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ORIGINAL CONTRIBUTION

J. Stein
M. Zores
O. Schröder

Short-chain fatty acid (SCFA) uptake into Caco–2 cells by a pH-dependent and carrier mediated transport mechanism

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dedicated to W. F. Caspary on the occasion of his 60th birthday

Professor J. Stein, MD, PhD (✉)
M. Zores · O. Schröder
Division of Gastroenterology
2nd Department of Internal Medicine
Johann Wolfgang Goethe-University
Theodor-Stern-Kai 7
D-60590 Frankfurt, Germany
e-mail: J.Stein@em.uni-frankfurt.de

Summary The short-chain fatty acids, acetate, propionate, and butyrate, are the most abundant organic anions in the human colon. SCFA play a pivotal role in maintaining homeostasis in the colon. Particularly butyrate induces cell differentiation and regulates growth and proliferation of colonic mucosal epithelial cells, whereas it reduces the growth rate of colorectal cancer cell. Previous studies by several groups, including our own, using isolated membrane vesicles have demonstrated that the uptake of butyrate is at least in part mediated by a non-electrogenic $SCFA^-/HCO_3^-$ antiporter. The purpose of the present study was to determine (1) whether Caco–2 cells could serve as an experimental model to assess the mechanisms of SCFA transport, and (2) whether monocarboxlate transporters could play a role in SCFA transport in these cells. Caco–2 cells were found to transport ^{14}C-butyrate in a concentration and time dependent manner. The uptake was sodium independent, but was stimulated by lowering extracellular pH. The uptake of 500μM butyrate was reduced by 49.6 % ± 3.3 % in the presence of propionate and by 57.2 % ± 4.8 % in the presence of 10 mM L-lactate. The addition of 1 mM α-cyano–4-hydroxycinnamate and phloretin, both known to be potent inhibitors of MCT1, decreased the uptake of 500 μM ^{14}C-butyrate by 59.4 % ± 4.1 % and 48.9 % ± 3.3 %, respectively, whereas similar concentrations of DIDS did not have any effect. These data suggest that the uptake of butyrate in Caco–2 cells occurs via a carrier mediated transport system specific for monocarboxylic acids, which is in accordance with characteristics of the MCT 1.

Key words Butyrate – Caco–2 cells – monocarboxylate transporter – carrier mediated transport

Introduction

Short-chain fatty acids (acetate, propionate, and butyrate) are main end-products of anaerobic bacterial carbohydrate fermentation in the human colon. SCFA play a pivotal role in maintaining homeostasis in the colon. SCFA, particularly butyrate, represent a significant physiological energy source for the colon (1–3); butyrate induces cell differentiation and regulates growth and proliferation of colonic mucosal epithelial cells (1, 4), whereas it reduces the growth rate of colorectal cancer cells (5, 6).

Although the mechanisms of mammalian colonic SCFA absorption are still debated, previous studies by several groups (including our own) using isolated membrane vesicles have demonstrated that the uptake is at least in part mediated via a non-electrogenic $SCFA^-/HCO_3^-$ antiporter (7–9). This anion transport system is pH-dependent, differs from other known (DIDS-sensitive) anion exchange processes, and does not exhibit any involvement of an apical Na^+/H^+-exchange activity (7, 9).

However, the use of apical membrane vesicles to study intestinal transport might not adequately reflect the intesti-

nal absorption process. Contributions of various driving forces such as pH, inorganic ions, and membrane potential in energizing the carrier functions under physiological conditions are difficult to assess by in vitro membrane vesicle studies. The human colonic adenocarcinoma cell line, Caco–2, which forms monolayers of viable and polarized intestinale epithelial cells, mimics the physiological functions found in small intestinal absorptive cells (10). This cell line has several advantages for studies of intestinal absorption because it forms well-differentiated epithelial cells and exhibits several functional properties, such as carrier-mediated transport systems, receptors, and apical membrane enzyme activities. The purpose of the present study was to determine (1) whether Caco–2 cells could serve as an experimental model to assess the mechanisms of SCFA transport, and (2) whether monocarboxlate transporters could play a role in SCFA transport in these cells.

Materials and methods

Materials

The Caco–2 cell line was purchased from the American Type Culture Collection (ATCC) at *passage 40*, and confirmed to be mycoplasma negative using the Hoechst 33258 test. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and nonessential amino acids (NEAA) were obtained from GIBCO (Grand Island, NY). The polycarbonate membrane and Transwell clusters, 11.2-mm diameter and 3.0-µm pore size, were purchased from Costar (Bredford, MA). Unlabeled monocarboxylic acids (propionate, butyrate, benzoate, lactate), DIDS (4,4´-diisothiocyanatostilbene–2,2´-disulfonic acid), SITS (4,4´-dinitrostilbene–2,2´-disulfonic acid), and 4-CHC (2-cyano–4-hydroxycinnamate), Phloretin, and PMSF (phenylmethansulfonylfluoride) were obtained from Sigma (Deisenhofen, Germany), DPH from Molecular Probes Inc. (Eugene, OR). (^{14}C)-butyrate (0.56 GBq/mmol) and (^{3}H)-mannitol (1110 GBq/mmol) were purchased from Amersham (Braunschweig, Germany). The radiopurity of the labeled butyrate was found to be > 95 % using HPLC (12). All other reagents were of analytical grade.

Cell Culture

Caco–2 cells were grown routinely in 75-cm^2 plastic T-flasks at 37°C in a 5 % CO_2/95 % air atmosphere. The culture medium consisted of DMEM, supplemented with 10 % fetal calf serum (FCS), 1 % NEAA, 2 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells reached confluence after 5–7 days in culture. All cells used in this study were between passage 42 and 50. Cells were passaged using Dulbecco's PBS containing 0.25 % trypsin and 1 % EDTA and seeded on 6-well plates at a density of 2 × 105/cm^2. For uptake studies, Caco–2 cells were seeded at density of 1 × 105 cells/cm^2 on a polycarbonate membrane. Cells were cultured for 5 days, and the culture medium was replaced on alternate days. Cells were grown for 21–23 days for the transport experiment. Monolayers were used at a minimum of 21 days after seeding, by which time transepithelial electrical resistance (TEER) was stable at about 200–250 Ω ×cm2 and the (^{3}H)-mannitol leakage was usually less than 2 % of the dose/hr/well

Transport studies

Caco–2 cells were washed with HBSS containing 25 mM D-Glucose and 10 mM Hepes (pH 7.3) or Mes (pH 6.5, 6.0, 5.5). Test solution (0.25 mL) was added to initiate uptake. The cells were incubated at 37°C for time indicated. To determine uptake, the test solution was removed by suction and the cells were washed three times with ice-cold HBSS. For quantification of absorbed butyrate cells were solubilized with 0.5 mL Solvalable (NEN) and the radioactivity was measured using a standard liquid scintillation technique. Cellular protein was measured by the method of Bradford et al. (16) using bovine serum albumin as a standard. After correction for medium radioactivity extracellularly bound to the cells, absolute uptake was calculated and expressed in picomoles per milligram of protein. Details of the conditions for each experiment are given in the figure legends or table footnotes.

Metabolism measurements

Metabolism of (^{14}C)-butyrate acid by Caco–2 cells was assessed by HPLC and on column radioactivity measurement. The HPLC system used was as follows (11): analytical column Aminex 87H 7.8 x 300 mm (Biorad, München); mobile phase 0. 02 mM sulphuric acid, pH 2.0; flow rate 0.7 mL/min; radioactivity detector (LB 501 C–1, Berthold, Germany).

Data analysis

To estimate kinetic parameters of saturable transport into the Caco–2 monolayers, transport rate (J) was fitted to the following equation, consisting of both saturable and non-saturable linear terms, using the nonlinear least-squares regression:

$$J = J_{max} \times S/(K_t + k_d \, x \, S)$$

where J_{max} is the maximum transport rate for the carrier-mediated process, S is the concentration of substrate, K_t is the half-saturation concentration (Michaelis constant), and k_d is the first order rate constant. All values are expressed

as means ± SE Statistical analysis was done by the nonparametric U-Test Mann-Whitney-Wilcoxon for unpaired data. Differences were considered significant at $p < 0.05$.

Results

Time course and pH dependence

Figure 1 shows the effect of pH on cellular uptake of (^{14}C)-butyrate from the apical side of a Caco–2 cell monolayer. The pH on the apical side was changed from 5.0 to 7.3, while the basolateral pH was set at pH 7.3, which is close to the intracellular pH of Caco–2 cells (12). The absorbed amount of (^{14}C)-butyrate increased linearly with time, as shown in the inset to Fig. 1. HPLC analysis indicated that the absorbed (^{14}C)-butyrate was not significantly metabolized within 10 min (data not shown).

The absorption coefficient of (^{14}C)-butyrate increased markedly with decreasing pH on the apical side to an acidic from neutral pH. At pH lower than 5.5, no further increase was observed. In the presence of 10 mM unlabeled butyric acid, cellular uptake of (^{14}C)-butyrate was significantly reduced. The extent of this inhibitory effect increased with lowering of the pH on the apical side, suggesting that a carrier-mediated mechanism partly contributes to the transport. Since (^{3}H)-mannitol transport was constant at various pH (data not shown), pH-dependent transport of (^{14}C)-butyrate cannot be ascribed to a nonspecific effect of pH change, such a membrane perturbation.

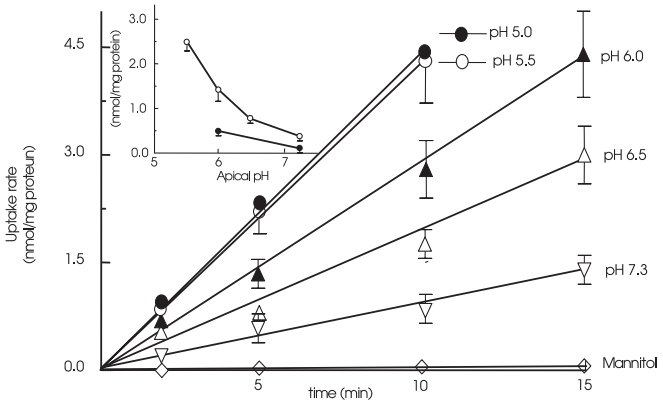

Fig. 1 Time course and pH dependence of [^{14}C]-butyrate uptake
Uptake by Caco–2 cells was measured at 5 min by incubating Caco–2 monolayers in HBSS at an apical side pH of 5.0, 5.5, 6.0, 6.5 or 7.3 and at basolateral-side pH of 7.3 in the presence (●) or absence (○) of unlabeled butyrate (10 mM). Each point represents the mean ±SE of six experiments.

Concentration dependence

Figure 2 represents the relationship between the initial uptake rate of (^{14}C)-butyrate and its concentration in the apical medium from 10 µM to 10 mM. The results indicate that the absorption of (^{14}C)-butyrate consists of two components, a saturable process evident at lower concentrations and an apparently nonsaturable process observed at higher concentrations. They are analyzed according to Eq. (1) as described under Materials and Methods. The evaluated kinetic parameters V_{max} and K_m were 89±7.82 mmol/mg protein/5 min and 2.4±0.32 mmol/mg protein/5 min, respectively. Furthermore, cellular uptake of (14C)-butyrate acid was remarkably temperature dependent.

Effect of potential inhibitors on butyrate transport

Effects of anion exchange process inhibitors on butyrate uptake. Many anion exchange process are inhibited by the classical anion exchange inhibitors, 4,4´-diisothiocyanatostilbene–2,2´-disulfonic acid (DIDS) and 4,4´-dinitrostilbene–2,2´-disulfonic acid SITS. In contrast to previous studies in rat distal colon (7–9), incubation of Caco–2 cells with DIDS and SITS (0.1 and 1 mmol/l) did not reduce the cellular uptake of butyrate in Caco–2 cells (Table1).

Furthermore, known reversible potent inhibitors of the MCT, phloretin and 2-cyano–4-hydroxycinnamate, were tested for their effect on butyrate uptake into apical membrane vesicles of the rat distal colon. As demonstrated in Table 1, butyrate uptake was inhibited both in the presence of 4-CHC and phloretin.

Structural specificity of the transporter. To examine the properties of the transporter responsible for butyrate acid uptake into Caco–2 cells, the effects of various other monocarboxylic acids were studied (Table 1). Many monocar-

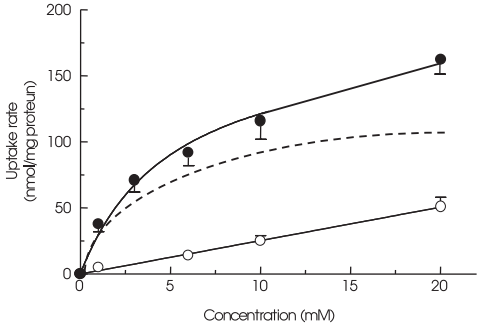

Fig. 2 Concentration and temperature dependence of [^{14}C]-butyrate uptake
Uptake by Caco–2 cells was measured at 5 min during incubation at 37°C (●) or 4 °C (○) and pH 6.0. Incubation conditions were identical to those described in Fig. 1. The dashed line represents the uptake rate for the saturable component calculated from the kinetic parameters obtained as mentioned under Results. The dotted line represents saturable uptake. Each point represents the mean ± SE of six experiments.

Table 1: Inhibitory effect of various compounds on [^{14}C]-Butyrate transport[a]

Inhibitor	Concentration (mM)	Relative permeability (% of control)[b]
Acetic acid	10	51.8 ± 4.3*
Propionic acid	10	49.6 ± 3.3*
Butyric acid	10	46.8± 4.2*
Valeric acid	10	68.7 ± 7.3*
Nicotinic acid	10	59.2 ± 6.3*
Benzoic acid	10	53.9 ± 4.1*
D-Lactic acid	10	105.2 ± 11.3
L-Lactic acid	10	57.2 ± 4.8*
DIDS	0.1	96.2 ± 9.3
	1.0	94.8 ± 10.2
4-CHC	0.1	73.8 ± 6.3
	1.0	59.4 ± 4.1*
Phloretin	0.1	63.3 ± 4.3*
	1.0	48.9 ± 3.3*

[a] The amount absorbed [^{14}C]-butyrate was measured at 37°C for 10 min by incubating Caco–2 cells (apical pH, 6.0; basolateral pH, 7.3) in the presence of each inhibitor; [b] Each value represents the mean of six experiments; * Significantly different from the control value by Student's *t* test ($p < 0.05$)

boxylic acids significantly reduced the uptake of (^{14}C)-butyrate acid. In contrast, dicarboxylic acids (oxalic acid, phthalic acid) and amino acids had no effect (data not shown). Moreover, monocarboxylic acid drugs such as benzoic acid and nicotinic acid also appear to share this transport system. Interestingly, our data demonstrated stereoselectivity for lactate, since the D-isomer had no influence on butyrate uptake.

Discussion

Among the SCFA, butyrate is of special interest because of its role as the main energy source for the colonocyte (1, 2). Butyrate promotes growth and proliferation of normal colonic mucosa while suppressing the growth of cancer cells (5, 6). The exact characterization of the transport pathway for butyrate in the colon helps elucidate the mechanism by which butyrate interacts with the colonic mucosa in health and disease. The mechanisms involved in the colonic transport of SCFA have been merely controversial. Early work suggested the presence of non-ionic diffusion as a major transport mechanism for SCFA in the colon. Such fluxes have been supported as the mechanism by which SCFA stimulate apical Na^+/H^+-cotransport to promote electroneutral sodium absorption (19, 20). However, due to their pK_a at the prevailing pH values of the colon, at least 90–95 % of the most abundant SCFA exist in their ionized form (18). Despite this relative abundance of the anionic form, it is nevertheless difficult to predict whether the transmembrane fluxes of any molecular form will predominate, since nonionic diffusion has been shown to be extremely rapid (21). On the other hand, a number of groups, including our own, has identified the presence of electroneutral $SCFA^-/Cl^-$ and/or $SCFA^-/HCO_3^-$ anion exchange (7,9,22, 23).

The present study establishes the existence of a carrier-mediated transport system for butyrate in Caco–2 cells. In agreement with several previous studies in isolated membrane vesicles, butyrate transport into Caco–2 cells was inhibited by structural analogues such as acetate and or propionate, but not by formate and heptanoate (data not shown) uptake, thus indicating that the butyrate-bicarbonate exchanger is specific for SCFA.

Butyrate transport was not inhibited by stilbene derivatives, DIDS and SITS, at a concentration up to 1 mmol/l indicating a stilbene-insensitive anion exchanger. Recently published data using apical membrane vesicles of the human and pig colon (25) additionally demonstrated structural difference to the erythrocyte Cl^-/HCO_3^- exchange protein, band–3, since an antibody to the band–3 protein did not cross-react with any protein in the colonic apical membrane.

Recently, the successful cloning of specific monocarboxylate transporter (MCT) in several mammalian tissues, including the rat intestine, was reported (13, 14). At least for the two isoforms MCT 1 and MCT 2 this transport process resembles a carrier-mediated transport system by an obligatory co-transport of a proton together with the organic anion a broad specificity for short-chain monocarboxylates, including lactate, with high affinity for those with 2-keto groups and stereoselectivity for the L-isomer of lactate. Other characteristics are its sensitivity to relatively specific inhibitors, such as α-cyano-hydroxycinnamate, as well as to less specific inhibitors such as stilbene derivatives and phloretin (14, 15).

In accordance with a number of studies demonstrating the steroselectivity of L-lactate over D-lactate as substrate for transport through apical plasma membranes in a number of tissues by MCT 1 and MCT 2 (14, 15, 24), the butyrate transport system in Caco–2 cells also possesses stereoselectivity for L- over D-lactate.

Derivatives of the α-cyano-cinnamate like 4-CHC are the most potent aromatic monocarboxylate inhibitors. Also the amphiphilic compound phloretin is known to be a potent inhibitor not only of the monocarboxylate transporter but also to the band-3 and other membrane transport processes. There is little information concerning the effect of such compounds on potential effects on the colonic butyrate transport. Ritzhaupt *et al.* (25) found that phloretin but not 4-CHC led to a significant inhibition of butyrate uptake in apical membrane vesicles of the human and pig colon. They concluded that the butyrate transporter may belong to the family of the monocarboxylate transporter proteins. In our studies, both phloretin and 4-CHC demon-

strated a significant inhibitory effect on butyrate uptake in Caco–2 cells.

In conclusion, our results suggest that the transport of butyrate into Caco–2 cells is mediated by a pH-dependent, a carrier-mediated transport system. By demonstrating some functional similarities with isoforms of the monocarboxylate transporter, it might be concluded that the butyrate exchanger belongs to the mammalian MCT isoforms.

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