Lactobacillus acidophilus counteracts enteropathogenic E. coli-induced inhibition of butyrate uptake in intestinal epithelial cells

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Kumar A, Alrefai WA, Borthakur A, Dudeja PK. Lactobacillus acidophilus counteracts enteropathogenic E. coli-induced inhibition of butyrate uptake in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 309: G602–G607, 2015. First published August 13, 2015; doi:10.1152/ajpgi.00186.2015.—Butyrate, a key short-chain fatty acid metabolite of colonic luminal bacterial action on dietary fiber, serves as a primary fuel for the colonocytes, ameliorates mucosal inflammation, and stimulates NaCl absorption. Absorption of butyrate into the colonocytes is essential for these intracellular effects. Monocarboxylate transporter 1 (MCT1) plays a major role in colonic luminal butyrate absorption. Previous studies (Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. Adv Immunol 121: 91–119, 2014.) showed decreased MCT1 expression and function in intestinal inflammation. We have previously shown (Borthakur A, Gill RK, Hodges K, Ramaswamy K, Hecht G, Dudeja PK. Am J Physiol Gastrointest Liver Physiol 290: G30–G35, 2006,) impaired butyrate absorption in human intestinal epithelial Caco-2 cells due to decreased MCT1 level at the apical cell surface following enteropathogenic E. coli (EPEC) infection. Current studies, therefore, examined the potential role of probiotic Lactobacilli in stimulating MCT1-mediated butyrate uptake and counteracting EPEC inhibition of MCT1 function. Of the five species of Lactobacilli, short-term (3 h) treatment with L. acidophilus (LA) significantly increased MCT1-mediated butyrate uptake in Caco-2 cells. Heat-killed LA was ineffective, whereas the conditioned culture supernatant of LA (LA-CS) was equally effective in stimulating MCT1 function, indicating that the effects are mediated by LA-secreted soluble factor(s). Furthermore, LA-CS increased apical membrane levels of MCT1 protein via decreasing its basal endocytosis, suggesting that LA-CS stimulation of butyrate uptake could be secondary to increased levels of MCT1 on the apical cell surface. LA-CS also attenuated EPEC inhibition of butyrate uptake and EPEC-mediated endocytosis of MCT1. Our studies highlight distinct role of specific LA-secreted molecules in modulating colonic butyrate absorption.

SCFA; Caco-2; probiotics; pCMBS; endocytosis

THE SHORT-CHAIN FATTY ACIDS (SCFA) are produced by bacterial fermentation of dietary fiber and are efficiently absorbed by the colonic epithelial cells. Butyrate, a key SCFA, serves as the major and preferred metabolic substrate for colonocytes, providing 60–70% of energy requirements necessary for their proliferation and differentiation (13, 40). As such, colonocytes of germ-free mice, deficient in SCFAs, are highly energy deprived, as indicated by decreased expression of key mitochondrial enzymes involved in energy metabolism (14). Butyrate also promotes colonic mucosal integrity (40), inhibits inflammatory pathways (43), and induces NaCl absorption (1), hence eliciting anti diarrheal effects. Besides, interest in SCFAs has recently been rekindled for their emerging roles in the regulation of metabolism (14, 24), barrier function (31), autophagy (14), and immune responses (25, 30).

Earlier reports in ulcerative colitis (UC) patients suggested that a major consequence of reduction in intracellular SCFA oxidation results in metabolic starvation and mucosal atrophy. In fact, UC has been suggested to be a local starving disease in the colon, associated with a decrease in nutrient availability (16, 23). Butyrate oxidation deficiency was mainly observed in the inflamed mucosa of patients with active disease, and not with quiescent UC, suggesting that impaired butyrate oxidation is not a primary defect, but is rather a consequence of defect in luminal butyrate absorption (41, 42).

SCFA absorption may involve passive diffusion of the undissociated form and specific carrier-mediated transport of SCFA anions (1, 17, 40). Previous studies from our laboratory and others suggested monocarboxylate transporter isofrom 1 (MCT1) to play a key role in mediating H+–coupled absorption of human colonic luminal butyrate (22, 33, 34). Various studies have also shown differential regulation of MCT1 to impact colonic epithelial health. In fact, downregulation of MCT1 expression and/or activity has been reported in mucosal inflammation (41, 42), colon cancer (12, 19), and in response to infection by enteropathogenic E. coli (4), an important human enteric pathogen causing infantile diarrhea. On the other hand, various studies showed upregulation of MCT1 expression and/or activity in response to various agents, such as luminal leptin (9), somatostatin (36), butyrate (7, 11), and via nutrient-sensing mechanisms (6). In this report, we have demonstrated upregulation of MCT1 activity by the bioactive soluble factors secreted by the probiotic Lactobacillus acidophilus (LA) via increasing the levels of the transporter at the apical cell surface. These factors also alleviated enteropathogenic E. coli-induced inhibition of MCT1 activity.

MATERIALS AND METHODS

Materials. Caco-2 cells and probiotic Lactobacilli species were obtained from American Type Culture Collection (ATCC; Manassas, VA). 14C-butyrate was obtained from American Radiochemicals, p-chloromercuri-benzene sulphonate (pCMBS) was purchased from Sigma-Aldrich (St. Louis, MO); sulfo-NHS-SS-biotin was obtained from Thermo Scientific (Rockford, IL).

Cell culture. Caco-2 cells were grown at 37°C in an atmosphere of 5% CO2. Cells were maintained in DMEM with 4.5 g/l glucose, 50 kU/l penicillin, 5 mg/l streptomycin, 2 mg/l gentamycin, and 20% fetal bovine serum. Butyrate uptake studies were performed using fully differentiated cells grown for 12–14 day postplating on 24-well plastic supports or on 0.4 μM polycarbonate membrane filters in 12-mm inserts.
Bacterial culture and preparation of conditioned culture supernatant. The following Lactobacillus species, with ATCC strain numbers given in parentheses, were grown in Mann-Rogosa-Sharpe broth (Difco Laboratories, Detroit, MI) for 24 h at 37°C without shaking: LA (4357), Lactobacillus rhamnosus (53103), L. plantarum (LP) (14917), L. casei (LC) (393), and L. reuteri (23272). The cultures were then centrifuged at 3,000 g × 10 min at 4°C. The supernatant, filtered through a 0.22-µm filter (Millex, Millipore, Billerica, MA) to sterilize and remove all bacterial cells, was designated as conditioned culture supernatant (CS). For treating the cell monolayers, the bacterial pellet was washed with DMEM/F-12 media (Invitrogen, Carlsbad, CA) containing 5 mg/l mannose and resuspended in the same medium. Heat-killed bacteria were prepared by resuspending pellets and heating to 95°C for 20 min.

Enteropathogenic E. coli culture and infection of cells. The enteropathogenic E. coli (EPEC) strain used in this study was wild-type EPEC strain E2348/69 (generously provided by Dr. Gail Hecht of the Department of Medicine, Loyola University, Maywood, IL). Strains were grown overnight in Millelva Luria Broth media. On the day of experiment, an aliquot of the overnight culture was inoculated in an appropriate volume of serum and antibiotic-free medium supplemented with 0.5% mannose. Bacteria were grown to midlog phase (optical density at 600 nm = 0.4). The culture was spun down and resuspended in the same volume of fresh media. Cell monolayers were then infected at a multiplicity of infection of 1:100. After infection for the desired time, media were removed, and cell monolayers were washed with PBS.

Measurement of 14C-butyrate uptake. Apical uptake of 14C-butyrate was measured as described previously (6), in the presence or absence of pCMBS, a specific inhibitor of MCT1. Uptake values were calculated as nanomoles of 14C-butyrate per milligram protein per 5 min.

Cell surface biotinylation and immunoblotting. Cell surface biotinylation was performed using sulfo-NHS-SS-biotin (Thermo Scientific, Rockford, IL) (0.5 g/l) in borate buffer (in mM: 154 NaCl, 7.2 KCl, 1.8 CaCl2, 10 H3BO3, pH 9.0), as previously described (18). Labeling was allowed to proceed for 60 min at 4°C to prevent endocytosis and internalization of antigens. After immunoprecipitation of biotinylated antigens with neutravidin agarose, biotinylated proteins were released by boiling in Laemmli buffer containing proteinase K (200 µg/ml). The samples were separated by SDS-PAGE and transferred to PVDF membrane. Antibodies to MCT1 antibody (Santa Cruz) were used for immunoblotting and Western analysis.

Endocytic internalization assay by reversible cell-surface biotinylation. To measure the extent of endocytosis, Caco-2 cells were plated at a density of 1 × 105 cells on six-well plates. Cell surface was labeled with sulfo-NHS-SS-biotin (1.5 mg/ml; Pierce, Rockford, IL) in borate buffer (in mM: 154 NaCl, 7.2 KCl, 1.8 CaCl2, 10 H3BO3, pH 9.0) at 4°C for 60 min. Following surface biotinylation, cells were incubated with the CS of LA (LA-CS) at 37°C for 3 h, followed by 30 min incubation with or without EPEC. Immediately after treatment, cells were rinsed with ice-cold 1× PBS twice at 4°C. Surface biotin was cleaved by 150 mM GSH in 1× PBS, and the biotinylated freshly washed cells were treated with various species (overnight cultures diluted to 600-nm absorbance = 0.2) for 3 h. Cont, control; LR, L. rhamnosus; LP, L. plantarum; LT, L. reuteri. MCT1 function [p-chloromercuri-benzene sulfonate (pCMBS)-sensitive 14C-butyrate uptake] was determined and calculated as mmol butyrate-mg protein−1·5 min−1. Values are means ± SE; n = 5. *Different from control, P ≤ 0.05.

RESULTS

LA and LC stimulate MCT1-mediated butyrate uptake. In the initial set of studies, we used five species of Lactobacilli, as outlined in MATERIALS AND METHODS, to examine their short-term treatment effects on MCT1-mediated butyrate uptake in post-confluent Caco-2 cell monolayers. Of the five species, LA and LC significantly stimulated pCMBS-sensitive butyrate uptake, whereas L. rhamnosus, LP, and L. reuteri had no effects (Fig. 1). Since LA showed maximal stimulatory effects, all subsequent studies were performed with this species. A time course experiment showed that LA did not affect pCMBS-sensitive butyrate uptake at 1 h, but by 2 h, butyrate uptake increased essentially to the level at 3 h (data not shown). A 3-h time point was used to treat cells in subsequent studies. We also compared the effects of LA added apically to Caco-2 monolayers grown on 0.4-µm polycarbonate membrane filters in 12-mm transwell inserts and obtained similar effects results on pCMBS-sensitive butyrate uptake (data not shown).

CS of LA, but not heat-killed bacteria, mimics the effects of live bacteria on pCMBS-sensitive butyrate uptake. We next examined whether the effects of LA on butyrate uptake required live organisms. Unlike live LA, however, the heat-killed bacteria did not stimulate apical butyrate uptake in Caco-2 cells (Fig. 2). Various earlier studies have shown that bacteria-free CS of certain probiotic bacteria could show beneficial effects similar to live bacteria on host intestinal epithelial cells (3, 44, 45). We, therefore, tested the effects of LA-CS prepared as described in MATERIALS AND METHODS, diluted 1:10 in DMEM/F-12 and pH adjusted to 7.4, on apical butyrate uptake. LA-CS stimulated butyrate uptake to the same extent, compared with control, as that of live bacteria (Fig. 2). Therefore, we used LA-CS instead of live LA for our subsequent studies. We have also determined LA-CS effects on MCT1 function in T-84 cells, another human intestinal epithelial cell line, and observed significant increase in pCMBS-sensitive 14C-butyrate uptake following LA-CS treatments for 3 h (in mmol butyrate-mg protein−1·5 min−1; control, 1.62 ± 0.08 vs. LA-CS, 2.90 ± 0.26; P ≤ 0.05). These results suggest that LA-CS effects in stimulating MCT1-mediated butyrate uptake are not cell line specific.
LA-CS increases $V_{\text{max}}$ of MCT1-mediated butyrate uptake and causes a decrease in $K_m$. To examine the mechanisms of butyrate enhancement of MCT1 function, we performed kinetic analysis of LA-CS effects on butyrate uptake. Effects of LA-CS on pCMBS-sensitive $^{14}$C-butyrate uptake were measured at different concentrations (0.5–15 mM) of cold butyrate added to the uptake buffer, and kinetic parameters $K_m$ and $V_{\text{max}}$ were calculated using GraphPad Prism software. As shown in Table 1, LA-CS treatment caused significant increase in $V_{\text{max}}$ of MCT1-mediated butyrate uptake and a decrease in $K_m$. Increased $V_{\text{max}}$ could imply increased MCT1 levels at the cell surface, whereas decreased $K_m$ suggests increased affinity of MCT1 for the substrate.

LA-CS increases surface MCT1 expression via decreasing endocytic internalization. We next utilized cell surface biotinylation to measure apical membrane levels of MCT1 in response to LA-CS treatments. Consistent with increased function, LA-CS treatment (3 h) significantly increased apical membrane MCT1 levels (Fig. 3A). The plasma membrane level of a protein is governed by the rates of its endocytic internalization and exocytic recycling pathways for its membrane insertion. To examine whether LA-CS-mediated increase in apical membrane MCT1 is via decreasing basal endocytosis of MCT1, we performed endocytic internalization assay to measure endocytosed MCT1 fraction in the presence or absence of LA-CS. As shown in Fig. 3B, LA-CS significantly decreased basal MCT1 endocytosis, suggesting that increased cell surface MCT1, at least partially, is due to decreased endocytosis in response to LA-CS treatments.

**DISCUSSION**

SCFAs are produced in the colonic lumen via the action of specific gut microbiota on dietary fiber. Of the SCFAs, butyrate has a prominent role at the colonic level, as it is the primary fuel for the colonocytes, and its oxidation is involved in various important metabolic processes in the colon (16). Be-
Recent studies have also shown LP stimulation of Na\(^+\)-coupled electrolyte absorption (5, 27, 32, 38, 39). Our laboratory has demonstrated distinct proabsorptive effects of Lactobacilli associated diarrheal disorders by virtue of their ability to modulate epithelial cell functions (8, 15, 29, 37). Pioneering previous studies from our laboratory have demonstrated distinct proabsorptive effects of probiotics in stimulating butyrate uptake in Caco-2 cells via increasing the level of MCT1 at the apical membrane. Probiotic bacterial species are known to exert positive effects on intestinal homeostasis via exerting several positive effects on epithelial cell functions. For example, they are known to contribute to intestinal homeostasis via exerting several positive effects on epithelial cell functions. For example, they are known to alleviate mucosal inflammation, improve barrier function, inhibit pathogen adherence, and modulate mucosal immune functions (8, 15, 29, 37). Pioneering previous studies from our laboratory have demonstrated distinct proabsorptive effects of Lactobacilli and Bifidobacteria to alleviate inflammation-associated diarrheal disorders by virtue of their ability to modulate electrolyte absorption (5, 27, 32, 38, 39). Our laboratory’s recent studies have also shown LP stimulation of Na\(^+\)-coupled butyrate uptake in human intestinal Caco-2 cells or apical membrane vesicles prepared from human colonic mucosa of organ donors did not exhibit Na\(^+\)-dependent butyrate uptake (2), although these models still showed H\(^+\)-gradient-coupled \(^{14}\)C-butyrate uptake (22). Therefore, it seemed logical to us to investigate the effects of probiotic Lactobacilli species on MCT1 activity. Interestingly, LP showing long-term effects in increasing

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\text{pmol butyrate mg protein}^{-1} \cdot \text{H}^{-1} \cdot \text{nmin}^{-1}
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so sides being the energy nutrient, butyrate also ameliorates mucosal inflammation (40), exerts antidiarrheal effects by stimulating electrolyte and fluid absorption (1), and enhances mucosal barrier integrity (40). Many of these beneficial effects of butyrate contributing to the maintenance of colonic epithelial homeostasis, however, are concentration dependent and require cellular absorption of butyrate. As such, efficient absorption of butyrate is of fundamental importance to colonic epithelial health. Indeed, disturbed energy homeostasis observed in chronically inflamed mucosa of inflammatory bowel disease patients has been attributed to impaired absorption of butyrate (41, 42). In this regard, studies from our laboratory and others have shown MCT1 to play a major role in the absorption of colonic luminal butyrate (17, 22, 33, 34). Impaired butyrate absorption secondary to downregulation of MCT1 has been reported in inflammation (41, 42) and colon cancer (12, 19). We have also shown inhibition of MCT1 function in response to infection by EPEC (4), a food-borne pathogen causing early diarrhea, more particularly in children. Therefore, agents that upregulate MCT1 activity and/or correct MCT1 dysfunction could be of therapeutic value for intestinal inflammatory disorders associated with impaired butyrate absorption. In the present report, we have shown short-term effects of the probiotic LA in stimulating butyrate uptake in Caco-2 cells via increasing the level of MCT1 at the apical membrane. Probiotic bacterial species are known to contribute to intestinal homeostasis via exerting several positive effects on epithelial cell functions. For example, they are known to alleviate mucosal inflammation, improve barrier function, inhibit pathogen adherence, and modulate mucosal immune functions (8, 15, 29, 37). Pioneering previous studies from our laboratory have demonstrated distinct proabsorptive effects of Lactobacilli and Bifidobacteria to alleviate inflammation-associated diarrheal disorders by virtue of their ability to modulate electrolyte absorption (5, 27, 32, 38, 39). Our laboratory’s recent studies have also shown LP stimulation of Na\(^+\)-coupled butyrate uptake in rat intestinal epithelial IEC-6 cells via upregulation of sodium-coupled MCT1 (SMCT1) expression (2). SMCT1 is a relatively recently characterized transport protein implicated in Na\(^+\)-coupled transport of butyrate across colonic luminal membrane (21). However, our studies in human intestinal Caco-2 cells or apical membrane vesicles prepared from human colonic mucosa of organ donors did not exhibit Na\(^+\)-dependent butyrate uptake (2), although these models still showed H\(^+\)-gradient-coupled \(^{14}\)C-butyrate uptake (22). Therefore, it seemed logical to us to investigate the effects of probiotic Lactobacilli species on MCT1 activity. Interestingly, LP showing long-term effects in increasing

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SMCT1 expression and function in IEC-6 cells, and mouse colon, as reported earlier (2), had no effects on MCT1 function. On the other hand, LA, another species of Lactobacilli, increased MCT1 function via increasing the level of MCT1 at the apical cell surface. Thus the effects of probiotics on these two SCFA transporters appeared to be species specific and to involve distinct mechanisms. Additionally, heat-killed LA did not affect MCT1 function, whereas bacteria-free LA-CS had the same stimulatory effects on MCT1 function as that of live LA, suggesting the role of LA-secreted soluble factors in mediating the beneficial effects on MCT1 function. Although some protective effects of probiotics require direct interactions of epithelial cells with live bacteria, various earlier and recent reports have shown that even the cellular components of these agents, or their secreted soluble effector molecules, may just be as effective and considerably safer for the host (3, 20, 35, 44, 45).

Short-term regulation of a transporter protein may involve its altered membrane abundance or changes in kinetic properties, such as substrate affinity or interaction with other molecules (28). LA-CS-induced activation of MCT1 function in polarized Caco-2 cells was kinetically manifested by increased $V_{\text{max}}$ and decreased $K_m$. Increased $V_{\text{max}}$ suggests that LA-CS-induced increase in the absorptive capacity of MCT1 was due to its increased apical membrane abundance, which was also supported by our cell surface biotinylation studies. Endocytic internalization studies further suggested that increased apical membrane localization of MCT1 could, at least partially, be due to LA-CS inhibition of basal MCT1 endocytosis. The observed decrease in $K_m$ in response to LA-CS treatments could indicate increased affinity of MCT1 for butyrate. However, potential alternative mechanisms of LA-CS effects, such as phosphorylation/dephosphorylation of MCT1, could also account for the observed effects on the $K_m$. Furthermore, altered interactions of MCT1 with CD147, an auxiliary glycoprotein known to play critical role in membrane targeting of MCT1 (26), need to be examined to account for LA-CS enhancement of apical membrane levels of MCT1.

Our results showed that LA-CS not only stimulates MCT1 function and membrane expression, but also counteracts EPEC inhibition of MCT1 function and EPEC-mediated endocytic internalization of MCT1 from apical cell surface. Counteracting enteric infections by probiotics involve varied mechanisms, such as competitive exclusion, secretion of anti-microbial compounds, and induction of host epithelial signaling pathways (10, 29). Since bacteria-free LA-CS itself could counteract EPEC inhibition of MCT1 activity and live LA was not required, the effects were presumably not due to pathogen exclusion, but rather via induction of signaling events that modulated MCT1 intracellular trafficking mechanisms. Our laboratory’s earlier studies (5) showed the role of phosphatidylinositol 3-kinase/Rac1 small GTPase signaling and involvement of lipid rafts in LA-CS-induced increase in apical membrane level of SLC26A3, the intestinal epithelial apical membrane Cl⁻/HCO₃⁻ exchanger protein. These and other relevant signaling events could also play important roles in mediating LA-CS effects on MCT1 endocytosis and recycling under basal conditions or in response to EPEC infection.

Based on the multiple beneficial effects on gut epithelial health and impact on overall systemic health, butyrate is currently being considered as an important nutrient. This is further supported by evidence from clinical trials that adding resistant starch (source of butyrate) increases efficacy of oral rehydration solution to ameliorate diarrhea (1). Therefore, maintaining optimal availability of butyrate to colonic epithelial cells via manipulation of production and absorption of butyrate appears to be critical for a healthy colon. Therefore, our studies showing positive effects of probiotic-secreted molecules on the functionality of MCT1, a major player in mediating cellular entry of luminally produced butyrate, have great implications in colonic epithelial health and integrity. Furthermore, precise molecular identification of the LA-secreted soluble factor(s) will be of critical importance for designing novel probiotic-based therapeutic strategies for intestinal inflammatory diseases associated with impaired SCFA absorption.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

A.K. and A.B. performed experiments; A.K., W.A.A., and A.B. analyzed data; A.K. and A.B. prepared figures; W.A.A., A.B., and P.K.D. interpreted results of experiments; W.A.A., A.B., and P.K.D. edited and revised manuscript; A.B. and P.K.D. conception and design of research; A.B. drafted manuscript; A.B. and P.K.D. approved final version of manuscript.

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