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Effects of Butyrate on Intestinal Barrier Function in a Caco-2 Cell Monolayer Model of Intestinal Barrier

LUYING PENG, ZHENJUAN HE, WEI CHEN, IAN R. HOLZMAN, AND JING LIN

Department of Pediatrics [L.P., W.C., I.R.H., J.L.], Mount Sinai School of Medicine, New York, New York 10029; Department of Pediatrics [Z.H.], Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China

ABSTRACT: Production of short-chain fatty acids (SCFA) in the intestinal lumen may play an important role in the maintenance of the intestinal barrier. However, overproduction/accumulation of SCFA in the bowel may be toxic to the intestinal mucosa and has been hypothesized to play a role in the pathogenesis of neonatal necrotizing enterocolitis (NEC). By using a Caco-2 cell monolayer model of intestinal barrier, we report here that the effect of butyrate on the intestinal barrier is paradoxical. Butyrate at a low concentration (2 mM) promotes intestinal barrier function as measured by a significant increase in transepithelial electrical resistance (TER) and a significant decrease in inulin permeability. Butyrate at a high concentration (8 mM) reduces TER and increases inulin permeability significantly. Butyrate induces apoptosis and reduces the number of viable Caco-2 cells in a dose-dependent manner. Intestinal barrier function impairment induced by high concentrations of butyrate is most likely related to butyrate-induced cytotoxicity due to apoptosis. We conclude that the effect of butyrate on the intestinal barrier is paradoxical; *i.e.* whereas low concentrations of butyrate may be beneficial in promoting intestinal barrier function, excessive butyrate may induce severe intestinal epithelial cell apoptosis and disrupt intestinal barrier. **(*Pediatr Res* 61: 37–41, 2007)**

Normal functioning of the intestines depends on the establishment and maintenance of mucosal barrier that are lined by a monolayer of intestinal epithelial cells. Establishing normal bacterial colonization in the intestine is essential for the development and maintenance of this robust epithelial barrier (1). SCFA, mainly acetic acid, butyric acid, and propionic acid, are the products of the bacterial fermentation of undigested carbohydrates and proteins in the intestine. SCFA are normally absorbed by the colonocyte and are either locally used as fuel for the colonic mucosal epithelial cells or enter the portal bloodstream (2,3). Production of SCFA in the bowel is important for normal intestinal biology such as water and salt absorption in the colon and energy salvation, and may also play important role in the maintenance of the intestinal barrier (4,5).

In premature infants, the maturation of the intestinal barrier function does not develop properly in the absence of enteral nutrients (6). Intestinal barrier function is significantly less developed in full-term newborn piglets receiving total parental nutrition compared with those receiving enteral nutrition (7). Production of SCFA in the bowel may be crucial for gastrointestinal adaptation and maturation in the early stage of postnatal life (8). However, overproduction and/or accumulation of SCFA in the bowel due to maldigestion and bacterial overgrowth may be toxic to mucosal cells and cause intestinal mucosal injury (9,10). Overproduction and/or accumulation of SCFA in the bowel and inability to clear the intraluminal SCFA because of poor gastrointestinal motility in premature infants have been hypothesized to play a role in the pathogenesis of neonatal NEC (11).

Several studies have previously looked at the effects of SCFA on the intestinal barrier *in vitro* as well as *ex vivo*. In physiologic concentrations, SCFA have been shown to promote intestinal epithelial restitution and induce a concentration-dependent, reversible decrease in paracellular permeability *in vitro* (12,13). On the other hand, in relatively higher concentration, butyrate induces an increase in paracellular permeability in an *ex vivo* model (14). However, the mechanisms underlying the differential effects of SCFA on the intestinal barrier function are poorly understood.

The present study further examined the effects of butyrate at different concentrations on the intestinal barrier function in a well-established Caco-2 intestinal epithelium model. We also determined that the detrimental effect of high concentration of SCFA on the intestinal barrier function may be related to the SCFA-induced intestinal epithelial cell apoptosis.

MATERIALS AND METHODS

Cell culture. Caco-2 cells, a human colonic epithelial cell line (ATCC, Rockville, MD), were maintained routinely in 100 × 15 mm Petri dishes at 37°C with 5% CO_2–95% air atmosphere and >95% humidity. The cell monolayers were grown in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin, L-glutamine (2 mM), and nonessential amino acids. All experiments were performed in serum-free medium.

Transepithelial electrical resistance assay. Twelve-well transwell system plates with collagen-coated insert membrane (Corning Inc., Corning, NY) were used for this assay. The culture plate inserts consisting of two chambers separated by a microporous transparent Biopore membrane (0.4 μm). Briefly, 1.5×10^6 Caco-2 cells in 0.5 mL medium were seeded in the apical chamber that bathed in the basal chamber with 1.5 mL of medium. Once grown to confluence, the cells were rinsed with PBS and incubated in serum-free medium alone or with sodium butyrate at concentrations of 2 or 8 mM for

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Correspondence: Jing Lin, M.D., Jack and Lucy Clark Department of Pediatrics, Division of Newborn Medicine, Mount Sinai School of Medicine, Box 1508, One Gustave L. Levy Place, New York, NY 10029-6574; e-mail: jing.lin@mssm.edu
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Abbreviations: NEC, necrotizing enterocolitis; **SCFA,** short-chain fatty acids; **TER,** transepithelial electrical resistance

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both chambers. The changes of TER were measured daily with an epithelial volt-ohmmeter (WPI, Sarasota, FL). Values were corrected for background resistance contributed by the insert membrane and the collagen layer, and calculated as $\Omega \cdot cm^2$.

Permeability assay. The permeability of Caco-2 cell monolayers was performed by measuring transepithelial passage of FITC-inulin (inulin-FITC, Sigma Chemical Co.) according to Basuroy *et al.* (15) with minor modification. Briefly, Caco-2 cell monolayers on transwell inserts were incubated under stimulation of different concentrations of butyrate at 37°C for 68 h. Inulin-FITC (6 μg/mL) was added to the basal chamber and cultured for an additional 4 h. Fifty microliters of the apical and basal medium were used respectively for fluorescence analysis in a microplate fluorescence reader (SPECTRAmax GEMINI XS, Molecular Devices, Menlo Park, CA). The permeability of the monolayers was expressed as flux into the apical chamber in the percent of total inulin-FITC administered into the basal chamber.

MTT assay. As a method of assessing butyrate-induced cytotoxicity, the colorimetric 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co.) assay was performed (16). Caco-2 cells were seeded on 96-well microplates (5000 cells/100 μL/well) and then cultured for 48 h. After this period, the medium from the 96-well plates was replaced with the serum-free medium alone or serum-free medium with different concentrations of sodium butyrate (2, 5, or 8 mM), and the plates were incubated for 72 h. At the end of the treatment, 50 μL of a 1 mg/mL solution of MTT in PBS was added to each well and the plates were incubated at 37°C for an additional 4 h. The supernatant was then discarded, and the purple formazan crystals formed were solubilized with 100 μL/well of DMSO (Sigma Chemical Co.). The absorbance of the 96-well plate was determined using an automatic microplate spectrophotometer at 570 nm. Cell viability was calculated as the percent ratio of absorbance of the samples against the control medium.

Apoptosis assay. The extent of apoptosis of cultured intestinal epithelial cells can be determined by measuring the proportion of the total cell population that has detached from the monolayers (17). Therefore, the proportion of floating cells was counted to quantify the apoptosis. After treatment of butyrate for 72 h in serum-free medium, the Caco-2 cells that had detached from the cell monolayer and were floating in the medium as well as that still attached in dish were harvested, and counted respectively using a hemacytometer. The fraction of the floating cells were stained for 10 min with acridine orange (2 μg/mL) in PBS and were viewed immediately by fluorescence microscopy. Acridine orange stained cells were imaged using fluorescence microscopy (Leica, Wetzlar, Germany).

DNA fragmentation assay. The Caco-2 cells (both floating and attached) treated by sodium butyrate at different concentrations were collected and centrifuged. Cell pellets were resuspended in the cell lysis buffer (10 mM Tris-HCl, pH 7.4; 10 mM EDTA, pH 8.0; 0.5% Triton X-100) and incubated. RNase A (0.5 mg/mL) and proteinase K (0.5 mg/mL) were added, respectively, and incubated for 2 h. DNA was precipitated by ethanol, and 5 μg sample was run on a 1.8% agarose gel contained ethidium bromide in TBE buffer. DNA was visualized by UV transilluminator.

Western blotting analysis. The Caco-2 cells were grown to 100% confluence and then treated with medium alone or with media with sodium butyrate at concentrations of 2, 5, and 8 mM for 72 h. The cells were rinsed with PBS, and then lysed in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) with protease inhibitor cocktail (Sigma Chemical Co.) at 4°C. Total cell lysate was separated with 8% SDS–polyacrylamide gel electrophoresis, and transferred to PVDF membrane. The target protein was probed using a rabbit anti-poly (ADP-ribose) polymerase (PARP) antibody and a goat anti-actin antibody (Santa Cruz Biotech), respectively, in combination with horseradish peroxidase–conjugated anti-rabbit or anti-goat IgG. Blots were developed using ECL Western blotting detection reagent kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Statistics. Most of the experiments were repeated at least three times. Group data from all experiments are presented as mean ± SE, except where noted, and the numbers in each group used for statistical analysis are mentioned respectively in the figure legends. ANOVA was used for statistical analysis. The difference between each group was compared with the Tukey test. A p value <0.05 was considered statistically significant in all cases. All statistical analyses were performed using Microcal Origin 6.0 for Windows (Microcal Software Inc., MA).

RESULTS

Butyrate induces alternation of TER and inulin permeability in Caco-2 monolayers. Different concentrations of sodium butyrate induced distinct changes of TER in Caco-2 monolayers (Fig. 1). TER of Caco-2 monolayers treated with media alone showed a spontaneous increase with time and reached a maximum of 200 ± 21 $\Omega \cdot cm^2$ by 72 h. Butyrate at 2 mM stimulated a significant increase of TER in Caco-2 cell monolayers after 24-h of butyrate treatment ($p < 0.01$, compared with the untreated cells). The TER continued to increase for an additional 24 h and remained constant from 48 h to 96 h with an average of 297 ± 28 $\Omega \cdot cm^2$. In monolayers treated with a high concentration (8 mM) of butyrate, no significant change in resistance was observed during first 24 h compared with the control cells. The resistance then decreased significantly by 48 h and continued to decline, reaching 23 ± 18 $\Omega \cdot cm^2$ by 96 h. In addition to the changes in TER, we examined the transepithelial permeability of the Caco-2 cell monolayers with fluorescent inulin probe of MW 2–5 kD. As expected, changes in permeability measured as the flux of inulin-FITC from basal chambers to apical chambers through Caco-2 monolayer after butyrate treatment were consistent with that of TER. Butyrate at 2 mM of concentration promoted barrier function as evidenced by the significant reduction of the inulin-FITC permeability of Caco-2 cell monolayers. However, butyrate at 8 mM of concentration impaired the barrier function and caused significant increase in inulin-FITC permeability (Fig. 2).

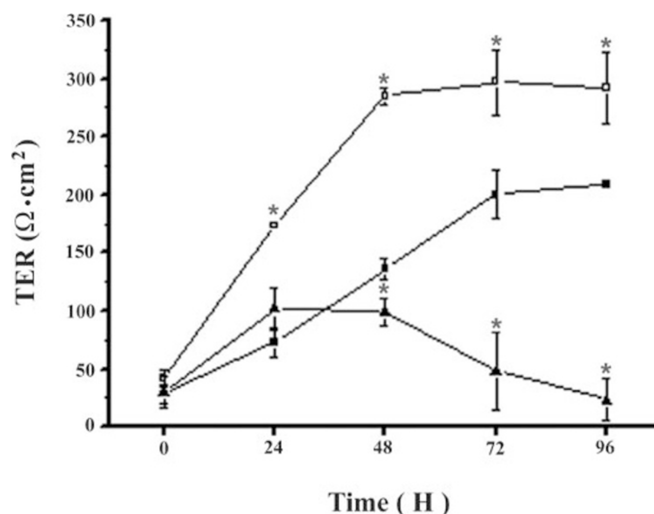

Figure 1. Effects of butyrate on TER in Caco-2 cell monolayers. TER was measured daily in Caco-2 cell monolayers treated with media alone (■), 2 mM of butyrate (□), or 8 mM of butyrate (▲). Butyrate at a low concentration (2 mM) promotes intestinal barrier function as measured by a significant increase in TER by 24 h. Butyrate at a high concentration (8 mM) reduces TER significantly by 48 h ($n = 6$ for each group, *$p < 0.05$).

Butyrate induces apoptosis and inhibits Caco-2 cell proliferation. Sodium butyrate inhibited Caco-2 cell proliferation as determined by the MTT assay. As seen in Figure 3, butyrate decreased cell viability in a dose-dependent manner. Although, lower concentrations of butyrate (2 mM) also inhibited proliferation of Caco-2 cells to some extent in comparison with the control cells, butyrate at concentrations of 5 or 8 mM reduced the Caco-2 cell viability by more than 50%. To determine whether reduced Caco-2 cell viability is due to butyrate-induced apoptosis, we performed apoptosis assays by measuring the proportion of the total cell population that

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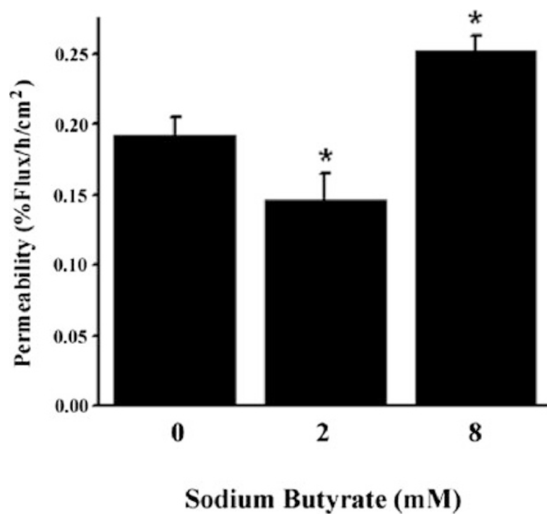

Figure 2. Effects of butyrate on transepithelial permeability of Caco-2 cell monolayers. Caco-2 cells of each group were grown in transwell inserts with serum-free medium only or containing 2 or 8 mM butyrate for 68 h. Transepithelial flux of fluorescent probe was measured after 4 h incubation in presence of inulin-FITC (6 μg/mL) in basal chambers. The permeability of monolayers was significantly decreased by stimulation of 2 mM of butyrate but was significantly increased by 8 mM of butyrate as compared with the media controls ($n = 4$ for each group, $^*p < 0.05$).

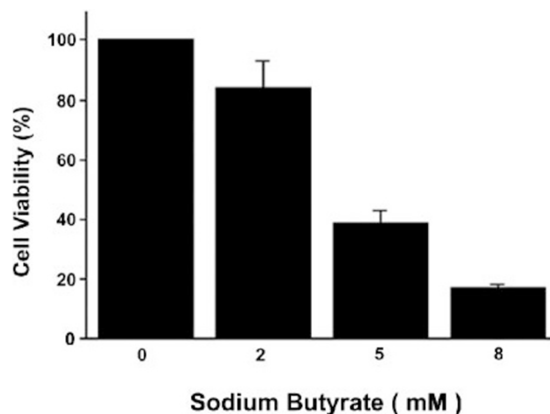

Figure 3. Butyrate inhibits proliferation of Caco-2 cells. Caco-2 cells were treated for 72 h with butyrate at the concentrations of 0, 2, 5, and 8 mM. The cell viability was determined by MTT assay. The data value are presented as means ± SD, which expressed as percentage of the results for control cells grown in serum-free DMEM without addition of sodium butyrate ($n = 12$ for each group, $p < 0.05$).

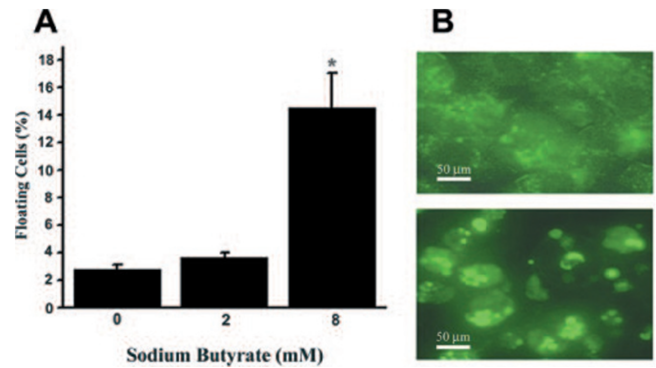

Figure 4. Butyrate induces Caco-2 cell apoptosis. (*A*) Floating Caco-2 cells as percentage of the total cell number (attached and floating cells) in media with different concentrations of butyrate. (*B*) Photos illustrate the attached cells stained with acridine orange after treatment with 8 mM of butyrate (*upper panel*), and the morphologic features of apoptosis in floating cells with bright and condensed chromatin (*lower panel*). Magnification ×100.

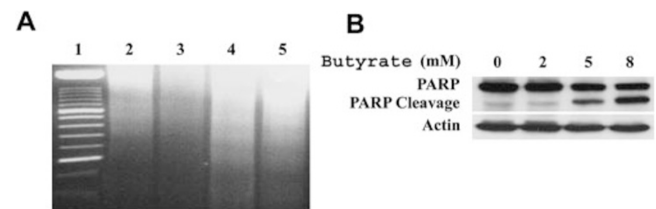

Figure 5. DNA fragments and PARP cleavage induced by butyrate in Caco-2 cells. (*A*) Electrophoretic pattern of DNA isolated from the Caco-2 cells treated for 72 h with butyrate at 0 mM (*lane 2*), 2 mM (*lane 3*), 5 mM (*lane 4*), and 8 mM (*lane 5*), respectively. The DNA was extracted from both attached and floating cells. *Lane 1* shows DNA marker. (*B*) PARP cleavage analysis by immunoblotting. Total proteins were isolated from the Caco-2 cells following 72-h treatment of butyrate (0, 2, 5, or 8 mM), and analyzed by electrophoresis on 8% SDS-PAGE gel. Full length of PARP (116 kD) and typical PARP cleavage (85 kD) were detected, respectively. Actin proteins were probed for equal loading and transfer control.

detached from the monolayers. In addition, morphologic evidence, DNA fragments assay, and Western blotting were used to confirm apoptosis. As showed in Figure 4, treatment with 8 mM of butyrate for 72 h resulted in 15.0 ± 3.0% of floating cells, which was significantly greater than that seen in medium control monolayer (3.0 ± 0.3%, $p < 0.01$). Following acridine orange staining, the floating cells show a typically condensed chromatin pattern (Fig. 4 *B, lower panel*), but attached cells lacked the apoptotic appearance (Fig. 4 *B, upper panel*). In contrast, treatment with 2 mM of butyrate did not induce significantly different numbers of floating cells compared with the medium control. Similarly, a DNA fragment assay, another index measurement for apoptosis, also showed no clear ladder present in the monolayers treated with 2 mM of butyrate or medium alone. On the other hand, 8 mM of butyrate induced the characteristic apoptotic DNA ladder by 72 h (Fig. 5 *A*). Furthermore, specific proteolytic cleavage of PARP (generating 85 kD and 26 kD fragments) has been demonstrated to be as a reliable marker for apoptosis in the intestinal epithelial cells (18). Using the antibody against PARP, a typical cleavage band of 85 kD was detected by Western blot analysis in the monolayers treated by 5 and 8 mM of butyrate. The effect of butyrate on the specific proteolytic cleavage of PARP was dose-dependent (Fig. 5 *B*). A weak cleavage band was also present in both Caco-2 cells treated with media alone and media containing with low-concentration (2 mM) of butyrate. This is expected since a small portion of Caco-2 cells undergo apoptosis spontaneously and similar results are seen from the floating cell assay and DNA fragmentation assay.

DISCUSSION

Postnatal maturation of the intestinal barrier occurs coincident with increasing enteral feeding and establishment of normal intestinal bacterial colonization (19,20). The mechanisms involved in the development of intestinal barrier function are probably multifactorial. Normal bacterial colonization

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is likely to be rather important in this process, as is indicated by the alterations of gastrointestinal tract gene expression and intestinal barrier function development by early treatment with broad-spectrum antibiotics (21). As the most important end products of bacterial fermentation in the intestinal lumen, SCFA may be the key molecules that mediate the regulatory effect of normal intestinal flora on intestinal barrier function as well as mucosal immune system (22).

In the present study, by using an *in vitro* model of intestinal epithelial barrier with Caco-2 cells grown in a trans-well system, we demonstrate that butyrate at low concentration (2 mM) promotes intestinal barrier function as measured by increases in TER and decreases in inulin permeability. Our results are consistent with the findings of Mariadason *et al.* (13), who have demonstrated previously that physiologically relevant SCFA induced a concentration-dependent, reversible increase in TER and reduced mannitol flux in the Caco-2 cell monolayer model of intestinal epithelium. They suggested that the effect of butyrate on TER might be a manifestation of the promotion of a more differentiated Caco-2 phenotype. They also speculated that tight junction specific proteins might be involved as one of the mechanisms.

Whereas low concentrations of butyrate may be beneficial in promoting mucosal barrier function, excessive luminal butyrate may cause intestinal injury by disrupting the mucosal barrier. We have previously demonstrated that high concentrations of SCFA can induce concentration and maturation-dependent intestinal mucosal injury in newborn rats and proposed a hypothesis that overproduction/accumulation of SCFA in the bowel and inability to clear the intraluminal SCFA because of poor gastrointestinal motility in premature infants may play a role in the pathogenesis of NEC (23,24). In the present study, we demonstrate that butyrate at high concentration (8 mM) causes interruption of the intestinal barrier, which correlates well with butyrate-induced apoptosis. Butyrate is well known to induce apoptosis in several colon cancer cell lines, including Caco-2 cells. Butyrate-induced apoptosis in Caco-2 cells has been reported to be mediated *via* the mitochondrial pathway, and caspase-3 is a key enzyme in the process (25). Another potential mechanism involves transcriptional stimulation of the Bax gene *via* activation of the JNK/AP1 pathway has also been reported in other colonic epithelial cells (26). It has been shown that almost all SCFA are capable of causing cytotoxicity by inducing apoptosis in cultured intestinal epithelial cells (27). Here we present evidence that severe apoptosis induced by butyrate is associated with the decrease of TER and the increase of inulin permeability in the Caco-2 cell model, suggesting that intestinal barrier may be impaired by cell death due to severe apoptosis in the presence of high concentration of butyrate. Apoptosis and shedding occur normally in the tip of mucosal villi as part of the physiologic renewal of the intestinal epithelium (28). The elimination of cells from the normal epithelium is followed by a physiologic rearrangement of the tight junction, which is generally sufficient to prevent transepithelial leaks of macromolecules and maintain the intestinal barrier (29). It was therefore assumed that apoptosis of isolated epithelial cells occurs without relevant disruption of epithelial barrier (30). Indeed, our data demonstrate that apoptosis can be induced by butyrate in Caco-2 cells even at low concentration, but the barrier function as measured by changes in TER and inulin permeability are even promoted by a mechanism yet to be determined. However, with increased apoptosis, intestinal epithelial barrier impairment occurs. This result is consistent with the recent findings by others. By measuring transepithelial conductivity and unidirectional tracer fluxes in monolayers of human colonic HT-29/b6 cells and the association with camptothecin-induced apoptosis, Bojarski and colleagues (31) concluded that the formerly "tight" colonic epithelium became leaky at 12% apoptosis. Furthermore, Jones *et al.* (32) reported recently that an inhibitor of caspases-8 was able to block both morphologic and biochemical features of apoptosis induced by tumor necrosis factor (TNF)-α/butyrate, and also block TNF-α/butyrate–induced loss of transmembrane resistance in Caco-2 cells. These data further support our findings that severe apoptosis induced by a high concentration of butyrate can impair the monolayer integrity of epithelial cells and consequently lead to disruption of the intestinal barrier.

It remains to be determined whether butyrate has similar effects on intestinal barrier *in vivo*. Butyrate inhibits Caco-2 cell proliferation but accelerates epithelial proliferation and increases crypt cell production rate in rat intestine (3). Increasing luminal SCFA concentration by dietary fiber in normal rat improves paracellular permeability of the colon (33). It has been assumed that postnatal development of intestinal barrier may be mediated by SCFA (11). However, an *ex vivo* study using adult rat distal colon mucosa mounted in a Ussing chamber demonstrated that acute exposure to butyrate increased paracellular permeability in rat colon, indicating the complexity of butyrate's potential effects on epithelial barrier function (14). In newborn rats, we have previously shown that luminal administration of high concentrations of SCFA can induce concentration-dependent intestinal mucosal injury (23). The severity of the colonic mucosal injury induced by luminal SCFA is maturation dependent (24). Whether SCFA-induced apoptosis and interruption of intestinal barrier function as one of the mechanisms underlying the SCFA-induced intestinal mucosal injury in newborn rat is unknown.

In summary, by using a Caco-2 cell monolayer model of intestinal barrier, we demonstrate that the effect of butyrate on the intestinal mucosa barrier is paradoxical, *i.e.* whereas low concentrations of butyrate may be beneficial in promoting mucosal barrier function, excessive butyrate may disrupt mucosal barrier function. The increased intestinal epithelial cell apoptosis induced by butyrate at high concentration, which interrupts intestinal barrier, may explain the detrimental effect of SCFA on intestinal barrier when SCFA in the intestinal lumen are excessive. Further studies are needed to determine whether the effect of SCFA on the intestinal mucosa *in vivo* is also paradoxical, especially in an immature intestinal mucosa, which may help us to understand the role of SCFA in the postnatal development of intestinal barrier function in premature infant and the possible role of overproduction/accumulation of SCFA in the pathogenesis of NEC.

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