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Inhibition of Butyrate Uptake by the Primary Bile Salt Chenodeoxycholic Acid in Intestinal Epithelial Cells

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ABSTRACT

Colorectal cancer (CRC) is one of the most common cancers worldwide. Epidemiological and experimental studies suggest that bile acids may play a role in CRC etiology. Our aim was to characterize the effect of the primary bile acid chenodeoxycholic acid (CDCA) upon ^{14}C-BT uptake in tumoral (Caco-2) and non-tumoral (IEC-6) intestinal epithelial cell lines. A 2-day exposure to CDCA markedly and concentration-dependently inhibited ^{14}C-BT uptake by IEC-6 cells ($IC_{50} = 120\ \mu M$), and, less potently, by Caco-2 cells ($IC_{50} = 402\ \mu M$). The inhibitory effect of CDCA upon ^{14}C-BT uptake did not result from a decrease in cell proliferation or viability. In IEC-6 cells: (1) uptake of ^{14}C-BT involves both a high-affinity and a low-affinity transporter, and CDCA acted as a competitive inhibitor of the high-affinity transporter; (2) CDCA inhibited both Na^+-coupled monocarboxylate cotransporter 1 (SMCT1)- and H^+-coupled monocarboxylate transporter 1 (MCT1)-mediated uptake of ^{14}C-BT; (3) CDCA significantly increased the mRNA expression level of SMCT1; (4) inhibition of ^{14}C-BT uptake by CDCA was dependent on CaM, MAP kinase (ERK1/2 and p38 pathways), and PKC activation, and reduced by a reactive oxygen species scavenger. Finally, BT (5 mM) decreased IEC-6 cell viability and increased IEC-6 cell differentiation, and CDCA (100 μM) reduced this effect. In conclusion, CDCA is an effective inhibitor of ^{14}C-BT uptake in tumoral and non-tumoral intestinal epithelial cells, through inhibition of both H^+-coupled MCT1- and SMCT1-mediated transport. Given the role played by BT in the intestine, this mechanism may contribute to the procarcinogenic effect of CDCA at this level. J. Cell. Biochem. 113: 2937–2947, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: BUTYRATE UPTAKE; CHENODEOXYCHOLIC ACID; IEC-6 CELLS; Caco-2 CELLS; MCT1; SMCT1

Colorectal cancer (CRC) is a leading cause of cancer death in occidental countries [Jemal et al., 2011]. The causes of CRC are multifactorial, and a good correlation between a diet high in saturated fats and low in dietary fiber, fruit, and vegetables, and an increased risk of CRC has been well established by epidemiological studies [Martínez et al., 2008].

Short-chain fatty acids [(SCFAs) acetate, propionate, and butyrate (BT)] are organic acids produced in the intestinal lumen by bacterial fermentation of mainly undigested dietary fiber. Among SCFA, BT plays a key role in colonic epithelium homeostasis, by having multiple regulatory roles at that level, including: (1) being the main energy source for colonocytes; (2) inhibition of colon carcinogenesis (by suppressing growth of cancer cells, inducing differentiation and apoptosis and inhibiting cell proliferation); (3) promotion of growth and proliferation of normal colonic epithelial cells; (4) stimulation of fluid and electrolyte absorption; (5) stimulation of mucus secretion and increase of vascular flow and motility; (6) reduction of visceral perception, intestinal discomfort, and pain; (7) inhibition of colon inflammation and oxidative stress; and (8) improvement of the colonic defense barrier function [Hamer et al., 2008].

As stated, one of the proposed beneficial effects of BT on human colonic health is the prevention/inhibition of colon carcinogenesis

Abbreviations used: ALP, alkaline phosphatase; BT, butyrate; Caco-2, human epithelial colon adenocarcinoma cell line; CaM, Ca^{2+}/calmodulin; CaMK II, CaM-dependent protein kinase II; CDCA, chenodeoxycholic acid; CRC, colorectal cancer; IEC-6, rat non-tumoral small intestinal epithelial cell line; LDH, lactate dehydrogenase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MCT1, H^+-coupled monocarboxylate transporter 1; MTT, 3-{4,5-dimethyl-2-thiazolyl}-2,5-diphenyl-2H-tetrazolium bromide; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PTK, protein tyrosine kinases; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; SCFA, short-chain fatty acid; SMCT1, Na^+-coupled monocarboxylate cotransporter 1; SRB, sulforhodamine B.

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[Hamer et al., 2008]. BT is transported into intestinal epithelial cells by two specific carrier-mediated transport systems, the electroneutral H^+-coupled monocarboxylate transporter 1 (MCT1) [Cuff et al., 2005] and the Na^+-coupled monocarboxylate cotransporter 1 (SMCT1) [Gupta et al., 2006]. MCT1 [Cuff et al., 2005] and SMCT1 [Gupta et al., 2006] were recently proposed to function as tumor suppressors, most probably due to their ability to mediate the entry of BT into intestinal epithelial cells. Therefore, factors that interfere with BT uptake into intestinal epithelial cells are potentially detrimental to intestinal health and integrity by promoting CRC.

Primary bile acids, predominantly cholic acid and chenodeoxycholic acid (CDCA), are produced in the liver by the metabolism of cholesterol and are delivered into the intestinal tract as glycine or taurine conjugates. Most bile acids are reabsorbed in the ileum as conjugates. However, during each enterohepatic cycle, about 10% of the bile acids escape into the colon where they are exhaustively converted by the intestinal flora, and 1–3% of the bile acids will eventually be excreted in the feces. Fecal bile acids are almost completely deconjugated and have undergone several other reactions, such as dehydroxylation, dehydrogenation, and epimerization [Ridlon et al., 2006]. Intestinal epithelial cells are thus exposed to various concentrations and compositions of bile acids.

Several epidemiological studies have found that fecal bile acid concentrations are elevated in populations with a high incidence of CRC [Reddy et al., 1977, 1978], and high-fat, high-cholesterol diets, which increase the risk of CRC, also increase the total of bile acids in the gut [Stadler et al., 1988]. In agreement with these observations, secondary bile acids have been shown to be tumor promoters, both in vitro and in animal studies [Reddy et al., 1977; Mahmoud et al., 1999]. Their mechanism of action is still poorly understood, although PKC, MAP kinases (MAPK), and nuclear factor-kappa B (NF-κB) were identified as molecular targets for these compounds [Qiao et al., 2000; McMillan et al., 2003].

In relation to primary bile acids, less information is available. However, a significantly higher fecal concentration of the primary bile salt CDCA was seen in patients with CRC/adenoma [Tong et al., 2008] and CDCA was also shown to be tumor promoting in some animal and cell culture studies [Mahmoud et al., 1999; McMillan et al., 2003].

The majority of studies concerning the effects of BT and bile acids on intestinal epithelial cells have considered these factors individually. However, in vivo, both are present in the colon and may directly or indirectly influence each other's actions. Very recently, we demonstrated a discrete interference of the bile salt deoxycholic acid upon uptake of ^{14}C-BT by intestinal epithelial cells [Gonçalves et al., 2011d]. So, the aim of the present study was to investigate the hypothesis that interference with the apical uptake of BT may be one of the mechanisms contributing to the effect of the primary bile acid CDCA upon colon carcinogenesis.

MATERIALS AND METHODS

CACO-2 AND IEC-6 CELL CULTURE

The Caco-2 and IEC-6 cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and were used between passage numbers 53–58 and 17–30, respectively. The cells were maintained in a humidified atmosphere of 5% CO_2–95% air. Caco-2 cells were cultured in minimum essential medium (Sigma, St. Louis, MO) containing 5.55 mM glucose and supplemented with 15% fetal calf serum, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Sigma). IEC-6 cells were cultured in Dulbecco's modified Eagle's 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 U/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 7 days. For sub-culturing, the cells were removed enzymatically (0.25% trypsin–EDTA, 5 min, 37°C), split 1:3, and sub-cultured in plastic culture dishes (21 cm^2; Ø 60 mm; Corning Costar, Corning, NY). For use in experiments, cells were seeded on 24-well plastic cell culture clusters (2 cm^2 Ø 16 mm; TPP®), and experiments were performed 7–9 days after the initial seeding (90–100% confluence).

TREATMENT OF THE CELLS

The effect of CDCA was tested by cultivating cells in culture medium containing CDCA (or the respective solvent) for 1, 2, 3, or 7 days. The effect of BT was tested by cultivating cells in culture medium containing BT or the respective solvent for 2 days. The effect of inhibitors of signaling pathways was tested by cultivating cells in culture medium containing CDCA, these compounds or the respective solvents for 1 day. The medium was renewed daily and the end of the treatment period was always days 7–9 of cell culture.

TRANSPORT STUDIES

Transport experiments were performed with Caco-2 and IEC-6 cells incubated in glucose-free Krebs (GFK) buffer containing (mM): 125 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 25 $NaHCO_3$, 1.6 KH_2PO_4, 0.4 K_2HPO_4, and 20 MES, pH 6.5 [Gonçalves et al., 2009]. Initially, the culture medium was aspirated and the cells were washed with 0.3 ml GFK buffer at 37°C. Then, uptake was initiated by the addition of 0.3 ml GFK buffer at 37°C containing ^{14}C-BT (10 μM, except in kinetic experiments). Incubation was stopped after 3 min by removing the incubation medium, placing the cells on ice and rinsing the cells with 0.5 ml ice-cold GFK buffer. The 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 room temperature overnight. Radioactivity in the cells was measured by liquid scintillation counting. In the Na^+ dependence experiments, NaCl was substituted by an isotonic concentration of LiCl.

DETERMINATION OF CELLULAR VIABILITY

Two different methods were used to determine cell viability.

MTT (3-{4,5-dimethyl-2-thiazolyl}-2,5-diphenyl-2H-tetrazolium bromide) assay. After 45 h of treatment, 50 μl MTT solution (5 mg/ml) were added to each well. The cells were then further incubated for 3 h at 37°C. The formazan crystals derived from MTT cleavage were then measured as described by Mosmann [1983].

Lactate dehydrogenase (LDH) assay. After the treatment period (48 h), cellular leakage of the cytosolic enzyme LDH into the extracellular (culture) medium was measured spectrophotometrically, by quantification of the decrease in absorbance of NADH during the reduction of pyruvate to lactate, as described by Bergmeyer and Bernt [1974].

DETERMINATION OF CELLULAR PROLIFERATION [SULFORHODAMINE B (SRB) ASSAY]

After the period of treatment (48 h), the whole cell protein was determined, as described previously by our group [Gonçalves et al., 2011c].

DETERMINATION OF CELLULAR DIFFERENTIATION (ALKALINE PHOSPHATASE ACTIVITY ASSAY)

After the period of treatment (48 h), cell differentiation was measured by quantification of alkaline phosphatase (ALP) activity, as previously described [Gonçalves et al., 2011a]. ALP activity was determined spectrophotometrically, by using *p*-nitrophenylphosphate as substrate. The results are expressed as nmol *p*-nitrophenol/min/mg protein.

QRT-PCR

Total RNA was extracted from IEC-6 cells using the Tripure® isolation reagent, according to the manufacturer's instructions (Roche Diagnostics, Germany). cDNA synthesis and real-time PCR was carried out as described by Gonçalves et al. [2011abc]. Annealing temperatures (AT) and sequence of primers are indicated in Table I. 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 the 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. Statistical analysis of the difference between various groups was evaluated by the ANOVA test, followed by the Student–Newman–Keuls test. Statistical analysis of the difference between two groups was evaluated with Student's *t*-test. Differences were considered to be significant when $P < 0.05$.

TABLE I. Primers Used in qRT-PCR

Gene name	Primer sequence (5′–3′)	AT (°C)
rGAPDH	F: GGC ATC GTG GAA GGG CTC ATG AC R: ATG CCA GTG AGC TTC CCG TTC AGC	62
rMCT1	F: CAG TGC AAC GAC CAG TGA ATG TG R: ATC AAG CCA CAG CCA GAC AGG	69
rSMCT1	F: CGG GAT CAC CAG CAC CTA C R: GCA GGG GCA TAA ATC ACA ATC	62

rGAPDH, rat glyceraldehyde-3-phosphate dehydrogenase; rMCT1, rat monocarboxylate transporter type 1; rSMCT1, rat Na^+-coupled monocarboxylate transporter type 1; F, forward; R, reverse.

MATERIALS

^{14}C-BT ([1-^{14}C]-*n*-butyric acid, sodium salt; specific activity 30–60 mCi/mmol; Biotrend Chemikalien GmbH, Köln, Germany); 4′,5,7-trihydroxyisoflavone (genistein), 5,5′-dimetil-BAPTA-AM, antibiotic/antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B), calmidazolium, chelerythrine chloride, H7, H-89 dihydrochloride hydrate, KN-62, MES (2-[*N*-morpholino]ethanesulfonic acid hydrate), nicotinamide adenine dinucleotide (NADH), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), 4-(hydroxymercuri)benzoic acid sodium salt (pCMB), *p*-nitrophenylphosphate, serum albumin, sodium BT, sodium chenodeoxycholate (CDCA), SRB, trichloroacetic acid sodium salt, trypsin–EDTA solution (Sigma); fetal calf serum (Invitrogen Corporation, Carlsbad, CA); PD 98058 and SB 203580 (Research Biochemicals International, Natick); dimethylsulfoxide (DMSO), triton X-100 (Merck, Darmstadt, Germany).

Drugs to be tested were dissolved in water, DMSO, or ethanol; the final concentration of these solvents in the culture medium and GFK buffer was 1% and 0.1%, respectively. Controls for these drugs were run in the presence of the respective solvent.

RESULTS

TIME- AND CONCENTRATION-DEPENDENCE OF THE EFFECT OF CDCA UPON ^{14}C-BT APICAL UPTAKE IN CACO-2 AND IEC-6 CELLS

Our group had previously shown that the apical uptake of ^{14}C-BT in both Caco-2 cells [Gonçalves et al., 2009] and IEC-6 cells [Gonçalves et al., 2011b] was linear with time for up to 3 min of incubation. So, in the present work, cells were incubated with ^{14}C-BT for 3 min in order to measure initial rates of uptake.

The effect of CDCA upon ^{14}C-BT apical uptake by Caco-2 and IEC-6 cells was investigated in a first series of experiments. For this, CDCA (100 μM) was tested over different time periods (1, 2, 3, and 7 days). As shown in Figure 1A, CDCA was devoid of a significant effect upon the apical uptake of ^{14}C-BT in Caco-2 cells. By contrast, in IEC-6 cells, CDCA strongly decreased (≈50%) ^{14}C-BT apical uptake, in a time-independent manner up to 7 days of exposure (Fig. 1B).

We further investigated the effect of a 2-day treatment with CDCA upon the apical uptake of ^{14}C-BT, by characterizing its concentration-dependency. As shown in Figure 2, CDCA inhibited ^{14}C-BT uptake in both Caco-2 and IEC-6 cells in a concentration-dependent manner, but with a higher potency in IEC-6 cells. Its IC_{50} was found to be 402 (255–634) μM and 120 (88–164) μM in Caco-2 and IEC-6 cells, respectively.

EFFECT OF CDCA UPON THE KINETICS OF ^{14}C-BT APICAL UPTAKE IN IEC-6 CELLS

Next, we decided to investigate the effect of a 2-day exposure of IEC-6 cells to CDCA (100 μM) upon the kinetic parameters of ^{14}C-BT

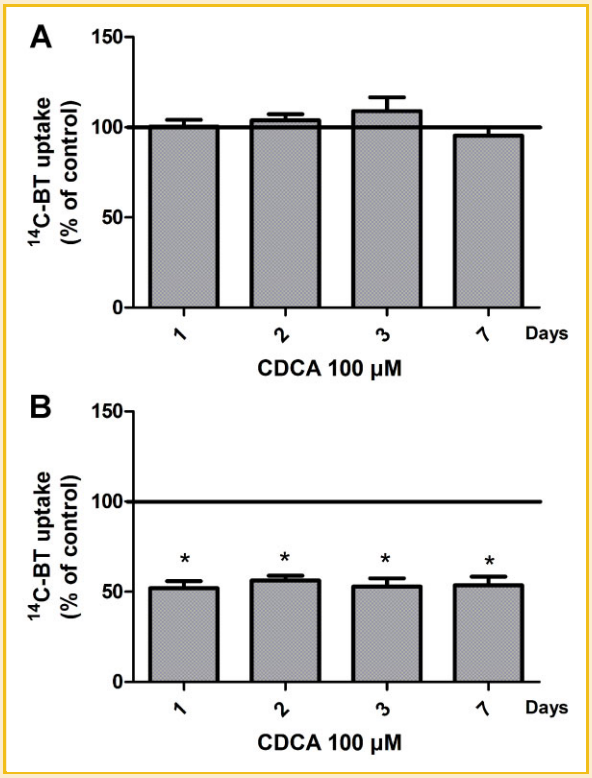

Fig. 1. Time-dependence of the effect of chenodeoxycholic acid (CDCA; 100 µM) upon the apical uptake of ^{14}C-BT by Caco-2 cells (A) and IEC-6 cells (B). Initial rates of uptake were determined in cells incubated for 3 min with 10 µM ^{14}C-BT after treatment for 1, 2, 3, or 7 days with CDCA 100 µM (n = 6–9). Shown are arithmetic means ± SEM. *Significantly different from the respective control ($P < 0.05$).

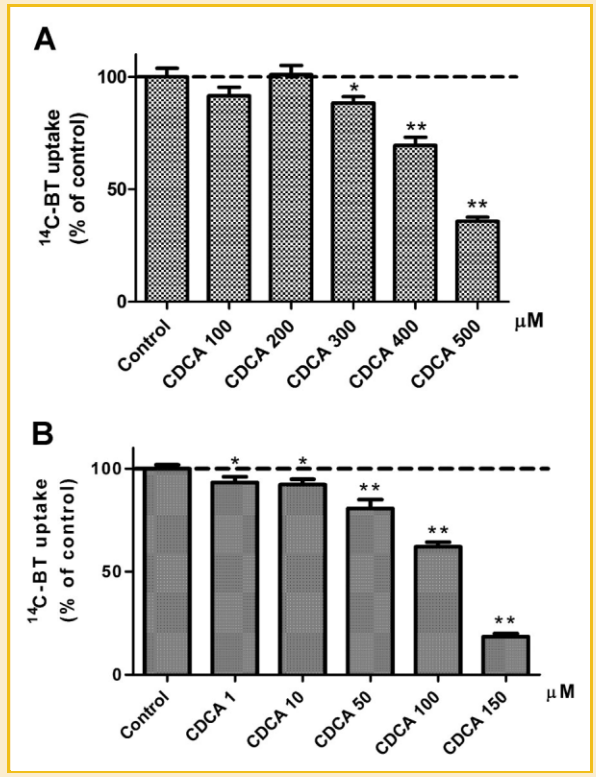

Fig. 2. Concentration-dependence of the effect of chenodeoxycholic acid (CDCA) upon the apical uptake of ^{14}C-BT by Caco-2 cells (A) and IEC-6 cells (B). Initial rates of uptake were determined in cells incubated for 3 min with 10 µM ^{14}C-BT after treatment for 2 days with CDCA (n = 8–16). Shown are arithmetic means ± SEM. *Significantly different from the respective control ($P < 0.05$). **Significantly different from the respective control ($P < 0.005$).

apical uptake. Kinetic experiments revealed the existence of a high affinity and a low-affinity transporter for ^{14}C-BT (Fig. 3). The high- and low-affinity transporters of BT in IEC-6 cells most probably correspond to SMCT1 and MCT1, respectively [Thangaraju et al., 2008; Gonçalves et al., 2011b]. We verified that CDCA was devoid of significant effect upon the kinetics of ^{14}C-BT uptake mediated by the low-affinity transporter ($V_{max} = 57.6 \pm 11.3$ and 78.0 ± 20.4 nmol mg prot^{-1} 3 min^{-1}, $K_m = 2.66 \pm 0.94$ and 3.39 ± 1.46 mM; in the absence and presence of CDCA, respectively; Fig. 3A). On the other hand, CDCA behaved as a competitive inhibitor of ^{14}C-BT apical uptake mediated by the high-affinity transporter, as it significantly increased the K_m of uptake (40.2 ± 3.7–95.4 ± 16.5 µM), without changing the V_{max} (3.91 ± 0.14 and 4.37 ± 0.42 nmol mg prot^{-1} 3 min^{-1} in the absence and presence of CDCA, respectively; Fig. 3B).

EFFECT OF CDCA UPON SMCT1 AND MCT1-MEDIATED ^{14}C-BT APICAL UPTAKE IN IEC-6 CELLS

In IEC-6 cells, Na^+-dependent BT uptake corresponds to SMCT1-mediated transport, and Na^+-independent uptake mainly corresponds to MCT1-mediated transport [Borthakur et al., 2010; Gonçalves et al., 2011b]. So, in order to distinguish between the effect of CDCA upon SMCT1- and MCT1-mediated ^{14}C-BT apical uptake, we compared the inhibitory effect of CDCA (100 µM) upon ^{14}C-BT uptake in the presence and absence of NaCl (which was substituted by LiCl). As shown in Figure 4A, about 60% of ^{14}C-BT apical uptake by IEC-6 cells is Na^+-dependent. CDCA (100 µM) caused a decrease of ^{14}C-BT uptake to ≈20%, both in the presence of NaCl or LiCl. This indicates that CDCA inhibits SMCT1-mediated ^{14}C-BT uptake and strongly suggests that it also inhibits MCT1-mediated ^{14}C-BT uptake. Confirmation that CDCA is able to inhibit MCT1-mediated ^{14}C-BT uptake was obtained by verifying that the inhibitory effect of CDCA upon Na^+-independent ^{14}C-BT uptake by IEC-6 cells disappeared in the presence of the classical MCT1 inhibitors, pCMB and NPPB (Fig. 4B,C).

REVERSE TRANSCRIPTION QUANTITATIVE REAL-TIME PCR IN IEC-6 CELLS

A comparison between the mRNA expression levels of MCT1 and SMCT1 in control and CDCA-treated IEC-6 cells was then evaluated. Quantification of mRNA levels shows that IEC-6 express 10× more SMCT1 than MCT1, and that SMCT1 mRNA levels were significantly higher (and MCT1 mRNA levels tended to be higher) in cells treated with CDCA, in relation to control cells (≈80%; Fig. 5).

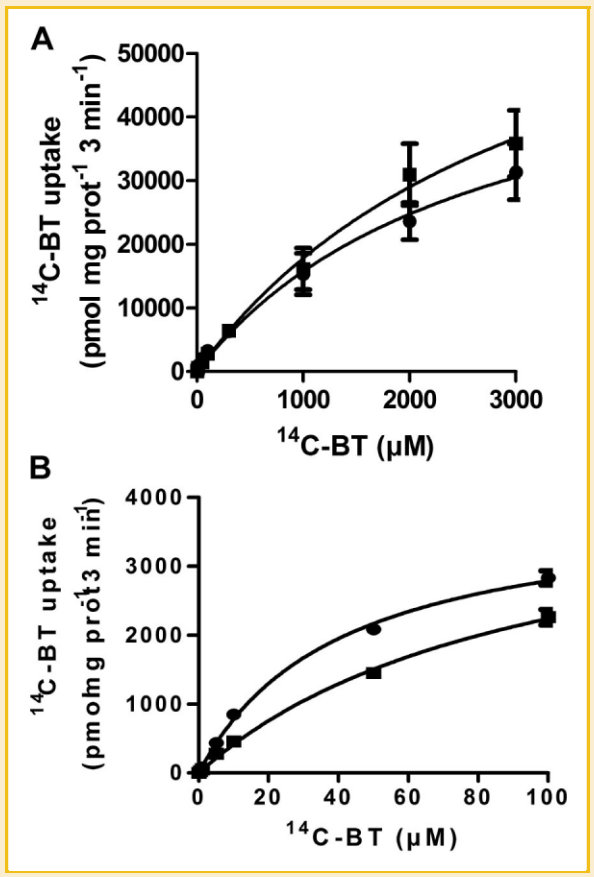

Fig. 3. Effect of chenodeoxycholic acid (CDCA; 100 μM) upon the kinetic parameters of the apical uptake of ^{14}C-BT by IEC-6 cells. Initial rates of uptake were determined in IEC-6 cells incubated at 37°C with 0.01–0.1 mM (high-affinity transport, A) or 0.01–3 mM (low-affinity transport, B) of ^{14}C-BT for 3 min, after treatment for 2 days with CDCA 100 μM (closed squares) or the respective solvent (control; closed circles; n = 4–8). Shown are arithmetic means ± SEM.

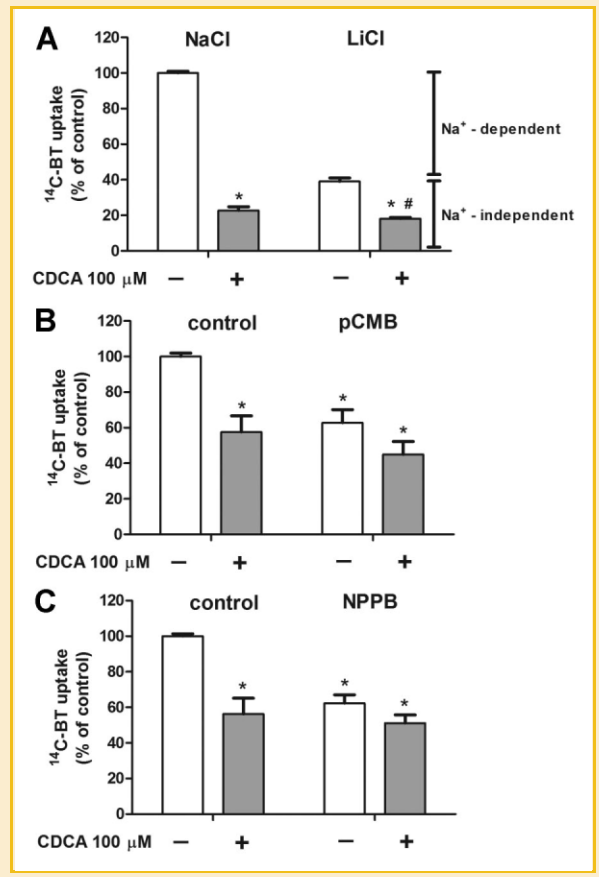

Fig. 4. Effect of chenodeoxycholic acid (CDCA; 100 μM) upon SMCT1- and MCT1-mediated apical uptake of ^{14}C-BT by IEC-6 cells. A: Effect of CDCA upon Na^+-dependent and Na^+-independent uptake. After treatment for 2 days with CDCA or the respective solvent (control), cells were incubated at 37°C with ^{14}C-BT 10 μM for 3 min in GFK buffer containing NaCl (control) or an isotonic concentration of LiCl (n = 10). B,C: Effect of CDCA and MCT1 inhibitors upon Na^+-independent uptake. After treatment for 2 days with CDCA or the respective solvent (control), cells were preincubated for 20 min and then incubated at 37°C with ^{14}C-BT 10 μM for 3 min in GFK buffer containing LiCl in the absence (control) or presence of *p*-chloromercuribenzoate (pCMB) 0.5 mM (n = 9) or 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) 0.5 mM (n = 9). *Significantly different from control ($P < 0.05$). #Significantly different from CDCA ($P < 0.05$).

EFFECT OF MODULATORS OF INTRACELLULAR SIGNALING PATHWAYS ON CDCA-INDUCED INHIBITION OF ^{14}C-BT UPTAKE IN IEC-6 CELLS

We next investigated the intracellular signaling mechanisms involved in the inhibition of ^{14}C-BT uptake caused by CDCA. For this, the effect of a 1-day exposure of IEC-6 cells to modulators of intracellular signaling pathways, to CDCA or to a combination of both was assessed.

First, the role of intracellular Ca^{2+} in CDCA-induced inhibition of ^{14}C-BT uptake was investigated, by testing the effect of the Ca^{2+} chelator BAPTA-AM. No significant change in ^{14}C-BT uptake was found with BAPTA-AM, and the effect of CDCA was also not changed in the presence of this agent (Fig. 6).

We then evaluated the role of the Ca^{2+}/calmodulin (CaM) inhibitor calmidazolium, and of the CaM-dependent protein kinase II (CaMK II) inhibitor KN-62 [Said et al., 1999]. Uptake of ^{14}C-BT was significantly reduced in the presence of calmidazolium, and slightly increased in the presence of KN-62 (Fig. 6), suggesting that BT uptake is dependent on intracellular CaM. The effect of CDCA upon

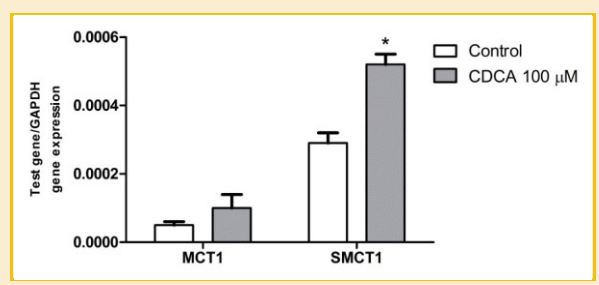

Fig. 5. Quantification of mRNA levels of MCT1 and SMCT1, by qRT-PCR, in IEC-6 cells after treatment for 2 days with chenodeoxycholic acid (CDCA; 100 μM) or its solvent (Control; n = 5). Results are shown as the expression of MCT1 or SMCT1 relative to GAPDH (arithmetic means ± SEM). *Significantly different from control ($P < 0.05$).

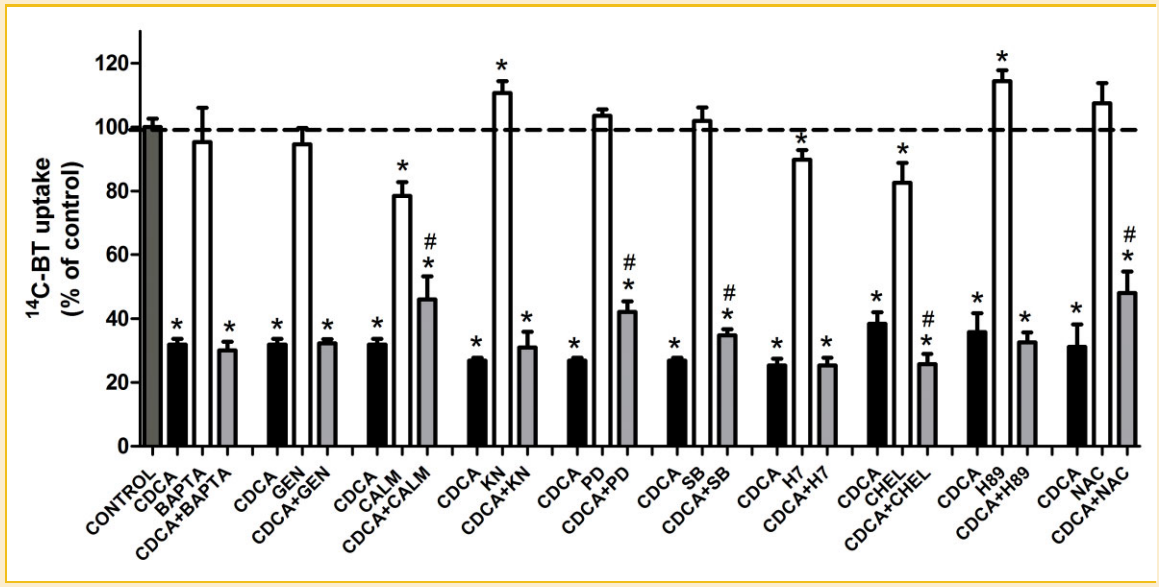

Fig. 6. Effect of inhibitors of intracellular signaling pathways upon the apical uptake of ^{14}C-BT and upon CDCA-induced inhibition of ^{14}C-BT uptake by IEC-6 cells. Initial rates of ^{14}C-BT uptake were determined in cells incubated for 3 min with ^{14}C-BT 10 μM after treatment for 1 day with CDCA 100 μM (CDCA), BAPTA AM 5 μM (BAPTA), CDCA 100 μM + BAPTA AM 5 μM (CDCA + BAPTA), genistein 10 μM (GEN), CDCA 100 μM + genistein 10 μM (CDCA + GEN), calmidazolium 0.5 μM (CALM), CDCA 100 μM + calmidazolium 0.5 μM (CDCA + CALM), KN-62 1.5 μM (KN), CDCA 100 μM + KN-62 1.5 μM (CDCA + KN), PD 98058 2.5 μM (PD), CDCA 100 μM + PD 98058 2.5 μM (CDCA + PD), SB 203580 2.5 μM (SB), CDCA 100 μM + SB 203580 2.5 μM (CDCA + SB), H-7 10 μM (H7), CDCA 100 μM + H-7 10 μM (CDCA + H7), chelerythrine 1 μM (CHEL), CDCA 100 μM + chelerythrine 1 μM (CDCA + CHEL), H-89 10 μM (H89), CDCA 100 μM + H-89 10 μM (CDCA + H89), *N*-acetyl-cysteine 1 mM (NAC), CDCA 100 μM + *N*-acetyl-cysteine 1 mM (CDCA + NAC), or the respective solvents (control) (n = 6–9). Shown are arithmetic means ± SEM. *Significantly different from the respective control ($P < 0.05$). #Significantly different from CDCA 100 μM ($P < 0.05$).

^{14}C-BT uptake was not changed in the presence of KN-62, but was reduced by calmidazolium (Fig. 6).

The involvement of MAPK was studied by testing the effect of specific inhibitors of MAPK ERK1/2 (PD 98059) [Alessi et al., 1995] and p38 MAPK (SB 203580) [Cuenda et al., 1995]. Interestingly enough, both PD 98059 and SB 203580 were devoid of effect upon ^{14}C-BT uptake, but they were able to partially reduce the inhibitory effect of CDCA upon ^{14}C-BT uptake (Fig. 6).

Next, we tested the effect of H-7, a non-selective inhibitor of PKA, PKC, and PKG [Hidaka et al., 1984]. Although H-7 was found to cause a small (≈10%) but significant decrease in ^{14}C-BT uptake, it was not able to change the effect of CDCA upon this parameter (Fig. 6). We then studied the effect of specific inhibitors of PKC (chelerythrine) and PKA (H-89) [Silva et al., 2010]. PKC inhibition was found to cause a significant decrease (≈17%) and PKA inhibition caused a significant increase (≈14%) in ^{14}C-BT uptake. This suggests that ^{14}C-BT uptake by IEC-6 cells is stimulated by PKC and inhibited by PKA activation, an observation in perfect agreement with previous publications [Alrefai et al., 2004; Narumi et al., 2010]. Interestingly enough, chelerythrine (PKC inhibitor) was able to significantly reduce the inhibition of ^{14}C-BT uptake induced by CDCA, but H89 (PKA inhibitor) had no effect (Fig. 6).

The involvement of protein tyrosine kinases (PTK) was studied by testing genistein, a known PTK inhibitor [Said et al., 1999]. As shown in Figure 7, neither ^{14}C-BT uptake nor CDCA-induced decrease of ^{14}C-BT uptake were affected by this drug (Fig. 6).

We also evaluated the putative involvement of oxidative stress in CDCA-mediated inhibition of ^{14}C-BT uptake [Song et al., 2007; Ignacio Barrasa et al., 2011], by testing the effect of the ROS scavenger *N*-acetyl-cysteine (NAC) [Aruoma et al., 1989]. NAC was devoid of effect upon ^{14}C-BT uptake but it was able to reduce the inhibitory effect of CDCA upon ^{14}C-BT uptake (Fig. 6).

The involvement of NF-κB signal transduction pathway was investigated by testing the effect of a specific inhibitor of IkBa phosphorylation and degradation (BAY 11-7082) [Pierce et al., 1997]. BAY 11-7082 (500 nM) was devoid of effect upon ^{14}C-BT uptake and did not change the inhibitory effect of CDCA upon this parameter (results not show).

Finally, the involvement of cyclooxygenases (COX) in CDCA-mediated inhibition of ^{14}C-BT uptake was tested by using quinacrine, a selective phospholipase A_2 inhibitor [Winocour et al., 1981]. Quinacrine (500 μM) was devoid of effect upon ^{14}C-BT uptake and also did not affect the inhibitory effect of CDCA upon ^{14}C-BT uptake (results not shown).

EFFECT OF CDCA ON VIABILITY AND PROLIFERATION OF IEC-6 AND CACO-2 CELLS

The effect of CDCA on cell viability and proliferation was evaluated in order to exclude these factors as responsible for its effect upon ^{14}C-BT uptake. For this, the effect of a 2-day exposure to increasing concentrations of CDCA upon Caco-2 cellular viability and upon IEC-6 cellular viability and proliferation was investigated. We verified that CDCA up to 500 μM did not affect Caco-2 cell viability

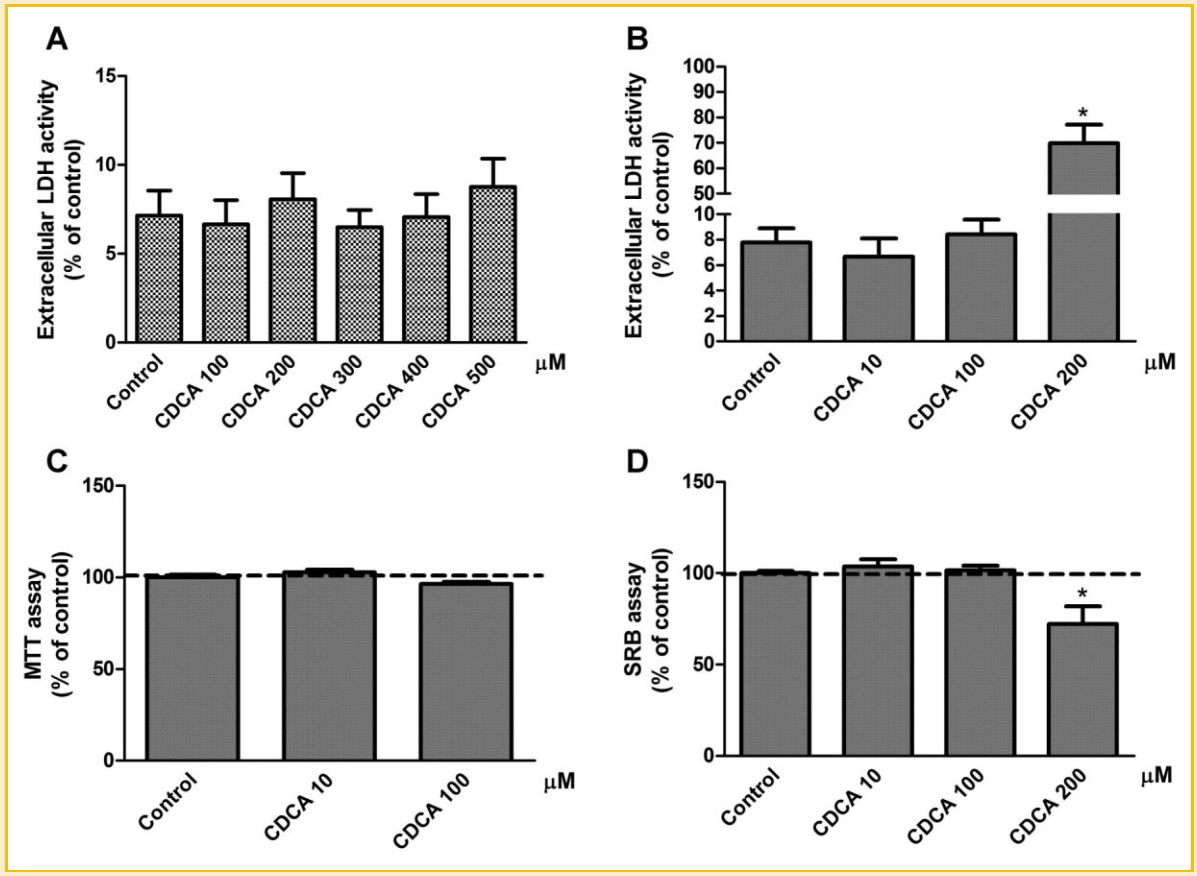

Fig. 7. Effect of a 2-day exposure to increasing concentrations of chenodeoxycholic acid (CDCA) upon Caco-2 cellular viability (A) and IEC-6 cellular viability (B,C) and proliferation (D). Cellular viability was determined by (A,B) quantification of extracellular lactate dehydrogenase activity ($n = 12–17$) and (C) the MTT assay ($n = 10$). Cell proliferation was quantified by (D) the SRB assay ($n = 6–12$). A,B: Cells were seeded on 24-well plates and cellular viability was determined by quantification of extracellular lactate dehydrogenase activity, as described in Materials and Methods Section. Results are shown as extracellular LDH activity (as % of total LDH activity). C: Cells were seeded on 24-well plates and cellular viability was determined by the MTT assay, as described in Materials and Methods Section. Results are shown as % of control. D: Cells were seeded on 24-well plates and cellular growth was determined by quantification of whole cellular protein with SRB, as described in Materials and Methods Section. Results are shown as absorbance (% of control). Results are presented as arithmetic means + SEM. *Significantly different from control ($P < 0.05$).

(Fig. 7A), and that CDCA up to 100 μM did affect neither cell viability (Fig. 7B,C) nor proliferation (Fig. 7D) of IEC-6 cells. However, 200 μM CDCA caused a significant decrease in IEC-6 cell viability (Fig. 7B) and proliferation (Fig. 7D).

THE EFFECT OF THE INTERACTION BETWEEN BT AND CDCA UPON IEC-6 CELLULAR VIABILITY, PROLIFERATION, AND DIFFERENTIATION

In this final series of experiments, we investigated whether CDCA was able to modify the effects of BT upon cell viability, proliferation, and differentiation. The effect of BT upon IEC-6 cell viability, proliferation, and differentiation was recently assessed by our group [Gonçalves et al., 2011c]. On the basis of these results, we selected BT 5 mM for further experiments.

As shown in Figure 8, BT (5 mM) caused a significant decrease in IEC-6 cell proliferation (≈20%) and viability (≈13%) and a very marked increase in cell differentiation (≈1,000%). CDCA (100 μM) did not affect cellular viability or differentiation, and caused only a small increase (≈10%) in cell proliferation. Interestingly enough, combination of CDCA with BT resulted in an attenuation of the effects of BT upon cell proliferation and differentiation. On the contrary, the effect of BT upon cell viability was potentiated by CDCA (Fig. 8).

DISCUSSION

BT plays a key regulatory role in colonic epithelium homeostasis. Therefore, factors that interfere with BT uptake into colonic epithelial cells are potentially detrimental to intestinal health and integrity. Very recently, we demonstrated a discrete interference of some n-3 polyunsaturated fatty acids (PUFAs; docosahexaenoic acid and eicosapentaenoic acid), n-6 PUFAs (linoleic acid, γ-linolenic acid, and arachidonic acid), conjugated linoleic acid, and the bile salt deoxycholic acid upon uptake of ^{14}C-BT by intestinal epithelial cells [Gonçalves et al., 2011d]. Secondary and primary bile acids have tumor promoting effects, but the

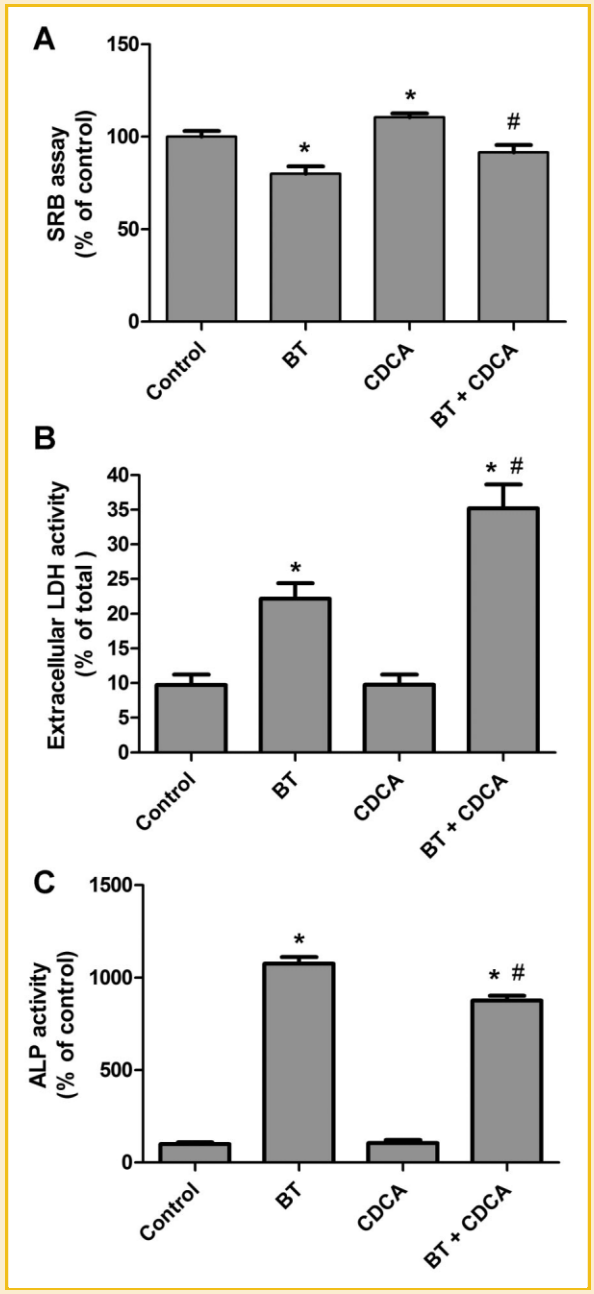

Fig. 8. Effect of a 2-day exposure to BT 5 mM (BT), chenodeoxycholic acid 100 μM (CDCA), or to a combination of both compounds (BT + CDCA) upon IEC-6 cellular proliferation (A), viability (B), and differentiation (C). A: Cellular proliferation was determined by quantification of whole cellular protein with SRB, as described in Materials and Methods Section. Results are shown as absorbance (% of control; n = 12). B: Cellular viability was determined by quantification of extracellular LDH activity, as described in Materials and Methods Section. Results are shown as extracellular LDH activity (% of total LDH activity; n = 17–18). C: Cell differentiation was determined by quantification of alkaline phosphatase (ALP) activity, as described in Materials and Methods Section. Results are shown as nmol *p*-nitrophenol/min/mg protein (% of control; n = 18). Results are presented as arithmetic means + SEM. *Significantly different from control ($P < 0.05$). #Significantly different from BT ($P < 0.05$).

mechanisms involved remain to be fully elucidated. So, the aim of the present study was to investigate the possibility that inhibition of the apical uptake of BT may be one mechanism contributing to the procarcinogenic effect of the primary bile acid CDCA at the intestinal level. We found important to compare the effect of CDCA in a colon adenocarcinoma cell line, the Caco-2 cells [Sambuy et al., 2006] and a non-tumoral intestinal epithelial cell line, the IEC-6 cells [Wood et al., 2003], as comparison between a carcinogenic and a non-carcinogenic cell line seemed interesting in the context of a possible distinct effect of CDCA in these cells. Although not a colonic cell line, the IEC-6 cells proved to be a good cellular model to study colonic BT uptake [Borthakur et al., 2010; Gonçalves et al., 2011b], because they express the BT transporters (MCT1, SMCT1, and BCRP) expressed in the human colon [Borthakur et al., 2010; Gonçalves et al., 2011b,c].

A 2-day exposure to CDCA markedly and concentration-dependently inhibited ^{14}C-BT uptake by IEC-6 cells ($IC_{50} = 120$ μM), mM), and, less potently, by Caco-2 cells ($IC_{50} = 402$ μM). The effect of CDCA in IEC-6 cells (up to 100 μM) and in Caco-2 cells (up to 500 μM) does not appear to be related to changes in cell viability or proliferation. The inhibitory effect of CDCA (100 μM) upon ^{14}C-BT uptake by IEC-6 cells was constant from 1 to 7 days of exposure, and CDCA behaved as a competitive inhibitor of the high-affinity uptake mechanism for ^{14}C-BT.

Absorption of BT from the intestinal lumen involves both the H^+-coupled MCT1 [Cuff et al., 2005] and the Na^+-coupled SMCT1 [Gupta et al., 2006], both of which are functionally present in IEC-6 cells [Borthakur et al., 2010; Gonçalves et al., 2011b]. Interestingly enough, the observation that CDCA reduced both the Na^+-dependent and the Na^+-independent uptake of ^{14}C-BT, and that inhibition of the Na^+-independent uptake of ^{14}C-BT by CDCA disappeared in the presence of MCT1 inhibitors (NPPB and pCMB) clearly show that CDCA inhibits both MCT1- and SMCT1-mediated BT uptake. It is important to note that IEC-6 cells, similarly to normal human colonic epithelium, functionally express both MCT1- and SMCT1-mediated transport, and CDCA was able to inhibit both transport mechanisms. Moreover, the lower inhibitory potency of CDCA in relation to ^{14}C-BT uptake by Caco-2 cells ($IC_{50} = 402$ μM), in which BT uptake is mainly MCT1-mediated [Gonçalves et al., 2009] and the fact that, in IEC-6 cells, CDCA (100 μM) inhibits only ^{14}C-BT uptake mediated by the high-affinity transporter (SMCT1 and MCT1 being high- and low-affinity BT transporters, respectively [Thangaraju et al., 2008]), suggests that the inhibitory effect of CDCA is more pronounced for SMCT1 than for MCT1.

Next, we evaluated the effect of CDCA on the mRNA expression levels of MCT1 and SMCT1 in IEC-6 cells. Contrary to the effect of CDCA upon ^{14}C-BT uptake, treatment of IEC-6 cells for 2 days with CDCA caused a marked (≈80%) increase in the steady-state mRNA levels of SMCT1, and a tendency to an increase in the steady-state mRNA levels of MCT1. Colchicine, an agent well known to affect the membrane trafficking of cytoplasmic proteins, did not change the effect of CDCA upon ^{14}C-BT uptake (results not show), and so CDCA appears to have no effect in the amount of transporters inserted in the cell membrane. We thus conclude that CDCA is acting possibly through decreased SMCT1 and/or MCT1 intrinsic activity, and we speculate that the increase in SMCT1 mRNA level is a compensation

mechanism in response to the marked decrease in SMCT1 activity caused by CDCA.

In relation to the mechanism(s) involved in the inhibitory effect of CDCA upon ^{14}C-BT uptake, several hypothesis can be advanced. These include: (1) changes in membrane structure, as bile acids cause the release of cell membrane components (e.g., proteins and phospholipids) prior to the occurrence of significant cell lysis [Coleman and Holdsworth, 1976]; (2) changes in membrane fluidity, as bile acids increase membrane fluidity [Zhao and Hirst, 1990], and changes of the physical state of plasma membrane lipids can influence the function of membrane carriers [Lundbaek et al., 2010]; (3) mitochondrial damage resulting in cellular ATP depletion, as this mechanism was found to be associated with the strong inhibitory effect of CDCA upon some ion transporters [Maléth et al., 2011]; (4) changes in intracellular phosphorylation/dephosphorylation mechanisms, as the amount/activity of many membrane transporters, including MCT1 [Alrefai et al., 2004; Narumi et al., 2010], is modulated by these mechanisms, and intracellular signaling mechanisms, such as PKC, MAPK, Ca^{2+}, and phosphoinositide-3 kinase have been identified as molecular targets for bile acids [Qiao et al., 2000; McMillan et al., 2003]; and (5) increased production of reactive oxygen species (ROS) and oxidative stress [Song et al., 2007; Ignacio Barrasa et al., 2011].

We decided to clarify the role of intracellular regulatory pathways in the regulation of ^{14}C-BT uptake by CDCA in IEC-6 cells, by examining the effect of inhibitors of various intracellular regulatory pathways, including pathways mediated by CaM, PTK, PKA, PKC, PKG, MAPK ERK1/2, and p38 MAPK. Our study revealed that the inhibitory effect of CDCA upon ^{14}C-BT uptake was partially reversed by inhibition of CaM, PKC, and the MAPK pathways ERK1/2 and p38. This observation suggests that CDCA-induced inhibition of ^{14}C-BT uptake is dependent on CaM, PKC, MAPK ERK1/2, and p38 activation. The stimulatory effect of CDCA upon MAPK is in agreement with previous reports obtained with some other bile acids [McMillan et al., 2003], and PKC is a known molecular target for bile acids [Qiao et al., 2000; McMillan et al., 2003].

We also decided to investigate the role of oxidative stress and ROS in the inhibitory effect of CDCA upon ^{14}C-BT uptake, because we recently demonstrated that oxidative stress decreases BT uptake in IEC-6 cells [Gonçalves et al., unpublished results], and proinflammatory cytokines, which inhibit SMCT1- and MCT1-mediated BT uptake [Thibault et al., 2007; Borthakur et al., 2010], also lead to the production of ROS [Seidelin and Nielsen, 2005; Babbar and Casero, 2006]. Increased production of ROS appears to be involved in the inhibitory effect of CDCA upon BT uptake, as NAC reduced the inhibitory effect of CDCA upon ^{14}C-BT uptake. This observation is of great importance, because oxidative stress is associated with the process of initiation and progression of colon carcinogenesis and inflammatory bowel disease [Seril et al., 2003; Almenier et al., 2012].

The ability of BT to modulate gene expression is often attributed to histone hyperacetylation through inhibition of histone deacetylases (HDACi) [Hamer et al., 2008]. Because these effects are dependent on the intracellular concentration of BT, it is expected that inhibition of BT cellular uptake will change its cellular effects. So, in the last part of this work, we evaluated the effect of BT in conjunction with CDCA upon viability, proliferation, and differentiation of IEC-6 cells.

BT (5 mM; 2 days) caused a significant reduction in IEC-6 cell proliferation and viability and a very marked increase on cell differentiation. This observation is in perfect agreement with our recent report obtained in this same cell line [Gonçalves et al., 2011c], and with the known effect of HDACi such as BT upon these parameters [Hamer et al., 2008]. CDCA (100 μM; 2 days) did not affect cell viability and differentiation, and caused only a discrete increase in IEC-6 proliferation. Interestingly enough, combination of CDCA with BT caused a reduction in the inhibitory effect of BT upon cell proliferation and differentiation. Unexpectedly, CDCA potentiated the effect of BT upon cell viability, a result for which we have at present no explanation.

In summary, we demonstrate that CDCA is an effective inhibitor of ^{14}C-BT uptake in both tumoral (Caco-2) and non-tumoral (IEC-6) intestinal epithelial cells. In IEC-6 cells: (1) CDCA inhibits ^{14}C-BT uptake in a concentration-dependent manner, acting as a competitive inhibitor of the high-affinity transporter of ^{14}C-BT; (2) CDCA decreases both MCT1- and SMCT1-mediated uptake of ^{14}C-BT (although the inhibitory effect upon SMCT1 appears more pronounced); (3) CDCA induces an increase in SMCT1 mRNA steady-state levels; (4) ^{14}C-BT uptake is regulated by CaM, CaMKII, PKC, and PKA; (5) inhibition of ^{14}C-BT uptake by CDCA is dependent on CaM, MAPK ERK1/2, and p38 and PKC activation; and (6) CDCA significantly reduces the effects of BT upon cell proliferation and differentiation.

Given the very important homeostatic role played by BT at the intestinal epithelial level, knowledge on the interaction between an endogenous compound present in high amounts at that level (in cecum, 7–20% of total bile acids are CDCA [Hamilton et al., 2007] and in colon high concentrations are also present) and BT uptake into epithelial cells is very important. Indeed, the results of this study are very interesting in the context of the well-known anticarcinogenic role played by BT in the intestinal epithelium, as they suggest that inhibition of BT uptake into the intestinal epithelium might contribute to the procarcinogenic effect of CDCA at this level.

REFERENCES

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 270:27489–27494.

Almenier HA, Menshawy HH, Maher MM, Gamal SA. 2012. Oxidative stress and inflammatory bowel disease. Front Biosci (Elite Ed) 4:1335–1344.

Alrefai WA, Tyagi S, Gill R, Saksena S, Hadjiagapiou C, Mansour F, Ramaswamy K, Dudeja PK. 2004. Regulation of butyrate uptake in Caco-2 cells by phorbol 12-myristate 13-acetate. Am J Physiol Gastrointest Liver Physiol 286:G197–G203.

Aruoma OI, Halliwell B, Hoey BM, Butler J. 1989. The antioxidant action of N-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Rad Biol Med 6:593–597.

Babbar N, Casero RA, Jr. 2006. Tumor necrosis factor-A increases reactive oxygen species by inducing spermine oxidase in human lung epithelial cells: A potential mechanism for inflammation-induced carcinogenesis. Cancer Res 66:11125–11130.

Bergmeyer HU, Bernt E. 1974. Lactate dehydrogenase. In: Bergmeyer HU, editor. Methods in enzymatic analysis. New York: Academic Press. pp 574–579.

Borthakur A, Anbazhagan AN, Kumar A, Raheja G, Singh V, Ramaswamy K, Dudeja PK. 2010. The probiotic *Lactobacillus plantarum* counteracts TNF-{alpha}-induced downregulation of SMCT1 expression and function. Am J Physiol Gastrointest Liver Physiol 299:G928–G934.

Bradford MM. 1976. A rapid method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Coleman R, Holdsworth G. 1976. The release of membrane components prior to haemolysis during extraction of intact erythrocytes with bile salts. Biochim Biophys Acta 426:776–780.

Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. 1995. 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 364: 229–233.

Cuff MA, Dyer J, Jones M, Shirazi-Beechey SP. 2005. The human colonic monocarboxylate transporter isoform 1: Its potential importance to colonic tissue homeostasis. Gastroenterology 128:676–686.

Gonçalves P, Araújo JR, Pinho MJ, Martel F. 2009. Modulation of butyrate transport in Caco-2 cells. Naunyn Schmiedebergs Arch Pharmacol 379:325–336.

Gonçalves P, Araújo JR, Pinho MJ, Martel F. 2011a. In vitro studies on the inhibition of colon cancer by butyrate and polyphenolic compounds. Nutr Cancer 63:282–294.

Gonçalves P, Araújo JR, Martel F. 2011b. Characterization of butyrate uptake by nontransformed intestinal epithelial cell lines. J Membr Biol 240:35–46.

Gonçalves P, Gregório I, Martel F. 2011c. The short-chain fatty acid butyrate is a substrate of breast cancer resistance protein. Am J Physiol Cell Physiol 301:C984–C999.

Gonçalves P, Araújo JR, Martel F. 2011d. Effect of polyunsaturated fatty acids and bile salts on butyrate uptake by intestinal epithelial cells. In: Schulz MA, editor. Caco-2 cells and their uses. New York: Nova Science Publishers Inc. pp 89–99.

Gupta N, Martin PM, Prasad PD, Ganapathy V. 2006. SLC5A8 (SMCT1)-mediated transport of butyrate forms the basis for the tumor suppressive function of the transporter. Life Sci 78:2419–2425.

Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. 2008. Review article: The role of butyrate on colonic function. Aliment Pharmacol Ther 27:104–119.

Hamilton JP, Xie G, Raufman JP, Hogan S, Griffin TL, Packard CA, Chatfield DA, Hagey LR, Steinbach JH, Hofmann AF. 2007. Human cecal bile acids: Concentration and spectrum. Am J Physiol Gastrointest Liver Physiol 293: G256–G263.

Hidaka H, Inagaki M, Kawamoto S, Sasaki Y. 1984. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23:5036–5041.

Ignacio Barrasa J, Olmo N, Pérez-Ramos P, Santiago-Gómez A, Lecona E, Turnay J, Antonia Lizarbe M. 2011. Deoxycholic and chenodeoxycholic bile acids induce apoptosis via oxidative stress in human colon adenocarcinoma cells. Apoptosis 16:1054–1067.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. 2011. Global cancer statistics. CA Cancer J Clin 61:69–90.

Lundbaek JA, Collingwood SA, Ingólfsson HI, Kapoor R, Andersen OS. 2010. Lipid bilayer regulation of membrane protein function: Gramicidin channels as molecular force probes. R Soc Interface 7:373–395.

Mahmoud NN, Dannenberg AJ, Bilinski RT, Mestre JR, Chadburn A, Churchill M, Martucci C, Bertagnolli MM. 1999. Administration of an unconjugated bile acid increases duodenal tumors in a murine model of familial adenomatous polyposis. Carcinogenesis 20:299–303.

Maléth J, Venglovecz V, Rázga Z, Tiszlavicz L, Rakonczay Z, Jr., Hegyi P. 2011. Non-conjugated chenodeoxycholate induces severe mitochondrial damage and inhibits bicarbonate transport in pancreatic duct cells. Gut 60:136–138.

Martínez ME, Marshall JR, Giovannucci E. 2008. Diet and cancer prevention: The roles of observation and experimentation. Nat Rev Cancer 8:694–703.

McMillan L, Butcher SK, Pongracz J, Lord JM. 2003. Opposing effects of butyrate and bile acids on apoptosis of human colon adenoma cells: Differential activation of PKC and MAP kinases. Br J Cancer 88:748–753.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63.

Muzyka A, Tarkany O, Yelizanof V. 2005. Non-linear regression (curve fit). GraphPad Prism for Windows. San Diego, CA: GraphPad Software.

Narumi K, Furugen A, Kobayashi M, Otake S, Itagaki S, Iseki K. 2010. Regulation of monocarboxylate transporter 1 in skeletal muscle cells by intracellular signaling pathways. Biol Pharm Bull 33:1568–1573.

Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, Gerritsen ME. 1997. Novel inhibitors of cytokine-induced I kappa B alpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. J Biol Chem 272:21096–21103.

Qiao D, Chen W, Stratagoules ED, Martinez JD. 2000. Bile acid-induced activation of activator protein-1 requires both extracellular signal-regulated kinase and protein kinase C signaling. J Biol Chem 275:15090–15098.

Reddy BS, Watanabe K, Weissburger JH, Wynder EL. 1977. Promoting effect of bile acids in colon carcinogenesis in germ-free and conventional F344 rats. Cancer Res 37:3238–3242.

Reddy BS, Hedges AR, Laakso K, Wynder EL. 1978. Metabolic epidemiology of large bowel cancer: Fecal bulk and constituents of high-risk North American and low-risk Finnish population. Cancer 42:2832–2838.

Ridlon JM, Kang DJ, Hylemon PB. 2006. Bile salt biotransformations by human intestinal bacteria. J Lipid Res 47:241–259.

Said HM, Ortiz A, Kumar CK, Chatterjee N, Dudeja PK, Rubin S. 1999. Transport of thiamine in human intestine: Mechanism and regulation in intestinal epithelial cell model Caco-2. Am J Physiol 277:C645–C651.

Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F. 2006. The Caco-2 cell line as a model of the intestinal barrier: Influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol 21:1–26.

Seidelin JB, Nielsen OH. 2005. Continuous cytokine exposure of colonic epithelial cells induces DNA damage. Eur J Gastroenterol Hepatol 17:363–369.

Seril DN, Liao J, Yang GY, Yang CS. 2003. Oxidative stress and ulcerative colitis-associated carcinogenesis: Studies in humans and animal models. Carcinogenesis 24:353–362.

Silva E, Pinto V, Simão S, Serrão MP, Afonso J, Amaral J, Pinho MJ, Gomes P, Soares-da-Silva P. 2010. Renal aging in WKY rats: Changes in Na+, K+-ATPase function and oxidative stress. Exp Gerontol 45:977–983.

Song S, Guha S, Liu K, Buttar NS, Bresalier RS. 2007. COX-2 induction by unconjugated bile acids involves reactive oxygen species-mediated signalling pathways in Barrett's oesophagus and oesophageal adenocarcinoma. Gut 56:1512–1521.

Stadler J, Stern HS, Yeung KS, McGuire V, Furrer R, Marcon N, Bruce WR. 1988. Effect of high fat consumption on cell proliferation activity of colorectal mucosa and on soluble faecal bile acids. Gut 29:1326–1331.

Thangaraju M, Cresci G, Itagaki S, Mellinger J, Browning DD, Berger FG, Prasad PD, Ganapathy V. 2008. Sodium-coupled transport of the short chain fatty acid butyrate by SLC5A8 and its relevance to colon cancer. J Gastrointest Surg 12:1773–1781.

Thibault R, De Coppet P, Daly K, Bourreille A, Cuff M, Bonnet C, Mosnier JF, Galmiche JP, Shirazi-Beechey S, Segain JP. 2007. Down-regulation of the

monocarboxylate transporter 1 is involved in butyrate deficiency during intestinal inflammation. Gastroenterology 133:1916–1927.

Tong JL, Ran ZH, Shen J, Fan GQ, Xiao SD. 2008. Association between fecal bile acids and colorectal cancer: A meta-analysis of observational studies. Yonsei Med J 49:792–803.

Winocour PD, Kinlough-Rathbone RL, Mustard JF. 1981. The effect of phospholipase inhibitor mepacrine on platelet aggregation, the platelet release reaction and fibrinogen binding to the platelet surface. Thromb Haemost 45:257–262.

Wood SR, Zhao Q, Smith LH, Daniels CK. 2003. Altered morphology in cultured rat intestinal epithelial IEC-6 cells is associated with alkaline phosphatase expression. Tissue Cell 35:47–58.

Zhao DL, Hirst BH. 1990. Comparison of bile salt perturbation of duodenal and jejunal isolated brush-border membranes. Digestion 47:200–207.