Enteropathogenic *Escherichia coli* inhibits butyrate uptake in Caco-2 cells by altering the apical membrane MCT1 level

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Butyrate is the most important SCFA in colonocyte metabolism and is used preferentially over propionate and acetate. We have previously shown (12) that SCFA absorption across the epithelial membranes of the human ileum and colon involves an SCFA/HCO$_3^-$ exchange mechanism. We and others also demonstrated the involvement of monocarboxylate transporter 1 (MCT1) in the luminal absorption of SCFAs in human intestinal epithelial cells (11, 28). Butyrate is known to stimulate water and NaCl absorption via activation of Na$^+/H^+$ exchanger (NHE; see Refs. 6 and 25) and apical Cl$^-$/HCO$_3^-$ exchangers (24). The anti-inflammatory actions of butyrate are supported by both clinical and animal studies, implicating its role in suppressing mucosal inflammation (16). Also, butyrate-containing retention enemas have proven beneficial in the treatment of ulcerative and diversion colitis (6). Although decreased SCFA production resulting from impaired colonic fermentation has been shown to impair colonic absorption of sodium and water, little information is available on the factors that might influence efficient absorption of SCFAs by colonicocytes. Particularly, no information is available on the modulation of SCFA uptake by pathogenic microorganisms that cause secretory and inflammatory diarrhea in humans.

Enteropathogenic *Escherichia coli* (EPEC) is an important noninvasive human enteric pathogen associated with diarrhea, particularly in infants. Its infection causes specific histopathological alterations of the intestinal enterocytes, called attaching and effacing (A/E) lesions, characterized by effacement of microvilli, close adherence of the bacteria to the host cell membrane, and recruitment of filamentous actin and other cytoskeletal proteins resulting in pedestal formation beneath the sites of attachment (32). The gene products required for producing A/E lesions are encoded by an ~35-kb pathogenicity island in the bacterial chromosome, known as the Locus of Enterocyte Effacement (LEE). The LEE encodes a number of virulence genes, including the components of a type III secretion system (TTSS) that allows direct transfer of bacterial effector molecules into host cells (22). Several effectors, which are translocated into host cells via the TTSS, have been identified and characterized (21). These include Tir, EspF, EspG, EspH, and Map. The complete mechanism(s) of EPEC-induced diarrhea is not fully understood and appears to be multifactorial. One such factor may be impairment of ion and solute transport. Diarrhea associated with infection by enteric pathogens could result from either increased Cl$^-$/OH$^-$ secretion, decreased NaCl absorption, or both. We have previously shown that Cl$^-$/OH$^-$ exchange activity is inhibited in response to EPEC infection in Caco-2 cells (13). Also, EPEC infection increased the activity of NHE2, whereas the activity of NHE3, the predominant Na$^+$-absorbing isoform, was inhibited (14). Based on the fact that EPEC-induced diarrhea is multifactorial, and that SCFAs maintain mucosal integrity and influence water and electrolyte absorption, it was of interest to determine if butyrate absorption by mucosal cells is affected by EPEC.
infection. We report here that butyrate uptake by intestinal epithelial Caco-2 cells is significantly decreased by EPEC infection. We further show that membrane expression of MCT1, the SCFA transporter in the intestinal epithelial cells, is also decreased in response to EPEC infection.

MATERIALS AND METHODS

Materials. [14C]sodium butyrate was obtained from NEN Life Science Products (Boston, MA). Caco-2 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). Sulfo-NHS-SS-biotin for biotinylation of cell surface proteins and streptavidin agarose were from Pierce (Rockford, IL). All other reagents were of at least reagent grade and were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

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 U/ml penicillin, 100 g/ml streptomycin, 2 µg/ml gentamicin, and 20% FBS. Cells used for these studies were plated on 24-well plates at a density of 2 x 10^4 cells/well. Cells were used for bacterial infection and butyrate uptake on day 12–14 postplating and were grown overnight in a medium free of serum and antibiotic before infection.

Bacterial culture and infection of cells. The EPEC strains used in this study were as follows: wild-type EPEC strain E2348/69, CVD452 (E2348/69 escN:: Kanamycin; see Ref. 17), UMD864 (E2348/69 espB1:: aph-3; Km; see Ref. 20), and E2348/69 espD1:: Kanamycin; see Ref. 18). The nonpathogenic E. coli strain ATCC 104 (EspB1:: Kanamycin; see Ref. 17), UMD864 (E2348/69 espN/H11003, UMD864 (E2348/69 espD/H11006, UMD864 (E2348/69 espH/H11021, UMD864 (E2348/69 espP/H9262). Caco-2 cells were starved overnight in a medium free of serum and antibiotic before infection. We further show that membrane expression of MCT1, the SCFA transporter in the intestinal epithelial cells, is also decreased in response to EPEC infection.

RESULTS

EPEC infection inhibits butyrate uptake in Caco-2 cells. To determine whether EPEC infection had any effect on butyrate uptake, Caco-2 cells were infected for 1 h, and butyrate uptake was determined as pH-driven [14C]butyrate uptake, as described in MATERIALS AND METHODS. Figure 1 shows that butyrate uptake was decreased significantly (~60%) in EPEC-infected cells compared with uninfected controls. In contrast, infection with nonpathogenic E. coli had no significant effect.

Time course of EPEC inhibition of butyrate uptake. The time course of EPEC-mediated inhibition of butyrate uptake was determined by infecting Caco-2 monolayers with EPEC for 15, 30, 60, 90, and 120 min. As shown in Fig. 2, at earlier time
EPEC INHIBITS BUTYRATE UPTAKE

Experiment is shown. Occasions of EPEC infection. A Michaelis-Menten plot from a representative tion (0.5–15 mM) of butyrate. The experiment was performed on 3 separate effects of controls was reduced significantly (15.9 ± 0.11 mmol·mg protein−1·min−1: 15.9 ± 0.1 for control vs. 9.1 ± 0.1 in response to EPEC infection). In contrast, the apparent Michaelis constant (Km) did not change significantly (control 2.52 ± 0.04 mM vs. EPEC infected 2.71 ± 0.02 mM).

TTSS is required to inhibit butyrate uptake. The role of various EPEC virulence proteins on the EPEC-induced decrease in butyrate uptake by Caco-2 cells was investigated using specific mutant strains. First, involvement of the TTSS was studied using an escN mutant strain. The product of this gene is the putative ATPase that drives type III secretion of various virulence proteins. As shown in Fig. 4, there was no inhibition of butyrate uptake by Caco-2 cells when infected with the escN mutant strain, in contrast to significant inhibition by wild-type EPEC. These results reveal that a functional TTSS is required for EPEC to inhibit butyrate uptake by Caco-2 cells.

EPEC secreted proteins are involved in modulating butyrate uptake. We further analyzed the role of the TTSS in EPEC-modulated butyrate uptake by infecting cells with either espA, espB, or espD mutant strains. Each of these virulence genes encodes a structural component of the TTSS; therefore, mutation of any one of them renders TTSS ineffective. Figure 5 shows that infection of cells with these mutants had no effect on butyrate uptake compared with uninfected controls. Infection with wild-type EPEC, as expected, resulted in a marked decline in butyrate uptake. These results indicate that the structural components of the translocation apparatus themselves or the secreted effector molecules are required for the observed effect of EPEC on butyrate uptake.

Effector proteins EspF, EspG, EspH, and Map are not involved. EPEC infection results in the delivery of a number of effector proteins in the host cytosol. The role of these effector molecules in modulating butyrate uptake was also studied. Figure 6 shows that mutation of espF, -G, and -H, map, or of a double mutation of espG and its homolog orfβ had no impact on the inhibition of butyrate uptake caused by wild-type EPEC. These results suggest that these effector proteins are not involved in EPEC’s effect on butyrate uptake.

Membrane expression of MCT1 is decreased by EPEC infection. It has been previously shown by us and others that MCT1 is involved in the uptake of butyrate by Caco-2 cells. Therefore, to examine the effect of EPEC infection on MCT1 protein, cell surface proteins from control and infected cells were biotinylated and pulled down from the cell lysate by avidin, and separated proteins were probed with an anti-MCT1 antibody. As shown in Fig. 7A, surface expression of MCT1 in infected cells was decreased considerably compared with uninfected controls or those infected with nonpathogenic E. coli (HS4), whereas levels of total cellular MCT1 remained constant in all groups. Densitometric scanning of the band inten-

Fig. 3. Effect of EPEC infection on the kinetics of butyrate uptake in Caco-2 cells. Caco-2 cells were infected with wild-type EPEC for 60 min, and [14C]butyrate uptake was determined in the presence of increasing concentrations (0.5–15 mM) of butyrate. The experiment was performed on 3 separate occasions of EPEC infection. A Michaelis-Menten plot from a representative experiment is shown.

Fig. 4. A functional type III secretion system (TTSS) is required for EPEC-mediated inhibition of butyrate uptake. Caco-2 cells were infected with EPEC or escN mutant strain for 60 min, and then [14C]butyrate uptake was measured. Results represent means ± SE of 3 independent experiments performed in triplicate. *P < 0.05 compared with control.

Fig. 5. EPEC-secreted proteins EspA, EspB, and EspD are required for EPEC inhibition of butyrate uptake. Caco-2 cells were infected with EPEC or one of the mutant strains espA, espB, or espD for 60 min, and butyrate uptake was determined. Results represent means ± SE of 4 separate experiments performed in triplicate. *P < 0.05 compared with control.
studies of EPEC inhibition of butyrate uptake showed effective inhibition as early as 60 min postinfection that persisted until 120 min.

EPEC is noninvasive and does not produce toxins. Instead, it employs the TTSS to deliver virulence factors directly in the host cells (32). Several mutational studies have enabled identification of individual components of the TTSS. Requirement of an intact TTSS for EPEC to inhibit butyrate uptake is supported by the fact that mutation of the putative ATPase (EscN) blocked the inhibitory effect of EPEC infection. This mutant has also been shown to be ineffective in modulating Na\(^{+}\) (14) and Cl\(^{-}\) (10) uptake by Caco-2 cells. Mutant strains carrying mutations in espA, espB, or espD, all of which interfere with type III secretion, also failed to inhibit butyrate uptake in infected Caco-2 cells. EspA forms a filamentous organelle on the bacterial surface that is postulated to act as a channel for the type III system to deliver proteins to the host cell (9). EspB and EspD are translocated to the host cell membrane and together are thought to form the translocation pore. EspB also has been reported to be present in the cytoplasm; however, its function here is not known (9).

The EPEC-secreted effector molecules have previously been reported to alter host cell functions via different mechanisms. For example, EspF is known to disrupt tight junctions (23); EspG, and its homolog Orf3, both disrupt microtubules and produce subtle alterations in barrier functions (8, 30); EspH is known to alter pedestal morphology and filopodia formation (31), whereas Map has been reported to alter mitochondrial membrane potential (18). However, our current studies suggested that the secreted effector molecules EspF, EspG, EspH, and Map were not involved in mediating the observed decrease.

**DISCUSSION**

Absorption of SCFAs is important for colonocyte health and metabolism, epithelial integrity, as well as colonic fluid and electrolyte balance. Butyrate, a key SCFA, is known to have multiple regulatory roles in the mammalian colon, including stimulation of fluid and electrolyte absorption (24) by increasing electroneutral NaCl absorption (3) and inhibiting Cl\(^{-}\) secretion (27). Recent studies (19) have also demonstrated that butyrate stimulates promoter activity and expression of the apical NHE3 in the human adenocarcinoma cell line Caco-2. Butyrate has also been implicated in suppressing mucosal inflammation (16). Decreased production or availability of butyrate has been shown to result in chronic inflammation and acute diarrhea (6). However, to date, the effects of enteric pathogens on the absorption of SCFAs have not been examined. EPEC is a human enteric pathogen infecting primarily infants and young children. The specific mechanisms by which EPEC causes early diarrhea in infected hosts, however, remain unclear. Diarrhea results from either increased secretion, impaired absorption, or both. EPEC infection has intact been shown to decrease secretagogue-induced Cl\(^{-}\) secretion (15). We have also shown that EPEC infection increases NHE2 activity, whereas the activity of NHE3, the predominant Na\(^{+}\)-absorbing isoform, is inhibited (14). Cl\(^{-}\)/OH\(^{-}\) exchange activity was also shown to be inhibited in Caco-2 cells infected with EPEC (13). We have also reported that EPEC infection induces inflammation (29) and disrupts the structure and barrier function of tight junctions after prolonged infections (26). These findings suggest that the mechanism(s) of EPEC-induced diarrhea are multifactorial and may also involve modulation of SCFA uptake. Caco-2 cell monolayers were selected to investigate this question, since previous studies from our laboratory (11) demonstrated that this cell line was a suitable in vitro model to study butyrate uptake. The data presented here suggest that EPEC infection of confluent differentiated Caco-2 monolayers results in a significant decrease in butyrate uptake. This effect was specific, since infection with nonpathogenic *E. coli* (HS4) had no effect on butyrate uptake. Time course studies of EPEC inhibition of butyrate uptake showed effective inhibition as early as 60 min postinfection that persisted until 120 min.

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in butyrate uptake in response to EPEC infection, since these mutants behaved similar to wild-type EPEC in inhibiting butyrate uptake. Orf3, the espG homolog, is encoded in the EPEC genome in a locus distinct from the LEE. Because both effectors (EspG and Orf3) are known to induce disruption of microtubule networks beneath adherent bacteria (21), we used a double mutant of espG/lorf3 to infect cells and observe the effects on butyrate uptake. There was again a significant inhibition of butyrate uptake, similar to that caused by wild-type EPEC, suggesting that these effector molecules are not involved in EPEC-mediated inhibition of butyrate uptake. However, the inability of nonpathogenic E. coli or type III secretion mutants to inhibit butyrate uptake seems to indicate that the observed effects are related to pathogenicity.

Our kinetic studies suggested that EPEC inhibited butyrate uptake via changes in the value of V_{max} without significantly altering the apparent K_{m}, indicating a decrease in the expression of the butyrate transporter on the plasma membrane. This was indeed found to be the case by performing biotinylation studies to quantitate the amount of surface MCT1, the butyrate transporter in Caco-2 cells. Although the surface expression of MCT1 was considerably lower in cells infected with EPEC compared with nonpathogenic E. coli, or uninfected control cells, total cellular MCT1 was the same in all the groups. These studies demonstrate the regulation of butyrate uptake either by retrieval of MCT1 from the apical plasma membrane or via translocation of MCT1 to the apical plasma membrane.

The effects of pathogenic organisms on fluid and electrolyte secretion by host intestinal epithelial cells have been well documented. Various enteric pathogens elicit a Cl\(^{-}\) secretory response. The studies from our laboratory showed profound early effects of EPEC infection on intestinal epithelial absorption, an aspect that has not been studied previously. The observed effects of EPEC infection on butyrate uptake reported here and previously published results from our laboratory on Na\(^{+}/H\(^{+}\) and Cl\(^{-}/OH\(^{-}\) exchange activities strongly suggest that EPEC infection has profound effects on the host intestinal absorptive ion transport processes. We speculate that decreased butyrate availability caused by EPEC infection might compromise colonic epithelial integrity, resulting in inflammation of the epithelium, and also might contribute to EPEC-mediated diarrhea by inhibiting ion absorption in the colon. Our results demonstrate that EPEC requires a TTSS to inhibit butyrate uptake via regulation of MCT1. In lieu of the important role of SCFA in stimulating human colonic electrolyte absorption and being the key nutrient in the colon, our studies not only add to increased understanding of the mechanisms regulating human intestinal SCFA absorption but also suggest the potential role of inhibition of this key ion as an important contributory factor in the pathophysiology of EPEC-associated diarrhea.

REFERENCES


GRANTS

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