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Role of monocarboxylic acid transporters in the cellular uptake of NSAIDs

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Abstract

The present study investigated the cellular uptake mechanism of non-steroidal anti-inflammatory drugs (NSAIDs) in Caco-2 cells. Diflunisal, diclofenac, ketoprofen and naproxen exhibited a strong inhibition effect on the cellular uptake of [^{14}C]-benzoic acid in Caco-2 cells with IC50 values of 0.05–0.44 mM. The inhibition of naproxen and ketoprofen against the membrane transport of [^{14}C]-benzoic acid appeared to be competitive, with Ki values of 0.22 and 0.38 mM, respectively. The membrane permeability of naproxen and ketoprofen was concentration dependent, implying that the cellular uptake pathway of ketoprofen and naproxen was saturable at the higher concentration. Furthermore, the cellular accumulation of ketoprofen was significantly reduced in the presence of benzoic acid and L-lactic acid, two known substrates of monocarboxylic acid transporter 1 (MCT1). These results suggest that MCT1 contributes at least in part to the carrier-mediated transport of NSAIDs containing a carboxylic acid moiety across the apical membrane in Caco-2 cells.

Introduction

The transport of monocarboxylates such as lactate and pyruvate across the plasma membrane of mammalian cells is facilitated by a family of monocarboxylate/H^+ co-transporters (MCT) (Poole & Halestrap 1993; Tamai et al 1995a; Juel & Halestrap 1999). So far, 14 MCTs have been identified but only MCT1–4 have been expressed in an active form and characterized as proton-linked monocarboxylic acid transporters (Halestrap & Price 1999; Enerson & Drewes 2003; Halestrap & Meredith 2004; Makuc et al 2004). Of these, only the MCT1 isoform plays a major role in the transport of various monocarboxylates across the gastrointestinal epithelia, whereas other MCT isoforms seem to be of little or no importance (Ritzhaupt et al 1998a, b; Halestrap & Price 1999; Orsenigo et al 1999; Enerson & Drewes 2003). Several studies have reported that MCT1 is located in the brush-border membranes of both the upper and lower intestines and has an important role in the intestinal absorption of pharmacologically active compounds such as β-lactam antibiotics (phenethicillin, propicillin, carindacillin, etc.), atorvastatin and pravastatin (Kang et al 1990; Tamai et al 1995b; Li et al 1999; Wu et al 2000).

Many non-steroidal anti-inflammatory drugs (NSAIDs) have a monocarboxylic acid group in their structures. Those NSAIDs are in general rapidly absorbed from the gastrointestinal tract; however, the mechanism of transport across the intestinal epithelia is not clear yet. Some studies have proposed that the membrane transport of NSAIDs should be facilitated by MCTs (Takanaga et al 1994; Tamai et al 1995a; Tsuji & Tamai 1996; Emoto et al 2002), while other authors have suggested the pH-dependent but non-carrier-mediated absorption of NSAIDs (Legen & Kristl 2003; Takagi et al 1998). The transport mechanism of NSAIDs is therefore still uncertain. Considering the clinical significance of drug–drug interactions mediated by drug transporters, it is important to evaluate the contribution of a carrier-mediated mechanism to the membrane transport of drugs. In particular, interactions between NSAIDs and other anionic drugs, including nucleoside antiviral drugs, antibiotics and hippurates, may occur relatively frequently since NSAIDs are widely used as prescription or over-the-counter drugs. The present study aims to clarify the cellular uptake mechanism of NSAIDs, particularly the potential contribution of a carrier-mediated mechanism to the overall absorption of NSAIDs.

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Caco-2 cells express the five isoforms of MCTs (MCT1 and MCT3–6), of which MCT1 is the most abundant isoform in Caco-2 cells (Hadjiagapiou et al 2000). In the present study, a Caco-2 cell monolayer was used as an appropriate in-vitro model to examine the role of MCT1 in the transport of NSAIDs across the intestinal epithelial membrane.

Materials and Methods

Materials

Diclofenac, diflunisal, naproxen, ketoprofen, benzoic acid, [^{14}C]-benzoic acid (13.1 mCi mmol^{-1}), L-lactic acid and BCA protein assay kit were purchased from Sigma Chemical Co. (St Louis, MO, USA). Caco-2 cells were purchased from ATCC (Rockville, MD, USA). 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 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 2–3 weeks post-seeding, the cells were washed twice with pH 6.0 uptake buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 5 mM D-glucose, and 5 mM MES. Each test solution (0.1–1000 μM) containing [^{14}C]-benzoic acid (20 μM, 0.1 μCi mL^{-1}) was added to each well and incubated for 15 min. At the end of incubation, the drug solution was removed and the cells were washed three times with ice-cold phosphate buffer solution (PBS). One millilitre 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.

Uptake studies in Caco-2 cells

The studies were carried out in six-well plates with confluent cells as described for the inhibition studies. Briefly, the initial uptake rates of ketoprofen (0.5 and 2.5 mM) and naproxen (0.5 and 5 mM) were determined in pH 6.0 uptake buffer to examine the concentration dependency in their cellular accumulation. The uptake of ketoprofen (0.5 mM) was also measured in the absence and presence of inhibitors. At the end of 15 min of incubation the cells were washed three times with ice-cold PBS and ruptured directly on the plate by adding 1 mL of Milli-Q water. Cells were harvested and sonicated for 1–2 min. Trichloroacetic acid (3–5%) was added to the cell lysate, vortexed rigorously, and centrifuged for 5 min at 3000 rpm. After filtration of the supernatant through a membrane filter (0.45 μm), samples were analysed by HPLC. The protein amount of each sample was determined with BCA protein assay kit following the manufacturer's instruction (Sigma Chemical Co., St Louis, MO, USA).

HPLC assay

Concentrations of ketoprofen and naproxen were determined by an HPLC assay as follows. Naproxen was used as the internal standard for the assay of ketoprofen and ketoprofen as the internal standard for the naproxen assay. 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, USA) was eluted with a mobile phase consisting of 0.01 M phosphate buffer (pH 7.0)/acetonitrile (75:25, v/v%) at a flow rate of 1.0 mL min^{-1}. Ketoprofen and naproxen were monitored at 258 and 229 nm, respectively. The calibration curve from the standard samples was linear over the concentration range of 0.01 to 10 μg mL^{-1}. The limit of detection was 0.01 μg mL^{-1}.

Data analysis

Estimate of IC50

IC50 is defined as the drug concentration to show the 50% inhibition on the uptake of benzoic acid. As described by De Lean et al (1978), it was determined from the non-linear regression of a dose–response curve using the SigmaPlot 9.0 (Systat Software Inc., Point Richmond, CA, USA).

Estimate of permeability

The permeability coefficient (P_{app}) was calculated from the linear portion of an uptake vs time plot using the follow equation:

$$P_{app} = (dm/dt) \times (1/(A \times C_0))$$

where A is diffusion area (cm^2), C_0 is the initial concentration and dm/dt is the initial uptake rate.

Statistical analysis

All the means are presented with their standard deviation. Statistical analysis was performed using a one-way ANOVA, followed by a posteriori testing with the use of the Dunnett correction. A P value < 0.05 was considered statistically significant.

Results and Discussion

Inhibition studies on the uptake of benzoic acid in Caco-2 cells

Previous studies have reported that the transport of benzoic acid and L-lactic acids were facilitated by MCT1 (Poole & Halestrap 1993; Tamai et al 1995a; Juel & Halestrap 1999). In the present study therefore benzoic

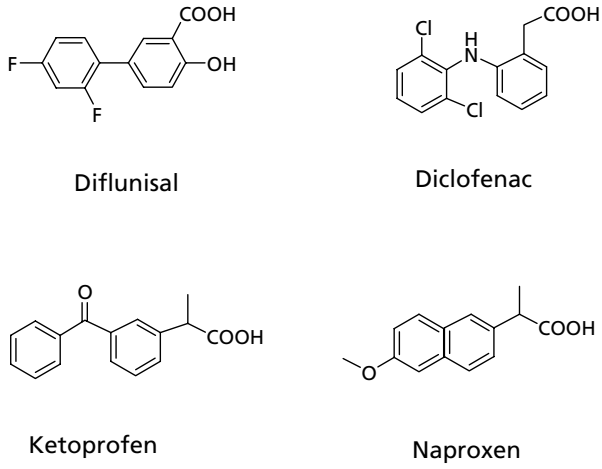

Figure 1 The structures of diflunisal, diclofenac, ketoprofen and naproxen.

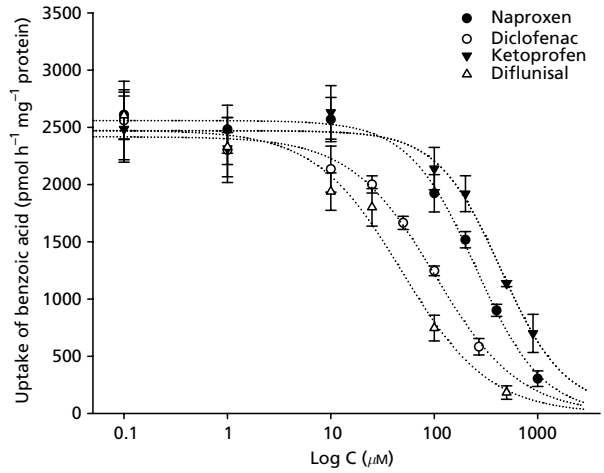

Figure 2 Inhibition effect of NSAIDs on the uptake of [^{14}C]-benzoic acid in Caco-2 cells (mean ± s.d., n = 6).

acid and L-lactic acids were selected as the representative substrates for MCT1 to examine the interaction between NSAIDs and MCT1 in Caco-2 cells. Four structurally diverse NSAIDs, such as diclofenac, diflunisal, ketoprofen and naproxen (Figure 1), were compared in Caco-2 cells with respect to their inhibitory effect on the uptake of benzoic acid. As summarized in Table 1 and Figure 2, all the tested drugs exhibited a strong inhibition effect on the uptake of [^{14}C]-benzoic acid with an IC50 value of 0.05 to 0.44 mM. These results support the previous report by Konishi et al (2002), suggesting that monoanionic carboxyl group and an unpolar side-chain or aromatic hydrophobic portion may be necessary to be recognized by MCTs. Diclofenac and diflunisal appeared to be more potent inhibitors against the uptake of benzoic acid than ketoprofen and naproxen, implying that the inhibitory potency might be influenced by the type of carboxylic acid. An acetic acid side-chain (diclofenac) seemed to be more favourable for the interaction with MCT1 than a propionic acid side-chain (naproxen and ketoprofen), suggesting that a methyl substituent in the immediate vicinity of a carboxylic acid group may decrease the affinity to MCT1. Furthermore, direct attachment of a carboxyl moiety to the hydrophobic aromatic ring (diflunisal) seemed to enhance the binding affinity to the monocarboxylic acid transporters.

Kinetic analysis using Lineweaver–Burk plots was also performed to clarify the inhibition mode of naproxen and ketoprofen on the accumulation of benzoic acid in Caco-2 cells. As illustrated in Figure 3, both naproxen and

Table 1 Inhibition of the uptake of [^{14}C]-benzoic acid in Caco-2 cells (mean ± s.d., n = 6)

Drug	IC50 (mM)
Diclofenac	0.10 ± 0.02
Diflunisal	0.05 ± 0.01
Ketoprofen	0.44 ± 0.09
Naproxen	0.25 ± 0.03

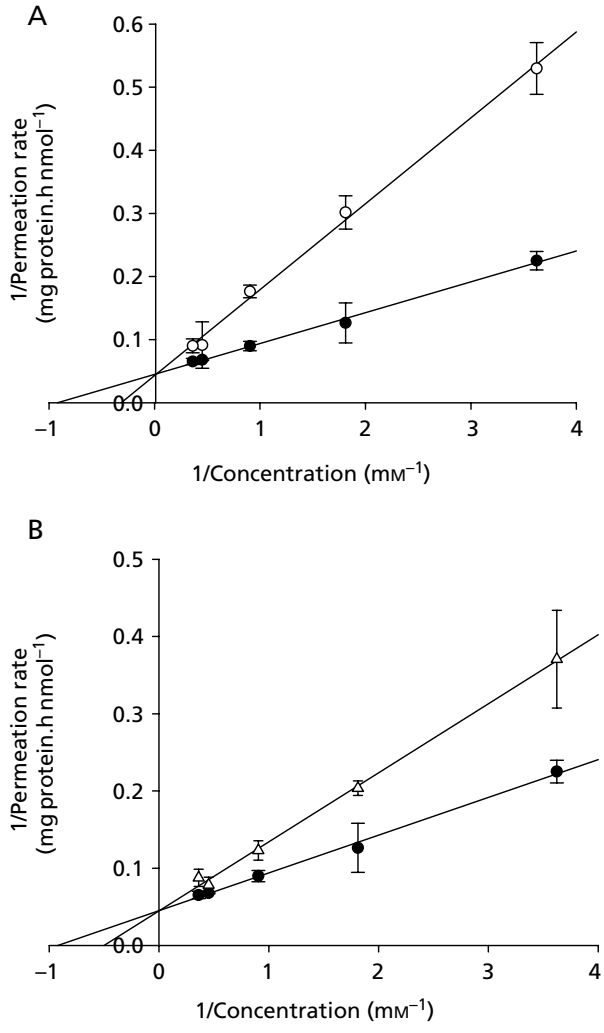

Figure 3 Lineweaver–Burk plots for the transport of benzoic acid across Caco-2 cell monolayers (mean ± s.d., n = 6). The transport was measured in the absence (●) and presence of (A) 0.5 mM naproxen (○) or (B) 0.5 mM ketoprofen (△).

Table 2 Concentration dependency in the transport of ketoprofen and naproxen across the apical membrane of Caco-2 cells (mean ± s.d., n = 6)

	Ketoprofen		Naproxen	
	0.5 mM	2.5 mM	0.5 mM	5 mM
P_{app} ($\times 10^{-6}$, cm s^{-1})	3.9 ± 0.29	1.6 ± 0.54	4.9 ± 0.61	1.5 ± 1.38

ketoprofen inhibited the cellular uptake of benzoic acid in a competitive manner with inhibition constant values (Ki) of 0.22 ± 0.05 and 0.38 ± 0.07 mM, respectively.

Collectively, NSAIDs containing a carboxylic acid moiety, such as diflunisal, diclofenac, naproxen and ketoprofen, were able to interact with MCT1, a transport system of benzoic acid.

Cellular uptake studies in Caco-2 cells

To evaluate the potential contribution of a carrier-mediated transport mechanism to the cellular uptake of monocarboxylic acid type NSAIDs, the concentration dependency in the membrane transport of naproxen and ketoprofen was examined in Caco-2 cells. As summarized in Table 2, the apparent permeability of naproxen and ketoprofen decreased significantly ($P < 0.05$) as the drug concentration increased by 5–10-fold, implying that the cellular uptake pathway of ketoprofen and naproxen was saturable at the high concentration. Furthermore, the transport of ketoprofen across the apical membrane of the Caco-2 cell monolayers was markedly inhibited by the presence of benzoic acid or L-lactic acid, supporting the idea that the cellular uptake of ketoprofen shares a common transport pathway at least partially with benzoic acid and L-lactic acid (Figure 4). This finding is contrary to the previous reports by Legen & Kristl (2003), while others (Takanaga et al 1994; Tamai et al 1995a; Ogihara et al 1996; Emoto et al 2002) are more supportive to our findings. By using the excised rat jejunal segment mounted in side-by-side diffusion cells, Legen & Kristl (2003) reported that ketoprofen transport was not saturable over the concentration range 0.125 to 5 mM and was not inhibited by benzoic acid or L-lactic acid. The explanation of this discrepancy is not clear yet. Maybe, the Km values for MCT1-mediated drug transport are much higher in the excised rat jejunal model than in Caco-2 cells and thus drug concentrations tested in their experiments might not be appropriate to observe the saturation or significant inhibition on the carrier-mediated transport of ketoprofen. Discrepancy in the results obtained from the different in-vitro settings should be further clarified based on the in-vivo relevance of in-vitro findings. The quantitative evaluation of the contribution of different mechanisms to the whole process of the NSAIDs absorption needs to be undertaken in vivo in future studies.

In conclusion, MCT1 contributes at least in part to the transport of NSAIDs containing a carboxylic acid moiety across the apical membrane in Caco-2 cells.

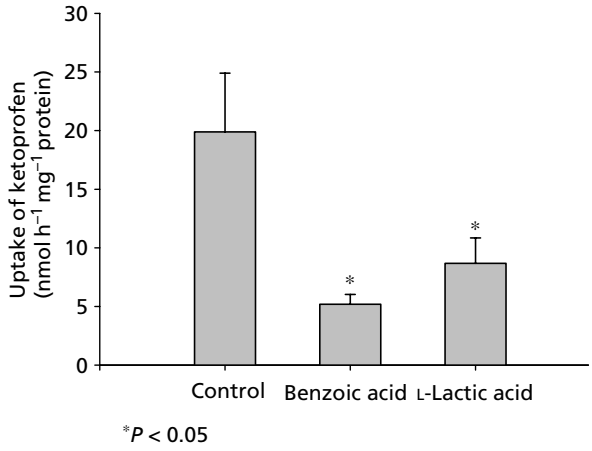

Figure 4 Cellular uptake of ketoprofen (0.5 mM) in the absence and presence of benzoic acid (2 mM) or L-lactic acid (2 mM) (mean ± s.d., n = 6).

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