 37 °C in a 5% CO_2–95% air atmosphere between passages 35 and 45. The culture medium consisted of DMEM F-12, 10% FBS, 100 μg/mL streptomycin and 70 μg/mL penicillin G. The confluent Caco-2 cells were cultured for 7–9 days for uptake experiments, and the culture medium was fed with fresh incubation medium three times every week.

2.3. Uptake experiments

Caco-2 cells were seeded in 100 ∗ 20 mm culture dishes by 30 × 104 cell/mL for uptake experiments. HBSS balanced salt solution (8 g/L NaCl, 0.4 g/L KCl, 0.14 g/L $CaCl_2$, 0.06 g/L $MgSO_4{\cdot}7H_2O$, 0.06 g/L KH_2PO_4, 0.12 g/L mM $Na_2HPO_4{\cdot}12H_2O$), 1.0 g/L D-glucose, pH 5.0, 6.8, 7.0 or 7.4, and the same concentration of KCl instead of Na^+ was used as Na^+ free HBSS balanced salt solution (pH 7.4). In time-, extracellular pH- and concentration-dependence on SPI uptake experiments, cells were washed twice with 5.0 ml of HBSS balanced salt solution (pH 7.4) then preincubated with 10 ml of fresh HBSS for 20 min at 37 °C to decrease interference. After preincubation, the supernatant was removed, and 10 ml of HBSS containing different concentration of SPI was added to each dish for certain times at 37 °C. SPI and other medications were dissolved in DMSO and the final concentration of DMSO in HBSS was lower than 1% for the uptake experiments.

In effect of low temperature, metabolic inhibitor and Na^+-dependence on SPI uptake experiments, Caco-2 cells were preincubated with 10 mM NaN_3, Na^+ free or 4 °C in the incubation medium (pH 7.4) at 37 °C or 4 °C for 20 min before incubating with SPI 10 min at the same temperature with preincubation. To investigate the effect of various monocarboxylic acids and other transporters inhibitors on SPI uptake, the cells were preincubated with 10 mM CHC, acetic acid, L-lactic acid, butyric acid, salicylic acid, nicotinic acid, succinic acid, citric acid, propionic acid, methanoic acid or 1 mM thymidine, inosine, uracil, 0.5 mM phlorizin (pH 7.4) for 20 min at 37 °C then incubated with SPI 10 min at the same temperature with preincubation.

After treatment with SPI, the supernatant was removed, and the cell surface was washed thirdly with 5 mL ice-cold HBSS. Then 2.5 mL of solution (water/methanol = 1:1) was added to each dish incubated for 60 min to extract SPI at room temperature, then using a cell scraper the cells were scraped off and collected in EP tubes. The suspension was centrifuged at 15,000*g* for 20 min, and a 30-μL aliquot of the supernatant was injected into the High Performance Liquid Chromatography (HPLC) system. The Bradford method was used to assay the concentration of protein in each dish (Bradford, 1976).

2.4. Instrumentation and chromatographic conditions

The extract SPI was determined by an HPLC system with a Waters 2695 pump and Waters 2489 UV detector, using an EMPORE-2000 workstation for data acquisition. And a XBP C18 (L) (5 μm, 4.6 mm × 250 mm) analytical column from Venusil Co. was used. The mobile phase was a binary mixture of acetonitrile–water (20:80, v/v) and at a flow rate