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Characterization of Butyrate Uptake by Nontransformed Intestinal Epithelial Cell Lines

Pedro Gonçalves · João R. Araújo · Fátima Martel

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Abstract Butyrate (BT) is one of the main end products of anaerobic bacterial fermentation of dietary fiber within the human colon. Among its recognized effects, BT inhibits colon carcinogenesis. Our aim was to characterize uptake of BT by two nontransformed intestinal epithelial cell lines: rat small intestinal epithelial (IEC-6) and fetal human colonic epithelial (FHC) cells. Uptake of ^{14}C-BT by IEC-6 cells was (1) time- and concentration-dependent; (2) pH-dependent; (3) Na^{+}-, Cl^{-}- and energy-dependent; (4) inhibited by BT structural analogues; (5) sensitive to monocarboxylate transporter 1 (MCT1) inhibitors; and (6) insensitive to DIDS and amiloride. IEC-6 cells express both MCT1 and Na^{+}-coupled monocarboxylate transporter 1 (SMCT1) mRNA. We conclude that ^{14}C-BT uptake by IEC-6 cells mainly involves MCT1, with a small contribution of SMCT1. Acute exposure to ethanol, acetaldehyde, indomethacin, resveratrol and quercetin reduced ^{14}C-BT uptake. Chronic exposure to resveratrol and quercetin reduced ^{14}C-BT uptake but had no effect on either MCT1 or SMCT1 mRNA levels. Uptake of ^{14}C-BT by FHC cells was time- and concentration-dependent but pH-, Na^{+}-, Cl^{-}- and energy-independent and insensitive to BT structural analogues and MCT1 inhibitors. Although MCT1 (but not SMCT1) mRNA expression was found in FHC cells, the characteristics of ^{14}C-BT uptake by FHC cells did not support either MCT1 or SMCT1 involvement. In conclusion, uptake characteristics of ^{14}C-BT differ between IEC-6 and FHC cells. IEC-6 cells demonstrate MCT1- and SMCT1-mediated transport, while FHC cells do not.

Keywords Butyrate uptake · Nontransformed intestinal epithelial cell · Monocarboxylate transporter type 1 · Xenobiotics

Introduction

The short-chain fatty acid butyrate (BT) is one of the main end products of anaerobic bacterial fermentation of dietary fiber within the human colon (Wong et al. 2006; Hamer et al. 2008). BT plays a key role in colonic epithelial homeostasis by having multiple regulatory roles at that level, including (1) being the main energy source for colonocytes, (2) inhibiting colon carcinogenesis (by suppressing growth of cancer cells, inducing differentiation and apoptosis and inhibiting cell proliferation), (3) promoting growth and proliferation of normal colonic epithelial cells, (4) stimulating fluid and electrolyte absorption, (5) inhibiting colon inflammation and oxidative stress and (6) improving the colonic defence barrier function (Wong et al. 2006; Hamer et al. 2008).

BT is transported into colonic epithelial cells by two specific carrier-mediated transport systems, an electroneutral H^{+}-coupled monocarboxylate cotransporter (MCT1, SLC16A1) (Halestrap and Meredith 2004; Morris and Felmlee 2008) and an Na^{+}-coupled monocarboxylate cotransporter (SMCT1, SLC5A8) (Gupta et al. 2006). As mentioned, one of the proposed beneficial effects of BT on human intestinal health is the prevention/inhibition of colon carcinogenesis (Park et al. 2005; Martínez et al. 2008). In agreement with this fact, both MCT1 (Cuff et al. 2005) and SMCT1 (Gupta et al. 2006) were recently proposed to function as tumor suppressors, the ability of these transporters to mediate the entry of BT into colonic epithelial

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cells underlying their potential tumor-suppressor effect. Interestingly enough, the characteristics of BT colonic epithelial transport have been almost exclusively studied by using colon adenocarcinoma cell lines (Caco-2 or HT-29) (Hadjiagapiou et al. 2000; Stein et al. 2000; Lecona et al. 2008; Gonçalves et al. 2009). However, knowledge on the characteristics of BT uptake in noncarcinogenic cell lines seems important in the context of the distinct effect of BT in carcinogenic and noncarcinogenic cells. Indeed, although BT presents, in many tumor cells, an anticarcinogenic effect which involves induction of differentiation and apoptosis and inhibition of proliferation, its effect on noncarcinogenic cells is contrary, this phenomenon being referred as the "BT paradox" (Hamer et al. 2008).

Additionally, because BT plays an essential role in the maintenance of colonic tissue homeostasis, an understanding of the regulation of its absorption by the colon mucosa appears particularly important (Cuff and Shirazi-Beechey 2004). In this context, MCT1-mediated intestinal epithelial absorption of BT is known to be upregulated by its substrate, BT (Cuff et al. 2002); enhanced by leptin (Buyse et al. 2002), phorbol 12-myristate 13-acetate (Alrefai et al. 2004), protein kinase C (Saksena et al. 2009a), somatostatin (Saksena et al. 2009b), and caffeine and acetylsalicylic acid (Gonçalves et al. 2009); and inhibited by enteropathogenic *Escherichia coli* (Borthakur et al. 2006), interferon-γ and tumor necrosis factor-α (Thibault et al. 2007), theophylline, tetrahydrocannabinol, MDMA (ecstasy), acetaldehyde and indomethacin (Gonçalves et al. 2009), as well as by some polyphenolic compounds (Konishi et al. 2003; Vaidyanathan and Walle 2003; Shim et al. 2007; Gonçalves et al. in press). Information on the regulation of SMCT1 is even more limited. SMCT1 is known to be inhibited by some nonsteroidal anti-inflammatory drugs (NSAIDs) (Itagaki et al. 2006), by the absence of gut commensal bacteria (Cresci et al. 2010) and by tumor necrosis factor-α (Borthakur et al. 2010) and stimulated by some other NSAIDs (Ananth et al. 2010), by activin A (Zhang et al. 2010) and by the probiotic *Lactobacillus plantarum* (Borthakur et al. 2010). However, the above-mentioned studies concerning regulation of BT intestinal uptake were also done using colon adenocarcinoma cell lines only. Because BT exerts distinct effects in carcinogenic and noncarcinogenic cells (see above) and SMCT1 expression has been reported to be silenced in colorectal carcinoma and colon cancer cell lines (Ganapathy et al. 2008), it seemed interesting to compare also the effect of some of these compounds upon BT uptake in nontransformed intestinal epithelial cell lines.

Thus, the aim of this study was to characterize the uptake of BT by nontransformed intestinal epithelial cell lines and to test the effect of a series of drugs upon it. For this, we characterized ^{14}C-BT uptake by a rat small intestinal epithelial cell line (IEC-6) and by a fetal human colonic epithelial cell line (FHC) and tested the acute and chronic effect of drugs upon it. IEC-6 and FHC cells were chosen based on their nontransformed intestinal epithelial origin. Knowledge of the characteristics of BT uptake by these cell lines was very scarce, and nothing was known concerning MCT1 and SMCT1 expression (Lecona et al. 2008; Borthakur et al. 2010).

Materials and Methods

IEC-6 Cell Culture

The IEC-6 cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (ACC-111; Braunschweig, Germany) and used between passages 19 and 34. The cells were maintained in a humidified atmosphere of 5% CO_2–95% air and cultured in Dulbecco's modified Eagle medium:RPMI 1640 medium (1:1), supplemented with 10% fetal bovine serum, 0.1 U/ml insulin, 5.96 g HEPES, 2.2 g $NaHCO_3$, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all from Sigma, St. Louis, MO). Culture medium was changed every 2–3 days, and the culture was split every 7 days. For subculturing, cells were removed enzymatically (0.05% trypsin-EDTA, 5 min, 37°C), split 1:3 and subcultured in plastic culture dishes (21 cm^2; ⌀ 60 mm; Corning Costar, Corning, NY). For uptake studies, IEC-6 cells were seeded on 24-well plastic cell culture clusters (2 cm^2; ⌀ 16 mm, Corning Costar), and the experiments were performed 9 days after the initial seeding (90–100% confluence). For 24 h before the experiments, the cell medium was made free of fetal calf serum and insulin.

FHC Cell Culture

The FHC cell line was obtained from the American Type Culture Collection (37-HTB; ATCC, Rockville, MD) and used between passages 21 and 26. The cells were maintained in a humidified atmosphere of 5% CO_2–95% air and cultured in DMEM:F12 Ham's nutrient mixture (1:1), supplemented with 10% fetal bovine serum, 10 mM HEPES, 1.2 g $NaHCO_3$, 10 ng/ml cholera toxin, 5 µg/ml insulin, 5 ng/ml transferrin, 100 ng/ml hydrocortisone, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all from Sigma). Culture medium was changed every 2–3 days, and the culture was split every 15–16 days. For subculturing, cells were removed enzymatically (0.25% trypsin–EDTA, 5 min, 37°C), split 1:2 and subcultured in plastic culture dishes (21 cm^2; ⌀ 60 mm, Corning Costar). For uptake studies, FHC cells were seeded on 24-well plastic cell culture clusters (2 cm^2; ⌀ 16 mm, Corning Costar), and

the experiments were performed 15–20 days after the initial seeding (90–100% confluence). For 24 h before the experiments, the cell medium was made free of fetal calf serum.

Determination of ^{14}C-BT Uptake by IEC-6 and FHC Cells

Uptake experiments were performed with cells incubated in glucose-free Krebs (GFK) buffer containing (in mM) 125 NaCl, 25 $NaHCO_3$, 4.8 KCl, 0.4 K_2HPO_4, 1.6 KH_2PO_4, 1.2 $MgSO_4$, 1.2 $CaCl_2$ and 20 HEPES (pH 7.0, 7.5 or 8.0) or 20 MES (pH 5.5 and 6.5). In most of the experiments, a buffer with pH 6.5 was used. Initially, the culture medium was aspirated and the cells were washed with 0.3 ml buffer at 37°C. Then, cell monolayers were preincubated for 20 min in 0.3 ml buffer at 37°C. Uptake was initiated by the addition of 0.3 ml medium at 37°C containing ^{14}C-BT (10 or 20 µM, except in kinetic experiments). Incubation was stopped after 3 min (except in time-course experiments) by removing the incubation medium, placing the cells on ice and rinsing the cells with 0.5 ml ice-cold buffer. Cells were then solubilized with 0.3 ml 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) and placed at 37°C overnight. Radioactivity in the cells was measured by liquid scintillation counting.

For characterization of ^{14}C-BT uptake by the cells, the effect of several drugs, medium pH or ionic composition was tested by preincubating and incubating cells with ^{14}C-BT in the presence of the compounds or conditions to be tested.

Acute and Chronic Effect of Drugs on ^{14}C-BT Uptake by IEC-6 Cells

The concentrations of compounds to test were chosen on the basis of previous works from our group (Araújo et al. 2008; Gonçalves et al. 2009, in press).

Acute Effect of Compounds

The acute effect of compounds on ^{14}C-BT uptake was tested by preincubating (20 min) and incubating (3 min) cells with ^{14}C-BT in the presence of the compounds to be tested.

Chronic Effect of Compounds

The chronic effect of compounds on ^{14}C-BT uptake was tested by cultivating cell cultures at 6–8 days of age (90–95% confluence) in culture medium in the presence of the compounds to be tested. The medium was renewed daily, and the transport experiments were performed after 48 h. Transport experiments were identical to the experiments described above, except that there was no preincubation period and cells were incubated with ^{14}C-BT in the absence of drugs.

Real-Time Quantitative Reverse-Transcription PCR

Total RNA was extracted from control FHC and IEC-6 cells and from chronically treated IEC-6 cells using the Tripure® isolation reagent, according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Before cDNA synthesis, total RNA was treated with DNase I (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and 10 µg of resulting DNA-free RNA was reverse-transcribed using Superscript Reverse Transcriptase II and random hexamer primers (Invitrogen) in 40 µl of final reaction volume, according to the manufacturer's instructions. Resulting cDNA was treated with RNase H (Invitrogen) to degrade unreacted RNA. For quantitative real-time PCR, 2 µl of the 40-µl reverse transcription reaction mixture was used. For the calibration curve, FHC and IEC-6 standard cDNA was diluted in five different concentrations.

Real-time PCR was carried out using a LightCycler (Roche, Nutley, NJ). We set up 20-µl reactions in microcapillary tubes using 0.5 µM of each primer and 4 µl of SYBR Green master mix (LightCycler FastStart DNA MasterPlus SYBR Green I, Roche). Cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantification (95°C for 10 s, annealing temperature [AT] for 15 s and 72°C for 10 s, with a single fluorescence measurement at the end of the 72°C for 10 s segment) repeated 50 times, a melting curve program ([AT + 10]°C for 15 s and 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement) and a cooling step to 40°C (30 s). ATs and primer sequences are indicated in Table 1. Data were analyzed using LightCycler® 4.05 analysis software (Roche, Mannheim, Germany).

Protein Determination

The protein content of cell monolayers was determined as described by Bradford (1976), using human serum albumin as standard.

Calculation and Statistics

For analysis of the time course of ^{14}C-BT uptake, the parameters of the equation $A(t) = k_{in}/k_{out}\ \left(1 - e^{-k_{out} \cdot t}\right)$ were fitted to the experimental data by a nonlinear regression analysis, using a computer-assisted method (Muzyka et al. 2005). A(t) represents the accumulation of

Table 1 Primer sequences and annealing temperatures (ATs) used for real-time qRT-PCR

Gene name	Primer sequence (5′–3′)	AT (°C)
hGAPDH	Fwd ATG GAG AAG GCT GGG GCT CAT	65
	Rev GAC GAA CAT GGG GGC ATC AG	
hMCT1	Fwd CAC CGT ACA GCA ACT ATA CG	60
	Rev CAA TGG TCG CCT CTT GTA GA	
hSMCT1	Fwd CTC CCG GTG TTC TAC AAA CTG	65
	Rev GGG CAG GGG CAT AAA TAA C	
rGAPDH	Fwd GGC ATC GTG GAA GGG CTC ATG AC	72
	Rev ATG CCA GTG AGC TTC CCG TTC AGC	
rMCT1[a]	Fwd CAG TGC AAC GAC CAG TGA ATG TG	69
	Rev ATC AAG CCA CAG CCA GAC AGG	
rSMCT1	Fwd CGG GAT CAC CAG CAC CTA C	66
	Rev GCA GGG GCA TAA ATC ACA ATC	

hGAPDH human glyceraldehyde-3-phosphate dehydrogenase, *hMCT1* human monocarboxylate transporter type 1, *hSMCT1* human Na^+-coupled monocarboxylate transporter type 1, *rGAPDH* rat glyceraldehyde-3-phosphate dehydrogenase, *rMCT1* rat monocarboxylate transporter type 1, *rSMCT1* rat Na^+-coupled monocarboxylate transporter type 1, Fwd forward, Rev reverse

[a] Primer sequence obtained from Thibault et al. (2007)

^{14}C-BT at time t; k_{in} and k_{out} are the rate constants for inward and outward transport, respectively; and t is the incubation time. A_{max} corresponds to the accumulation (A[t]) at steady state ($t \rightarrow \infty$). k_{in} is given in picomoles per milligram of protein per minute (pmol/[mg protein·min]) and k_{out} in minutes (min^{-1}). In order to obtain clearance values, k_{in} was converted to microliters per milligram of protein per minute (μl/[mg protein·min]). For analysis of the saturation curve of ^{14}C-BT uptake, the parameters of the Michaelis-Menten equation were fitted to the experimental data by a nonlinear regression analysis, using a computer-assisted method (Muzyka et al. 2005).

Arithmetic means are given with SEM, and geometric means are given with 95% confidence limits. The statistical significance of the difference between two groups was evaluated by Student's *t*-test; statistical analysis of the difference between various groups was evaluated by ANOVA, followed by the Bonferroni test. Differences were considered to be significant when $P < 0.05$.

Materials

^{14}C-BT (*n*-butyric acid, sodium salt, [1-^{14}C]; specific activity 30–60 mCi/mmol) was from Biotrend Chemikalien (Köln, Germany); acetylsalicylic acid, acetic acid sodium salt, alpha-cyano-4-hydroxycinnamic acid (4-CHC), amiloride hydrochloride, choline chloride, chrysin, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt (DIDS), dinitrophenol, ethanol, (–)epigallocatechin-3-gallate (EGCG), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 4-(hydroxymercuri)benzoic acid sodium salt (pCMB), indomethacin, luteolin, ketobutyric acid sodium salt hydrate, L-lactic acid sodium salt, lithium chloride, 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES), myricetin, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), propionic acid sodium salt, pyruvic acid sodium salt, sodium fluoride, quercetin dihydrate, resveratrol, rutin and trypsin-EDTA solution were all from Sigma; dimethylsulfoxide (DMSO) and Triton X-100 were from Merck (Darmstadt, Germany); fetal calf serum was from Invitrogen; acetaldehyde was from May & Baker (Dagenham, UK); and caffeine was from BDH Laboratory Chemicals (Poole, UK).

Drugs to be tested were dissolved in water, ethanol, DMSO or methanol, the final concentration of these solvents being 1% in the buffer or 0.1% in the culture media for acute or chronic treatments, respectively. Controls for these drugs were run in the presence of the solvent.

Results

Time and pH Dependence of ^{14}C-BT Uptake by IEC-6 Cells

In the first series of experiments, we determined the time course of ^{14}C-BT uptake by IEC-6 cells. For this, cells were incubated with ^{14}C-BT (10 μM) for various periods of time. As shown in Fig. 1a, IEC-6 cells accumulated ^{14}C-BT in a time-dependent way and uptake was linear with time for up to 3 min of incubation. Thus, in subsequent experiments, cells were exposed to ^{14}C-BT (10 μM) for 3 min in order to measure initial rates of uptake. Next, the pH dependence of ^{14}C-BT uptake was evaluated. Uptake of

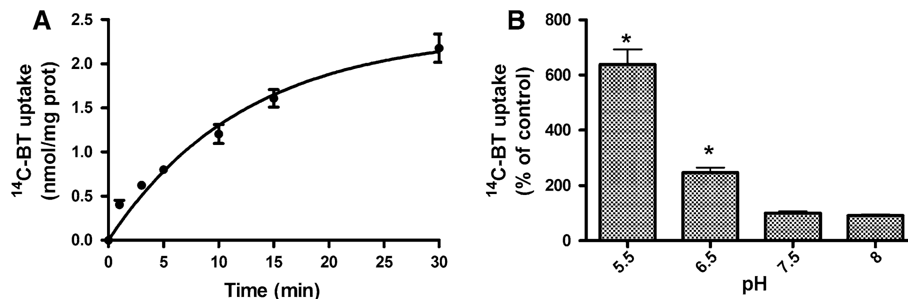

Fig. 1 Characteristics of ^{14}C-BT uptake by IEC-6 cells incubated at 37°C with 10 μM ^{14}C-BT. **a** Time course of ^{14}C-BT uptake by IEC-6 cells incubated in GFK buffer (pH 6.5, $n = 5$–6). **b** pH dependence of ^{14}C-BT uptake (3 min). IEC-6 cells were preincubated and incubated in GFK buffer with pH ranging 5.5–8.0 ($n = 6$). Shown are arithmetic means ± SEM. *Significantly different from uptake at physiological pH (7.5)

^{14}C-BT (10 μM) was found to be highly pH-dependent, increasing as the pH decreased from 7.5 to 5.5 (Fig. 1b). Thus, in subsequent experiments, a pH of 6.5 was used.

Kinetics of ^{14}C-BT Uptake by IEC-6 Cells

The relationship between the initial rates of uptake of ^{14}C-BT and its concentration in the medium is represented in Fig. 2a. Rates were analyzed according to the Michaelis-Menten equation (see Materials and Methods). The evaluated kinetic parameters V_{max} and K_m were 69.5 ± 16.7 nmol/(mg prot · 3 min) and 4.0 ± 1.3 mM, respectively.

Pharmacological Characterization of ^{14}C-BT Uptake by IEC-6 Cells

To test for the dependence of ^{14}C-BT uptake on extracellular Na^+ and Cl^-, we measured uptake in the absence of NaCl, which was substituted by either LiCl, choline chloride or NaF. As shown in Fig. 2b, substitution of Cl^- with F^- caused a dramatic decrease in the uptake of ^{14}C-BT (≈60%). On the other hand, substitution of Na^+ with Li^+ caused a small decrease in the uptake of ^{14}C-BT (≈17%); but when Na^+ was substituted with choline, no change in the uptake of ^{14}C-BT was observed (Fig. 2b).

The effect of BT structural analogues (acetate and propionate, which, together with butyrate, constitute the major SCFA present in the lumen of the colon, and other monocarboxylates [L-lactate, pyruvate and α-ketobutyrate]) on the initial rates of ^{14}C-BT uptake was next determined. As shown in Fig. 2c, ^{14}C-BT uptake was significantly reduced by all of the monocarboxylates tested, with propionate showing the greatest inhibition (77%).

Moreover, we also tested the effect of a series of inhibitors. We verified that pCMB and NPPB, typical MCT1 inhibitors, caused a dramatic decrease in the uptake of ^{14}C-BT (≈80%). Moreover, the MCT1 inhibitor luteolin was also able to reduce ^{14}C-BT uptake. On the other hand, CHC caused a small (20%) but significant increase in the uptake of ^{14}C-BT. The classical anion exchange inhibitor DIDS and the typical inhibitor of apical Na^+/H^+ exchanger amiloride had no effect on ^{14}C-BT uptake. Finally, ^{14}C-BT uptake by IEC-6 cells was found to be highly energy-dependent as it was greatly reduced in the presence of dinitrophenol (Fig. 2d).

Modulation of ^{14}C-BT Uptake by IEC-6 Cells by Several Drugs

In this series of experiments, the acute and chronic effects of several therapeutic agents, abuse substances and polyphenolic compounds upon the uptake of ^{14}C-BT by IEC-6 cells were investigated. The compounds tested were previously found to affect uptake of ^{14}C-BT in human colon adenocarcinoma Caco-2 cells (Gonçalves et al. 2009, in press). The aim is to compare their effect on IEC-6 nontransformed cells with their effect on Caco-2 tumor cells.

Effect of Therapeutic and Abuse Compounds upon the Uptake of ^{14}C-BT by IEC-6 Cells

Acute Effect

As shown in Fig. 3a, caffeine (10 and 100 μM) and acetylsalicylic acid (1 and 5 mM) were devoid of effect upon ^{14}C-BT uptake. On the other hand, indomethacin (0.1 and 0.3 mM) concentration-dependently reduced ^{14}C-BT uptake, to a maximum of 44% of control. Moreover, ethanol and its metabolite acetaldehyde (10 and 100 mM) also reduced ^{14}C-BT uptake in a concentration-dependent manner, to a maximum of 74 and 23% of control, respectively.

Chronic Effect

These same compounds were tested over a 48-h period. Interestingly enough, none of these compounds (ethanol,

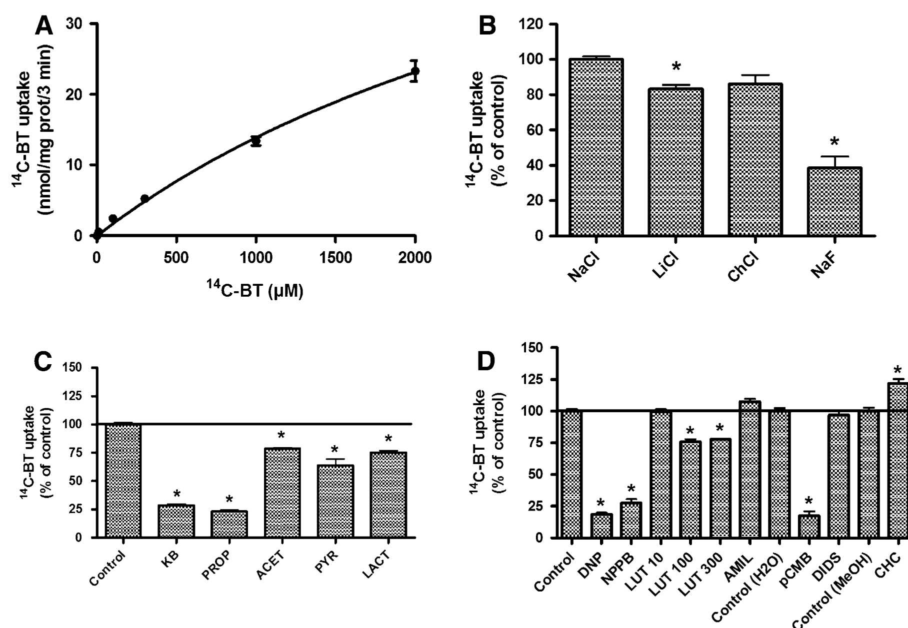

Fig. 2 Characteristics of ^{14}C-BT uptake by IEC-6 cells incubated at 37°C with 10 μM ^{14}C-BT (except in the kinetic experiments) for 3 min in GFK buffer (pH 6.5). **a** Kinetics ($n = 7$). **b** Ionic dependence. NaCl in the preincubation and incubation GFK buffer (control) was isotonically replaced by either LiCl, choline chloride (ChCl) or NaF ($n = 9$–13). **c** Effect of BT structural analogues. Cells were incubated in the absence (control) or presence of 10 mM α-ketobutyrate (*KB*, $n = 6$), propionate (*PROP*, $n = 6$), acetate (*ACET*, $n = 6$), pyruvate (*PYR*, $n = 7$) and lactate (*LACT*, $n = 7$). **d** Effect of inhibitors. Cells were incubated in the absence (control) or presence of dinitrophenol 0.5 mM (*DNP*, $n = 7$); NPPB 0.5 mM ($n = 6$); luteolin 10, 100 or 300 μM (*LUT*, $n = 4$–8); amiloride 0.5 mM (*AMIL*, $n = 4$); pCMB 0.5 mM ($n = 6$); DIDS 0.5 mM ($n = 11$); or CHC 1 mM ($n = 8$). Shown are arithmetic means ± SEM. *Significantly different from control

acetaldehyde, caffeine, acetylsalicylic acid and indomethacin) caused a significant change in the uptake of ^{14}C-BT by IEC-6 cells (Fig. 3b).

Effect of Polyphenols upon the Uptake of ^{14}C-BT by IEC-6 Cells

Acute Effect

The acute effect of several different polyphenolic compounds was next investigated (Fig. 4a). Of these, resveratrol and quercetin (10–100 μM) were found to concentration-dependently reduce ^{14}C-BT uptake, to a maximum of 47 and 76% of control, respectively. In contrast, chrysin and myricetin did not affect uptake of ^{14}C-BT by IEC-6 cells.

Chronic Effect

As shown in Fig. 4b, EGCG, rutin and chrysin were devoid of effect on ^{14}C-BT uptake. However, quercetin (10 μM) and resveratrol (1 and 10 μM) reduced uptake of ^{14}C-BT by IEC-6 cells (to a maximum of 83 and 85% of control, respectively).

Characteristics of ^{14}C-BT Uptake by FHC Cells

The characteristics of ^{14}C-BT uptake were also investigated in human fetal colonic FHC cells. Uptake of ^{14}C-BT by FHC cells was time-dependent (Fig. 5a), and initial rates of uptake (which were measured by incubating cells with ^{14}C-BT for 3 min) displayed saturable kinetics, having a K_m of 1.22 ± 0.45 mM and a V_{max} of 108.9 ± 16.8 nmol/mg prot/3 min (Fig. 5b). Additionally, the initial rate of ^{14}C-BT uptake was found to be pH-independent, Na^+- and Cl^--independent, energy-independent and insensitive to both BT structural analogues (propionate, lactate and α-ketobutyrate) and MCT1 inhibitors (pCMB and NPPB) (Fig. 5c–e).

Real-Time qRT-PCR of MCT1 and SMCT1 mRNA in IEC-6 and FHC Cells

MCT1 and SMCT1 mRNA expression was investigated in both IEC-6 and FHC cells. IEC-6 cells express low levels of both transporters (Fig. 6a) and FHC cells express MCT1, but not SMCT1, mRNA (Fig. 6b).

Quantification of MCT1 and SMCT1 mRNA expression was also investigated in IEC-6 cells chronically treated with resveratrol (10 μM) or quercetin (10 μM). Treatment

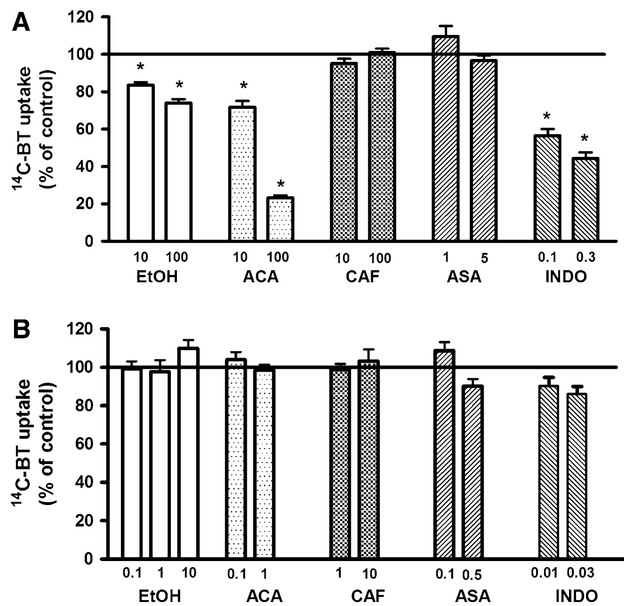

Fig. 3 Effect of several drugs upon the uptake of ^{14}C-BT by IEC-6 cells incubated at 37°C with 10 μM ^{14}C-BT for 3 min in GFK buffer (pH 6.5). **a** Acute effect. Cells were preincubated and incubated with ^{14}C-BT in the absence (control) or presence of ethanol (*EtOH*, 10–100 mM, $n = 9$), acetaldehyde (*ACA*, 10–100 mM, $n = 9$), caffeine (*CAF*, 10–100 μM, $n = 9$), acetylsalicylic acid (*ASA*, 1–5 mM, $n = 9$–12) or indomethacin (*IND*, 0.1–0.3 mM, $n = 9$). **b** Chronic effect. Cells were incubated with ^{14}C-BT after being cultivated for 48 h in the absence (control) or presence of EtOH (0.1–10 mM, $n = 10$–11), ACA (0.1–1 mM, $n = 11$–14), CAF (1–10 μM, $n = 9$), ASA (0.1–0.5 mM, $n = 15$) or IND (0.01–0.03 mM, $n = 15$). Shown are arithmetic means ± SEM. *Significantly different from control

with these compounds caused no change in expression levels of either MCT1 or SMCT1 (results not shown).

Discussion

The aim of this work was to characterize the uptake of BT in normal intestinal epithelial cells and to investigate its modulation by compounds previously found to affect BT uptake in human colon adenocarcinoma cells (Caco-2 cells).

Our first experiments were performed with the human fetal human colonic cell line FHC, which maintains the characteristics of normal human colonic cells (Siddiqui and Chopra 1984). This cell line was found to express MCT1 mRNA but not SMCT1 mRNA. However, characteristics of ^{14}C-BT uptake by FHC cells (namely, pH, Cl^- and energy independence and insensitivity to both BT structural analogues and MCT1 inhibitors) were quite distinct from those previously described in other intestinal epithelial cell lines, which are compatible with MCT1-mediated uptake (Hadjiagapiou et al. 2000; Stein et al. 2000; Lecona et al. 2008; Gonçalves et al. 2009). Thus, it is possible that although FHC cells express MCT1 mRNA, they do not express a functional protein (e.g., by lack of the chaperone CD147, which was recently found to be necessary for proper membrane expression and activity of MCT1 (Kirk et al. 2000; Su et al. 2009)) and that BT uptake in FHC cells involves a mechanism distinct from MCT1 and SMCT1 (e.g., a BT/HCO_3^- exchanger (Harig et al. 1996; Ritzhaupt et al. 1998; Schröder et al. 2000) or an organic anion transporter [OAT] (Anzai et al. 2006)). For this reason, FHC cells did not seem to be a good cell model to investigate BT uptake, and we decided to investigate ^{14}C-BT uptake in another nontransformed intestinal cell line.

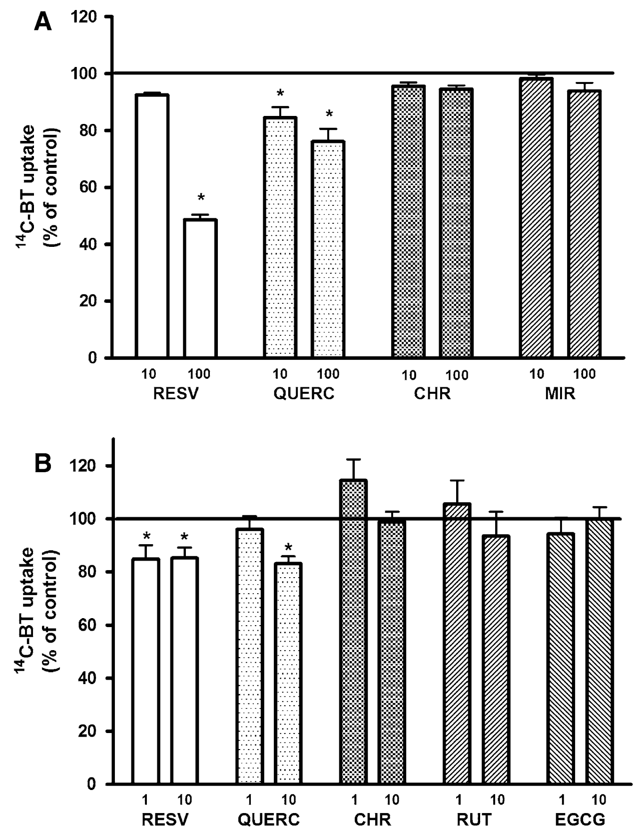

Fig. 4 Effect of several polyphenols upon uptake of ^{14}C-BT by IEC-6 cells incubated at 37°C with 10 μM ^{14}C-BT for 3 min in GFK buffer (pH 6.5). **a** Acute effect. Cells were preincubated and incubated with ^{14}C-BT in the absence (control) or presence of resveratrol (*RESV*, 10–100 μM, $n = 6$), quercetin (*QUERC*, 10–100 μM, $n = 6$), myricetin (*MYR*, 10–100 μM, $n = 9$) or chrysin (*CHR*, 10–100 μM, $n = 9$). **b** Chronic effect. Cells were incubated with ^{14}C-BT after being cultivated for 48 h in the absence (control) or presence of RESV (1–10 μM, $n = 9$), QUERC (1–10 μM, $n = 9$), rutin (*RUT*, 1–10 μM, $n = 8$), CHR (1–10 μM, $n = 8$–9) or EGCG (1–10 μM, $n = 8$–9). Shown are arithmetic means ± SEM. *Significantly different from control

The rat normal intestinal epithelial cell line IEC-6 was established from crypts of rat small intestinal cells by Quaroni et al. (1979). Although they were originally

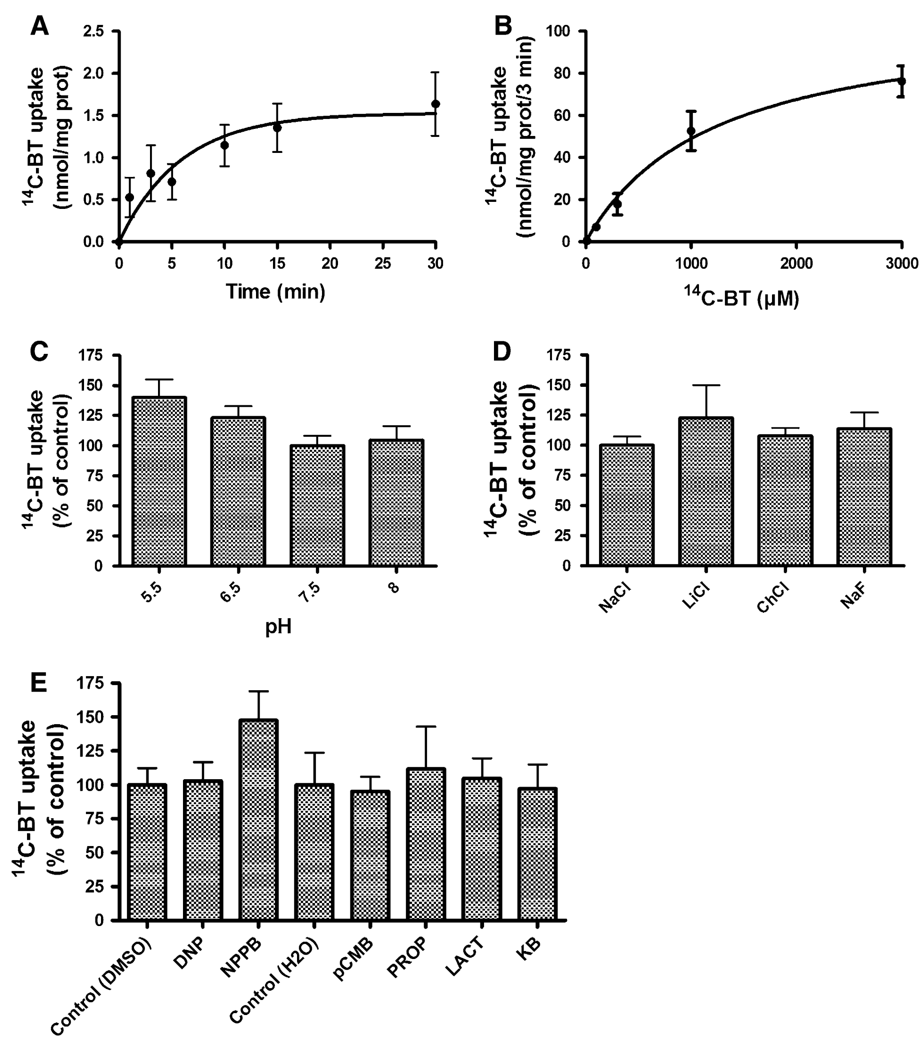

Fig. 5 Characteristics of ^{14}C-BT uptake by FHC cells incubated at 37°C in GFK buffer. **a** Time course of ^{14}C-BT uptake by cells incubated with 20 μM ^{14}C-BT (pH 6.5, $n = 5$–6). **b** Kinetics of ^{14}C-BT uptake by cells incubated for 3 min with increasing concentrations of ^{14}C-BT (pH 6.5) ($n = 4$). **c** pH dependence of ^{14}C-BT uptake by cells incubated with 20 μM ^{14}C-BT for 3 min. Extracellular pH in the preincubation and incubation GFK buffer ranged 5.5–8.0 ($n = 8$–9). **d** Ionic dependence of ^{14}C-BT uptake by cells incubated with 20 μM ^{14}C-BT for 3 min. NaCl in the preincubation and incubation GFK buffer (control. pH 6.5) was isotonically replaced by either LiCl, choline chloride (ChCl) or NaF ($n = 6$). **e** Effect of drugs on ^{14}C-BT uptake. Cells were incubated with 20 μM ^{14}C-BT for 3 min in the absence (control) or presence of dinitrophenol 10 mM (*DNP*, $n = 6$), 5-nitro-2-(3-phenylpropylamino)benzoate 0.5 mM (*NPPB*, $n = 5$), *p*-chloromercuribenzoate 0.5 mM (*pCMB*, $n = 6$), propionate 10 mM (*PROP*, $n = 5$), lactate 10 mM (*LACT*, $n = 6$) or α-ketobutyrate 10 mM (*KB*, $n = 6$). Shown are arithmetic means ± SEM

described as sharing many undifferentiated characteristics of immature intestinal cells, when IEC-6 cells are grown in postconfluent culture, they develop structural changes and differentiation from a crypt cell-like to an enterocyte-like phenotype (Wood et al. 2003). IEC-6 cells have been used in numerous studies, including studies on the intestinal absorption of nutrients (e.g., Inui et al. 1980; Jakobs and Paterson 1986; Said et al. 1997; Fujita et al. 2000; Murota et al. 2001; Fraga et al. 2002).

Uptake of ^{14}C-BT by IEC-6 cells was found to be (1) time- and concentration-dependent; (2) pH-dependent, with uptake increasing with decreasing pH; (3) Na^+- and Cl^--dependent; (4) energy-dependent; (5) inhibited by several BT structural analogues (propionate, lactate, acetate, pyruvate and α-ketobutyrate); (6) inhibited by the MCT inhibitors pCMB, NPPB and luteolin and enhanced by CHC; and (7) insensitive to a classic anion exchange inhibitor (DIDS) and to a typical inhibitor of apical Na^+/H^+ exchanger (amiloride).

As mentioned before, the characteristics of BT colonic epithelial transport have been almost exclusively studied by using colon adenocarcinoma cell lines (Caco-2 or HT-29 cells) (Hadjiagapiou et al. 2000; Stein et al. 2000; Lecona et al. 2008; Gonçalves et al. 2009). Interestingly

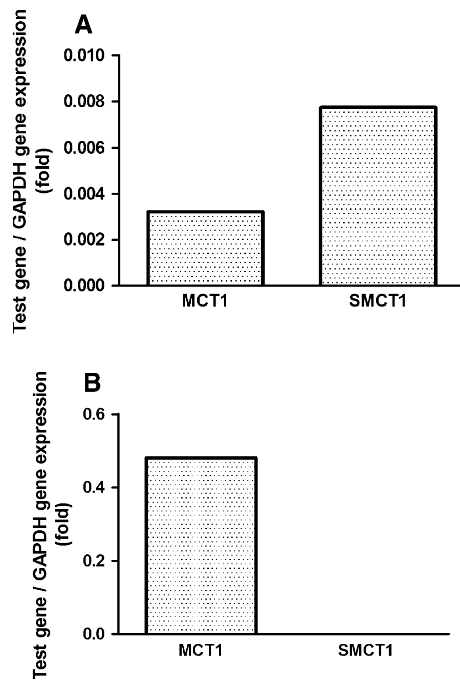

Fig. 6 Gene-expression levels of MCT1 and SMCT1 in IEC-6 cells (**a**) and FHC cells (**b**). MCT1 and SMCT1 levels were quantified by real-time qRT-PCR, as described in Materials and Methods. Results are shown as arithmetic means corresponding to the expression of MCT1 or SMCT1 relative to GAPDH ($n = 2$)

enough, the characteristics of ^{14}C-BT uptake in Caco-2 (recently described by our group (Gonçalves et al. 2009)) and IEC-6 cells show a high degree of similarity—namely, the time, pH and Cl^- dependence, the saturation kinetics with very similar K_m (4.0 and 2.8 mM for IEC-6 and Caco-2 cells, respectively), the inhibition by BT structural analogues and by the MCT1 inhibitors pCMB and NPPB and the lack of sensitivity to DIDS and CHC. The only difference between uptake of this compound by the two cell lines relates to its dependence on extracellular Na^+: Whereas uptake by Caco-2 cells is Na^+-independent, uptake by IEC-6 cells is slightly (17%) dependent on Na^+. Interestingly enough, we verified that IEC-6 cells express both MCT1 and SMCT1 mRNA. Thus, we conclude that uptake of ^{14}C-BT by IEC-6 cells seems to be mainly mediated by MCT1, with a small contribution of SMCT1.

It has been reported that SMCT1 is able to transport monocarboxylates in an Na^+-coupled manner when expressed in *Xenopus laevis* oocyes (Coady et al. 2004). However, most of the previous studies could not show Na^+-coupled BT transport in colonic cells (Hadjiagapiou et al. 2000; Stein et al. 2000; Gonçalves et al. 2009), with the exception of the work of Lecona et al. (2008), showing an Na^+-dependent high-affinity component of BT uptake in the human colon BCS-TC2 adenocarcinoma cell line. Interestingly enough, SMCT1 mRNA and Na^+-dependent BT uptake were very recently described in IEC-6 cells (Borthakur et al. 2010). Thus, our work fully confirms these findings. Nevertheless, we also think that the involvement of SMCT1 in BT transport in the normal colon is unlikely, given the low K_m (in the micromolar range) of SMCT1 for BT. Indeed, BT luminal concentrations in the normal colon are much higher (in the millimolar range), suggesting that SMCT1 could have a much less important role in BT transport than MCT1 (Thangaraju et al. 2008; Thibault et al. 2010).

Because BT plays an essential role in the maintenance of colonic tissue homeostasis, an understanding of the regulation of its absorption by the colon mucosa appears particularly important. In the past few years, important data concerning this subject have been obtained (see Introduction). However, these reports have employed colon adenocarcinoma cells only. Thus, it seemed important to investigate regulation of BT uptake also in normal intestinal epithelial cells, so the effect of a series of compounds recently found to affect BT uptake in Caco-2 cells (Gonçalves et al. 2009, in press) was investigated in the second part of this study.

Interestingly enough, we verified that several of the tested compounds affected ^{14}C-BT uptake by IEC-6 cells. Namely, acute exposure of IEC-6 cells to ethanol, acetaldehyde, indomethacin, resveratrol and quercetin and chronic exposure to resveratrol and quercetin reduced ^{14}C-BT uptake.

Epidemiological data have identified chronic alcohol consumption as a significant risk factor for colorectal cancer. Although ethanol is not carcinogenic in animal models, its bacterial fermentation in the colon produces acetaldehyde, which is highly toxic, mutagenic and carcinogenic (Pöschl and Seitz 2004; Bongaerts et al. 2006; Seitz and Homann 2007). In IEC-6 cells, acute exposure to ethanol and to its metabolite acetaldehyde reduced ^{14}C-BT uptake in a concentration-dependent manner. From these results, we can speculate that interference with BT uptake by the colonic epithelium might contribute to the colorectal cancer promoter effect of ethanol and acetaldehyde. Previously, acute exposure to acetaldehyde was also found to reduce ^{14}C-BT uptake in Caco-2 cells in a concentration-dependent and competitive manner (Gonçalves et al. 2009).

The NSAIDs emerged as a new perspective in tumor therapy as well as in cancer prevention (Tuynman et al. 2004; Elwood et al. 2009). Some NSAIDs are transportable substrates for MCTs but nontransportable blockers of SMCT1 (Coady et al. 2004; Choi et al. 2005; Itagaki et al. 2006). In Caco-2 cells, acute exposure to the NSAIDs acetylsalicylic acid and indomethacin concentration-dependently inhibited ^{14}C-BT uptake, and it was concluded that these two compounds are inhibitors of MCT1-mediated transport of BT (Gonçalves et al. 2009). Indomethacin also inhibited the uptake of ^{14}C-BT in IEC-6 cells (and

more potently that in Caco-2 cells). However, acetylsalicylic acid had no effect. Although we have at the present moment no explanation for this difference, it may be related to a differential regulation of MCT1 in these two cell lines (see below).

For ethanol, acetaldehyde and indomethacin, the observation of BT uptake inhibition after acute exposure but not after chronic exposure suggests that changes in the intrinsic activity of either MCT1 or SMCT1 induced acutely by these agents disappear after chronic exposure.

The flavonoid quercetin and the stilbene resveratrol were previously shown to be MCT1 inhibitors in Caco-2 cells (Shim et al. 2007; Gonçalves et al. in press), although their inhibitory effect disappeared after chronic exposure (Gonçalves et al. in press). In IEC-6 cells, these compounds were found to inhibit the uptake of ^{14}C-BT both acutely and chronically, thus demonstrating a more consistent inhibitory effect upon BT uptake in noncarcinogenic cells compared with carcinogenic ones. The lack of effect of chronic resveratrol and quercetin upon MCT1 and SMCT1 mRNA levels suggests that their effect does not result from changes in MCT1 or SMCT1 transcription rates but, rather, from changes in either functional protein levels or protein intrinsic activity.

Further comparison of the effect of the tested compounds on IEC-6 and Caco-2 cells gives interesting results. For some of the compounds, a similar effect in both cell lines was observed (acute acetaldehyde, indomethacin, resveratrol and quercetin). Moreover, and similar to what was verified with Caco-2 cells (Gonçalves et al. 2009), some compounds (ethanol, acetaldehyde and indomethacin) affected BT uptake when tested acutely but lost their effect after chronic exposure. However, for most of the compounds, the effect was found to be dramatically different in these two cell lines (acute ethanol, caffeine, acetylsalicylic acid, chrysin and myricetin and chronic caffeine, resveratrol, quercetin, chrysin, rutin and EGCG). Thus, ^{14}C-BT uptake in IEC-6 and Caco-2 cells seems to be differentially modulated by several distinct xenobiotics. These results are very interesting in the context of colon carcinogenesis as the effect of BT in many tumor cells (an anticarcinogenic effect which involves induction of differentiation and apoptosis and inhibition of proliferation) and noncarcinogenic cells is opposite, this phenomenon being referred as the "BT paradox" (Hamer et al. 2008). Thus, a comparison between the effect of a given compound upon BT uptake in carcinogenic and noncarcinogenic cell lines seems important. On the one hand, inhibition of BT uptake in tumor and nontumor colon cells will most probably have opposite effects in the context of carcinogenesis/anticarcinogenesis. On the other hand, a dramatic difference in the effect of a given compound on tumor and nontumor cells might be of interest. In this context, the effect of chronic caffeine, quercetin and EGCG (increasing BT uptake in Caco-2 while having no effect or decreasing uptake in IEC-6 cells) appears very interesting.

Finally, as uptake of BT by IEC-6 cells (present study) and Caco-2 cells (Gonçalves et al. 2009) seems to be mainly mediated by the same carrier-mediated mechanism, MCT1, the distinct effect of most of the xenobiotics tested upon ^{14}C-BT uptake in these two cell lines points to the possibility that MCT1 is differentially regulated in IEC-6 and Caco-2 cells. Knowing that these xenobiotics interfere with several distinct intracellular regulatory pathways, we hypothesize that this differential regulation might occur at several distinct levels (e.g., at the level of MCT1 gene transcription, protein synthesis or phosphorylation/dephosphorylation).

In conclusion, uptake of ^{14}C-BT by FHC and IEC-6 cells shows rather distinct characteristics. Uptake by IEC-6 cells seems to involve mainly MCT1, with a small contribution from SMCT1. Therefore, the IEC-6 cell line may be used to study regulation of SMCT1 expression and function. On the contrary, uptake by FHC cells seems to involve neither MCT1 nor SMCT1, and this cell line does not seem useful for examining BT intestinal transport. Moreover, uptake of ^{14}C-BT by IEC-6 cells is inhibited by either acute or chronic exposure to a series of xenobiotics (ethanol, acetaldehyde, indomethacin, resveratrol and quercetin). Finally, the distinct effect of some of the xenobiotics tested (chronic caffeine, quercetin and EGCG) upon ^{14}C-BT uptake by IEC-6 (no effect or decrease) and tumoral (Caco-2) cells (increase) (Gonçalves et al. 2009) might be of interest in the context of colon carcinogenesis.

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