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장상피세포, 대식세포 및 대장염에서 부틸레이트의 효과

Effect of Butyrates on Intestinal Epithelial Cells, Macrophages and Experimental Colitis In Mice

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August 2018

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Abstract

Effect of Butyrates on Intestinal Epithelial Cells, Macrophages and Experimental Colitis In Mice

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Introduction: Butyrate is a bacterial metabolite of dietary fiber in the colon that has been used to treat inflammatory disease. However, the effect of oral supplementation with butyrate on colitis has not been fully explored. We evaluated the effects of and mechanisms underlying oral supplementation with butyrate on experimental murine colitis.

Methods: The human intestinal epithelial cells (IECs) COLO 205, the murine macrophages RAW 264.7 and peritoneal macrophage from IL-10 deficient (IL-10−/−) mice were used. The production of cytokines was determined by ELISA. The effect of sodium
butyrate on LPS-induced NF-κB pathway and acetylation of histone H3 were examined by Western blot analysis. The DNA binding activity of NF-κB was assessed by an electrophoretic mobility shift assay. To confirm that butyrate plays a protective role in colitis, an acute colitis model induced by dextran sulfate sodium (DSS) and a chronic colitis model in IL-10$^{-/-}$ mice were used. Colitis was quantified by histologic evaluation and immunohistochemical staining was performed.

**Results:** Butyrate (100 μM and 500 μM) inhibited pro-inflammatory cytokine production (i.e., IL-8 in COLO205 and TNF-α, IL-6 and IL-12 in macrophages) but promoted anti-inflammatory cytokine (i.e., IL-10) production in RAW 264.7 cells. Butyrate attenuated both the LPS-induced degradation /phosphorylation of IκBa and DNA binding of NF-κB and enhanced histone H3 acetylation. The administration of oral butyrate (100 mg/kg) significantly improved histological scores in both colitis models, including the IL-10$^{-/-}$ mice. In immunohistochemical staining, IκBa phosphorylation was attenuated, and histone H3 acetylation was reversed in the treated colons of both colitis models.

**Conclusions:** Our results indicate that oral supplementation with butyrate attenuates experimental murine colitis by blocking NF-κB signaling and reverses histone acetylation. These anti-colitic effects of butyrate were IL-10 independent. Butyrate could be a candidate for a therapeutic agent for colitis.
Keywords: Butyrates; Histone Deactylase Inhibitors; Mice; NF-κappa B; Inflammatory Bowel Diseases;
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List of Abbreviations

ANOVA: One-way analysis of variance
DSS: Dextran sulfate sodium
EMSA: Electrophoretic mobility shift assays
HDAC: Histone deacetylase
IACUC: Institutional Animal Care and Use Committee
IBD: Inflammatory bowel disease
IEC: Intestinal epithelial cell
IL: Interleukin
IL-10\(^{-/-}\): IL-10 deficient
LPS: Lipopolysaccharides
PM: Peritoneal macrophage
SB: Sodium butyrate
SCFA: Short chain fatty acid
WT: Wide type
Introduction

Inflammatory bowel disease (IBD) is characterized by chronic, unregulated and relapsing intestinal inflammation. IBD has significant negative effects on quality of life and economic status because of its early onset and recurrence. Recently, the prevalence of IBDs has increased in East Asia, particularly in China and South Korea [1, 2]. This increase is believed to be related to changes in environmental factors, including diet during rapid urbanization and industrialization [3]. Dietary fiber is an important component of the diet that is associated with IBD, and the incidence of IBDs is inversely associated with the intake of dietary fiber [4]. In epidemiologic studies, the incidence of IBDs has increased as dietary fiber has decreased in diets [4, 5]. Furthermore, recent studies have shown that a high-fiber diet has a protective effect in IBD patients [6].

Dietary fiber is an indigestible carbohydrate that passes through the colon and is fermented to short-chain fatty acids (SCFAs) by anaerobic colonic microbiota [7]. Butyrate and other SCFAs help to maintain epithelial integrity and reduce luminal pH. Butyrate is also taken up by intestinal epithelial cells (IECs), which use them as a main energy source. Furthermore, butyrate has been shown to be a histone deacetylase (HDAC) inhibitor that suppresses the activation of nuclear factor kappa B (NF-κB) [8-10]. Butyrate also suppresses the production of pro-inflammatory cytokines and
promotes the production of anti-inflammatory cytokines [11]. Because of these anti-inflammatory effects, butyrate has been used in an attempt to treat different inflammatory diseases [8, 11, 12]. However, the effect of butyrate on colitis has not been fully explored [7]. Butyrate enema therapy has been shown to improve colitis in both mouse models and human clinical trials [7, 13]. However, orally administered sodium butyrate had only a limited effect on experimental colitis in a previous report [14].

In this study, we assessed the effect of butyrate on cytokine production in IEC and macrophages. We then evaluated the effect of butyrate on lipopolysaccharide (LPS)–induced IκBα degradation /phosphorylation, DNA–binding of NF–κB, and histone H3 deacetylation in IECs and macrophages. We next assessed the effect of butyrate on colitis in vivo by treating dextran sulfate sodium (DSS)–induced colitis in C57BL/6 wild type (WT) mice and piroxicam–accelerated colitis in IL–10 deficient (IL–10−/−) mice. The use of IL–10−/− mice and the peritoneal macrophages (PMs) of IL–10−/− mice helped us to show that the effects of butyrate on colitis are IL–10 dependent or IL–10 independent.
Materials and Methods

1. Ethical consideration
All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Seoul National University Hospital (No. 15-0043).

2. Mice and materials
Under specific pathogen-free conditions, IL-10\(^{-/-}\) C57BL/6 mice (9 weeks old, 21±1 g) and C57BL/6 WT mice (8 weeks old, 21±1 g) were used [15, 16]. Sodium butyrate (SB), piroxicam and LPS (\textit{Escherichia coli} 0127:B8) were provided by Sigma–Aldrich (St. Louis, MO). DSS was purchased from MP Biochemicals (Irvine, CA). Thioglycolate was supplied by Gibco (Invitrogen, Grand Island, NY). The Quantikine\textsuperscript{®} immunoassay Kit used for ELISA was obtained from R&D Systems (Minneapolis, MN), and the Lightshift\textsuperscript{®} Chemiluminescent EMSA Kit used for electrophoretic mobility shift assays (EMSA) was provided by Thermo Scientific Inc. (Rockford, IL). Anti-phosphorylated IκBα, anti–IκBα, anti-histone H3, and anti–acetyl-histone H3 (Lys9/Lys14) antibodies were supplied by Cell Signaling (Danvers, MA), and anti–β–actin and anti–NF–κB p50 antibodies were provided by
Santa Cruz Biotechnology (Santa Cruz, CA).

3. Preparation of cells
COLO 205 (human colon) and RAW 264.7 (mouse macrophage) cells were provided by the Korean Cell Line Bank (Seoul, Korea) [16]. The PMs of the IL-10$^{-/-}$ mice were induced using an intra-peritoneal injection of 4% thioglycolate and collected after 4 days via peritoneal lavage [17]. Pretreatment with or without butyrate (i.e., 100 or 500 μM/ml) was performed for 24 h.

4. ELISA for cytokine production
The production of IL-8 in COLO 205 cells and that of TNF-α, IL-6, IL-12 and IL-10 in RAW 264.7c cells were assessed after stimulation with 10 μg/ml of LPS for 4 h [17, 18]. To define the role played by the increase in IL-10 observed in macrophages, PMs of IL-10$^{-/-}$ mice were also tested. In addition, TNF-α, IL-6 and IL-12 production in PMs of IL-10$^{-/-}$ mice was assessed.

5. Western blot analysis of IκBα phosphorylation and histone acetylation
Anti-phosphorylated IκBα, anti-IκBα and anti-β-actin antibodies
were used to assess the change in the levels of phosphorylated 
IκBα and IκBα following stimulation with LPS [17]. The 
anti–acetyl–histone H3 (Lys9/Lys14) and anti–histone H3 
antibodies were also used to evaluate changes in histone H3 
acetylation following stimulation with LPS. To detect IκBα 
phosphorylation and degradation, cells were pretreated with or 
without butyrate (i.e., 100 or 500 μM/ml) and stimulated using 
LPS (10 μg/ml) for 30 min. To assess histone H3 acetylation, the 
pretreated cells were stimulated using LPS (10 μg/ml) for 8 h. 
For the quantitative analysis of IκBα phosphorylation and 
degradation, the ratio of the band density of the phosphorylated 
IκBα to that of the IκBα band was compared [19]. In the case of 
the acetylated histone H3, the density of the acetylated histone 
H3 band was compared to that of the β–actin. The band 
densities were measured using Image Gauge version 3.12 
(Fujifilm, Tokyo, Japan) and Luminescent Image Reader LAS 
1000–plus (Fujifilm) [20].

6. Electrophoretic mobility shift assay for 
detecting DNA binding of NF–κB

To detect changes in the DNA binding activity of NF–κB, a 
non–isotopic EMSA analysis was performed [21]. Pretreated 
COLO 205, RAW 264.7 cells and PMs of IL–10−/− mice were 
stimulated with LPS (10 μg/ml) for 1 h. A biotin–labeled probe
for the NF-κB consensus site was used to detect DNA-binding of NF-κB, and a supershift assay was performed using NF-κB p50 antibodies [18, 22]. A mutation test was performed using a mutated probe to determine the specific band that corresponded to NF-κB [18].

7. The effect of butyrate on acute DSS-induced colitis

An acute DSS-induced colitis model was used to confirm that butyrate had a protective effect in the colon. A total of 28 WT mice were allocated to the following four groups: 1) a control group (n=4), in which the mice were treated with only normal saline; 2) a vehicle group (n=8), in which the mice were administered 4% DSS in drinking water and treated with saline; 3) an SB 20 mg/kg group (n=8), in which the mice were administered 4% DSS and treated with butyrate (20 mg/kg/day); and 4) an SB 100 mg/kg group (n=8), in which the mice were administered 4% DSS and treated with butyrate (100 mg/kg/day). The mice were treated by oral gavage with butyrate beginning 2 days before 4% DSS administration [17]. During the experiment, body weight changes and changes in water consumption were measured among the groups. The mice were sacrificed on day 7, and colon length was then measured. The degree of colitis was assessed using a validated scoring scale [23, 24].
8. The effect of butyrate on colitis in IL-10\(^{-/-}\) mice

To determine whether the effect of butyrate on colitis was IL-10 dependent or IL-10 independent, we treated IL-10\(^{-/-}\) mice that had chronic colitis with oral butyrate for two weeks. To accelerate the induction of colitis, 200 ppm of piroxicam was added to the diet of IL-10\(^{-/-}\) mice for 2 weeks. The 28 mice were divided into the following four groups after colitis was induced using piroxicam: 1) a control group (n=4), in which no intervention was used; 2) a vehicle group (n=8), in which colitis was treated using normal saline for two weeks; 3) an SB 20 mg/kg group (n=8), in which colitis was treated using butyrate (20 mg/kg/day) for two weeks; and 4) an SB 100 mg/kg group (n=8), in which colitis was treated with butyrate (100 mg/kg/day) for two weeks. During the experiment, changes in body weight were measured. After the experiment, the mice were sacrificed by CO\(_2\) asphyxiation, and colon lengths were measured. The Berg’s grading system was used to assess the severity of colitis [25].

9. Immunoreactivity analysis of IκBα phosphorylation and histone H3 acetylation in the colon.
IkBa phosphorylation and histone H3 acetylation were assessed using immunohistochemical (IHC) staining to confirm that butyrate induced a protective effect in the colon. Immunoreactivity was determined using a visual scoring scale to evaluate the percentage of cells that were stained (0 to 4+) [18].

10. Statistical analysis
All data are presented as means ± SD. All statistical analyses were performed using Prism v5.0 software (GraphPad, La Jolla, CA). One-way analysis of variance (ANOVA) was used to compare differences among groups. Repeated measures ANOVA was used to compare changes in body weight among the groups. Tukey’s post hoc test was used to compare all pairs of groups. A p value less than 0.05 was considered to indicate statistical significance.
Results

1. Butyrate regulated the LPS-induced secretion of cytokines

The production of cytokines was assessed using ELISA to evaluate whether butyrate could inhibit LPS-induced cytokine production. These results are shown in Figure 1. LPS-induced IL-8 (Figure 1A) production was inhibited by butyrate (both 100 μM and 500 μM) in COLO 205 cells. In RAW 264.7 cells, the production of TNF-α (Figure 1B), IL-6 (Figure 1C) and IL-12 (Figure 1D) was strongly suppressed by butyrate (100 mM and 500 mM). However, we found that butyrate promoted the production of IL-10 (Figure 1E). To determine whether these anti-inflammatory effects were IL-10 dependent or IL-10 independent, we used PMs obtained from IL-10/− mice and found that the levels of TNF-α (Figure 1F), IL-6 (Figure 1G) and IL-12 (Figure 1H) were also strongly suppressed by butyrate (100 mM and 500 mM).
Figure 1. Butyrate regulates the LPS-induced secretion of cytokines.

(A) IL-8 in COLO 205 cells. COLO 205 cells were pretreated with two doses (i.e., 100 or 500 μM/ml) of butyrate for 24 h and then stimulated with 10 μg/ml lipopolysaccharide (LPS) for 4 h. The secretion of IL-8 from COLO 205 cells was measured using ELISA.

(B, C, D, E) ELISA of RAW 264.7 cells. RAW 264.7 cells were pretreated with two doses (i.e., 100 or 500 μM/ml) of
butyrate for 24 h and then stimulated with 10 μg/ml LPS for 4 h. The secretion of TNF-α (B), IL-6 (C), IL-12 (D) and IL-10 (E) was measured using ELISA.

(F, G, H) ELISA of peritoneal macrophages in IL-10 deficient mice. Peritoneal macrophages obtained from IL-10 deficient (PMs of IL-10−/−) mice were pretreated with two doses (i.e., 100 or 500 μM/ml) of butyrate for 24 h and then stimulated with 10 μg/ml LPS for 4 h. The secretion of TNF-α (F), IL-6 (F) and IL-12 (H) was measured using ELISA. Butyrate strongly suppressed pro-inflammatory cytokine production in these cells and induced the production of the anti-inflammatory cytokine IL-10 in RAW 264.7 cells. Butyrate suppressed the production of pro-inflammatory cytokines even in PMs obtained from IL-10−/− mice, indicating that the anti-inflammatory effect of butyrate on macrophages was IL-10 independent. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with LPS alone. These data are representative of three independent experiments.
2. Butyrate suppressed NF-κB signaling

To determine the effect of butyrate on NF-κB signaling, we assessed the levels of IκBα and phosphorylated IκBα using Western blot analysis. In COLO 205 and RAW 264.7 cells, IκBα was degraded, and the level of the phosphorylated form of IκBα was increased by LPS stimulation. These effects were markedly inhibited by treatment with butyrate (100 mM and 500 mM). Furthermore, the effects were also observed in PMs obtained from IL-10−/− mice. The results are shown in Figure 2A (COLO 205), Figure 2B (RAW 264.7) and Figure 2C (PMs of IL-10−/− mice). In Figure 2D (COLO 205), Figure 2E (RAW 264.7) and Figure 2F (PMs of IL-10−/− mice), the density ratio of phosphorylated IκBα to IκBα band increased with LPS stimulation and decreased significantly with butyrate treatment. Therefore, we found that inhibiting NF-κB signaling using butyrate was IL-10 independent. To confirm this inhibitory effect, the DNA binding activity of NF-κB was analyzed using EMSA. The results are shown in Figure 2G (COLO 205), Figure 2H (RAW 264.7) and Figure 2I (PMs of IL-10−/− mice). LPS stimulation increased DNA–binding activity (black arrow), and these DNA–binding activities were markedly inhibited by pretreatment with butyrate (100 mM and 500 mM) in those cells.
Figure 2. Butyrate suppresses the NF-κB signaling pathway.
(A, B, C) Western blot analysis of IκBα and phosphorylated IκBα. Western blot analysis of IκBα and phosphorylated IκBα levels in COLO 205 cells (A), RAW 264.7 cells (B) and peritoneal macrophages obtained from IL-10 deficient mice (C). The cells were pretreated with two doses (i.e., 100 or 500 μM/ml) of butyrate for 4 h and then stimulated with 10 μg/ml lipopolysaccharide (LPS) for 30 min. Pretreatment with butyrate attenuated LPS-induced IκBα phosphorylation and degradation.

(D, E, F) Relative band density ratio of phosphorylated IκBα to IκBα. The density ratios of phosphorylated IκBα to IκBα bands increased with LPS stimulation and decreased significantly with butyrate treatment in COLO 205 cells (D), RAW 264.7 cells (E) and peritoneal macrophages obtained from IL-10 deficient mice (F).

(G, H, I) Electrophoretic mobility shift assays of the DNA-binding activity of NF-κB. Electrophoretic mobility shift assays were used to analyze the DNA-binding activity of NF-κB in COLO 205 cells (G), RAW 264.7 cells (H) and peritoneal macrophages obtained from IL-10 deficient mice (I). The cells were pretreated with two doses (i.e., 100 or 500 μM/ml) of butyrate for 4 h and then stimulated with 10 μg/ml LPS for 1 h. A biotin-3′-end-labeled double-stranded DNA oligonucleotide probe (5′-agt tga ggg gac ttt ccc agg c-biotin-3′, 5′-gcc tgg gaa agt ccc ctc aac t-biotin-3′) corresponding to a consensus NF-κB binding site was used. The positions of the
NF-κB-specific complex are indicated by black arrows. The results showed that pretreatment with butyrate reduced LPS-induced DNA-binding of NF-κB. These data are representative of three independent experiments.
3. Butyrate reversed histone H3 acetylation

Butyrate is known to be an HDAC inhibitor [14]. We assessed the levels of acetylated histone H3 and histone H3 using Western blot analysis. Those results are shown in Figure 3A (COLO 205), Figure 3B (RAW 264.7) and Figure 3C (PMs of IL-10−/− mice). After the cells were stimulated with LPS, acetylated histone H3 levels were lower, and pretreatment with butyrate (100 mM and 500 mM) reversed histone H3 acetylation in these cells. In densitometry analysis, the density ratios of acetylated histone H3 to β-actin band decreased with LPS stimulation and increased with butyrate treatment. These results are also shown in Figure 3D (COLO 205), Figure 3E (RAW 264.7) and Figure 3F (PMs of IL-10−/− mice).
Figure 3. Butyrate inhibits histone H3 deacetylation.

(A, B, C) Western blot analysis of acetylated histone H3 and histone H3 levels. Western blot analysis of acetylated histone H3 and histone H3 levels in COLO 205 cells (A), RAW 264.7 cells (B) and peritoneal macrophages obtained from IL-10 deficient mice (C). The cells were pretreated with one of two doses (i.e., 100 or 500 μM/ml) of butyrate for 24 h and then stimulated with 10 μg/ml lipopolysaccharide (LPS) for 8 h. Pretreatment with butyrate increased the acetylation of histone H3.

(D, E, F) Relative band density ratio of acetylated histone
Histone H3 to β-actin. The density ratios of acetylated histone H3 to β-actin bands decreased with LPS stimulation and increased with butyrate treatment in COLO 205 cells (D), RAW 264.7 cells (E) and peritoneal macrophages obtained from IL-10 deficient mice (F). These data are representative of three independent experiments.
4. Butyrate attenuated acute DSS-induced colitis

In the Vehicle group, we observed a 15% loss in body weight, colon shortening, and higher colitis scores during the administration of 4% DSS. These results are presented in Figure 4. Oral treatment with butyrate (100 mg/kg/day) significantly prevented weight loss (Figure 4A), restored colon shortening (Figure 4B) and decreased colitis scores (Figure 4C).

Administration of DSS also induced the phosphorylation of IkBa and decreased histone H3 acetylation in the colonic mucosa. However, the phosphorylation of IkBa was attenuated (Figure 4D), and histone H3 acetylation was increased (Figure 4E) in mice treated with 100 mg/kg/day butyrate. Representative slides are shown in Figure 5.
Figure 4. Butyrate attenuates colitis by reducing IkBα phosphorylation and increasing histone H3 acetylation in dextran sulfate sodium (DSS)–induced acute colitis. (A) The effect of butyrate on changes in body weight. Oral administration of butyrate (100 mg/kg/day) resulted in
significantly lower body weight (*p < 0.05) than that of the vehicle group.

(B) Colon length changes. Oral administration of butyrate (100 mg/kg/day) reduced colon shortening. Bars indicate the means ± SD of the overall scores. **p < 0.01 compared with the vehicle group.

(C) Histological scores for colitis. Oral administration of butyrate (100 mg/kg/day) significantly attenuated histological scores in mice with acute colitis. Bars indicate the means ± SD of the overall scores. ***p < 0.001 compared with the vehicle group.

(D) Immunoreactivity index of phosphorylated IκBα in the colon. Oral administration of butyrate (100 mg/kg/day) inhibited IκBα phosphorylation. Bars indicate the means ± SD of the overall scores for phosphorylated IκBα. ***p < 0.001 compared with the vehicle group.

(E) Immunoreactivity index of acetylated histone H3 in laminar propria monocytes. Oral administration of butyrate (100 mg/kg/day) resulted in the induction of histone H3 acetylation. Bars indicate the means ± SD of the overall scores for phosphorylated IκBα. *p < 0.05 compared with the vehicle group. These data are representative of two independent experiments.
Figure 5. Representative slides obtained from mice with dextran sulfate sodium (DSS)-induced acute colitis. (A) Representative histological sections of colons obtained from each group (hematoxylin and eosin staining, x100). Colonic samples were obtained from mice in each group. The colons of DSS-treated mice showed that the epithelial architecture was destroyed, including the loss of crypts and epithelial integrity, and exhibited intense inflammatory cell infiltration. Oral administration of butyrate (100 mg/kg/day)
attenuated this damage.

(B) **Representative immunohistochemical staining** (phosphorylated IκBα [diluted 1:100], x200) in colons obtained from each group. In the vehicle group, tissues were heavily stained from phosphorylated IκBα in both destroyed epithelial cells and lamina propria inflammatory cells. Oral administration of butyrate (100 mg/kg/day) clearly attenuated the amount of phosphorylated IκBα staining in colon tissues.

(C) **Representative immunohistochemical staining** (acetylated histone H3 [diluted 1:800], x200) in colons obtained from each group. In the vehicle group, slight staining was observed for acetylated histone H3 in lamina propria inflammatory cells. Oral administration of butyrate (100 mg/kg/day) clearly increased the amount of acetylated histone H3 staining in lamina propria inflammatory cells.
5. Butyrate suppressed chronic colitis in IL-10⁻/⁻ mice

In a previous study, Chang et al. found that butyrate regulated intestinal macrophages [14], which are key players in chronic colitis in IL-10⁻/⁻ mice [26]. By using IL-10⁻/⁻ mice, we were able to estimate the therapeutic effect of butyrate in chronic colitis and determine whether the therapeutic effect of butyrate was IL-10 dependent or IL-10 independent. Oral treatment with 100 mg/kg of butyrate suppressed colitis. This group showed significantly lower histological scores than the vehicle group (Figure 6A). The phosphorylation of IκBα was also attenuated (Figure 6B), while the acetylation of histone H3 was increased (Figure 6C) in mice treated with 100 mg/kg/day butyrate. Representative slides are shown in Figure 7.
Figure 6. Butyrate attenuates colitis by reducing IkBα phosphorylation and increasing histone H3 acetylation in IL-10 deficient mice with chronic colitis.

(A) Histological grading scores in the proximal colon. Oral administration of butyrate (100 mg/kg/day) reduced chronic colitis in the proximal colon. The data are presented as means ± SD. **p < 0.01 compared with the vehicle group.

(B) Immunoreactivity index for phosphorylated IkBα in the colon. Oral administration of butyrate (100 mg/kg/day) significantly inhibited IkBα phosphorylation. Bars indicate the
means ± SD of the overall scores for phosphorylated IkBa. *p < 0.05 and **p < 0.01 compared with the vehicle group.

(C) Immunoreactivity index for acetylated histone H3 in lamina propria monocytes. Oral administration of butyrate (100 mg/kg/day) induced histone H3 acetylation. Bars indicate the means ± SD of the overall scores for phosphorylated IkBa. *p < 0.05 compared with the vehicle group. These data are representative of two independent experiments.
Figure 7. Representative slides showing tissues obtained from IL-10 deficient mice with chronic colitis.

(A) Representative histological sections of proximal colons obtained from each group (hematoxylin and eosin stain, x100). Colons extracted from mice treated with vehicle exhibited completely destroyed epithelial architecture, including deep ulcerations and intense submucosal inflammatory cell infiltration. Oral administration of butyrate (100 mg/kg/day) reduced the severity of chronic colitis in the proximal colon.
(B) Representative immunohistochemical staining (phosphorylated IκBa [diluted 1:300], x200) in colonic samples. Sections obtained from untreated mice with piroxicam–induced chronic colitis showed heavy staining for phosphorylated IκBa in both the destroyed epithelium and lamina propria inflammatory cells. The oral administration of butyrate (100 mg/kg/day) clearly reduced the intensity of phosphorylated IκBa staining in colon tissues.

(C) Representative immunohistochemical staining (acetylated histone H3 [diluted 1:800], x200) in colon tissues obtained from each group. In the vehicle group, lamina propria inflammatory cells were slightly stained for acetylated histone H3. The oral administration of butyrate (100 mg/kg/day) clearly enhanced the intensity of acetylated histone H3 staining in lamina propria inflammatory cells.
Discussion

We found that oral supplementation with butyrate suppressed experimental murine colitis, attenuated the phosphorylation of \( \text{IkB}\alpha \) and reversed the acetylation of histone H3 in colonic tissues. In the present study, butyrate attenuated pro-inflammatory cytokine production in both IECs and macrophages and induced the expression of IL-10, an anti-inflammatory cytokine, in macrophages. The phosphorylation of \( \text{IkB}\alpha \) and the DNA-binding activity of NF-\( \kappa \)B were suppressed, and histone H3 acetylation was increased by treatment with butyrate in both IECs and macrophages. These results were IL-10 independent because butyrate also had an anti-inflammatory effect on both chronic colitis in and PMs obtained from IL-10\(-/-\) mice. This study is the first to evaluate the mechanism underlying the effect of orally administered butyrate in piroxicam-induced chronic colitis in IL-10\(-/-\) mice.

As previously reported, we found that butyrate increased the production of IL-10 in macrophages [11, 27]. IL-10 is known as an anti-inflammatory cytokine that inhibits the production of pro-inflammatory cytokines by inflammatory cells. The IL-10 produced by intestinal macrophages regulates mucosal immune responses and prevents the progression of colitis. Furthermore, transferring IL-10-positive macrophages has been shown to reduce mortality in infectious colitis in IL-10\(-/-\) mice [28]. In
other studies, impaired function of IL-10 or the IL-10 receptor caused severe spontaneous colitis not only in IL-10−/− mice but also in humans [29, 30]. Butyrate also up-regulated histone H3 acetylation, which led to the expression of Foxp3 and induced the development of Treg cells [31]. IL-10 producing Treg cells are also important for maintaining homeostasis in the colon and preventing colitis [31]. Therefore, the up-regulation of IL-10 has been suggested as one of the mechanisms potentially underlying the anti-inflammatory effect of butyrate [11, 32]. However, in our study, we demonstrated that the anti-inflammatory effect of butyrate on colitis is mainly IL-10 independent. The production of pro-inflammatory cytokines and the activation of NF-κB were both inhibited by butyrate, even in PMs obtained from IL-10−/− mice. The direct comparison of cytokine production from PMs obtained from wild-type and IL-10−/− mice is shown in Figure 8. The PMs from IL-10−/− mice showed significantly higher production of TNF-α (Figure 8A) and IL-6 (Figure 8B) with LPS stimulation. However, butyrate significantly suppressed the production of cytokines in both macrophages. Furthermore, the colitis observed in IL-10−/− mice was improved by the oral administration of butyrate.
Figure 8. Direct comparison of cytokine production from peritoneal macrophages obtained from wild type and IL-10 deficient mice.
The secretion of TNF-α (A), IL-6 (B), IL-12 (C) was measured using ELISA. The peritoneal macrophages (PM) from IL-10−/− mice showed significantly higher production of TNF-α (A) and IL-6 (B) than PM from wild-type mice by LPS stimulation. However, the butyrate significantly suppressed the production of cytokines in both macrophages, indicating that the anti-inflammatory effect of butyrate on macrophages was IL-10 independent. **p < 0.01 and ***p < 0.001 compared to LPS alone. These data are representation of two independent experiments.
Butyrate was absorbed by the IECs and used as an energy source. In this study, butyrate attenuated the production of IL-8 and the activation of NF-κB in COLO 205 cells. The regulation of NF-κB is important for maintaining intestinal homeostasis, and NF-κB activity was up-regulated in the inflamed mucosa of DSS-induced colitis and IBD patients. Until now, the inhibition of NF-κB was considered a therapeutic strategy for treating IBD [33]. NF-κB is regulated by binding with inhibitory molecules such as IκBa. In the present study, butyrate prevented the degradation and phosphorylation of IκBa and attenuated the DNA-binding activity of NF-κB. Butyrate was previously reported to influence NF-κB by inhibiting the degradation of IκBa via attenuating the activity of the cellular proteasome [9, 10, 34]. Our study data support those results.

HDAC regulates the acetylation status of histones, and the epigenetic remodeling of histones regulates macrophages. Butyrate is known as a pan-HDAC inhibitor that mainly affects the activity of class I and II HDACs. HDAC3 (class I) is important for the activation of macrophages. HDAC3-deficient macrophages exhibited reduced expression levels of inflammatory cytokines following stimulation with LPS. In the present study, pretreatment with butyrate enhanced histone H3 acetylation and reduced inflammatory cytokine production in macrophages.
A previous study by Chang et al. indicated that butyrate had immunomodulatory effects in intestinal macrophages but that oral administration of butyrate did not ameliorate colitis [14]. This result was obtained because in the study by Chang et al., the authors used only 0.1 to 10 mg/kg of butyrate to treat murine colitis. In this present study, 100 mg/kg of butyrate was effective, while 20 mg/kg of butyrate was not. This discrepancy was observed because butyrate has a short half-life in mice. We overcame this issue by administering a relatively high dose of butyrate [35].

Recently, Zhang et al. reported that butyrate modified the composition of microbiota in IL-10−/− mice [36]. The authors induced IL-10−/− mice to develop spontaneous colitis and then treated them with oral butyrate. However, the spontaneously developed colitis was not consistent among the mice [25, 37]. The colitis depended on environmental conditions and the ages of the mice and manifested as different degrees of inflammation. To overcome this issue, in this study, we used piroxicam to accelerate the development of colitis. By using piroxicam, the disease course became predictable and resulted in similar degrees of colitis. Therefore, our piroxicam-accelerated chronic colitis model in IL-10−/− mice is more adaptable to tests of new therapies for IBD [17, 25, 37, 38].
To apply our results in humans, it is most important to establish safety and tolerability. Several human studies have shown that butyrate has beneficial effects in rectal enemas [12, 39–42]. However, enemas cause patient’s discomforts and possibly mucosal irritability resulting from their acidic properties. Furthermore, the use of orally administered butyrate is limited because of its unpleasant odor and taste. To overcome these disadvantages, other studies have reported that using a fermentable dietary fiber supplement resulted in increased fecal butyrate levels, which led to butyrate-releasing derivatives and microbiota that are capable of producing butyrate in the colon. These options are therefore alternatives to butyrate [43, 44].

In conclusion, our results indicate that an oral supplement containing butyrate attenuated experimentally induced murine colitis by blocking NF-κB signaling and enhancing histone acetylation. This anti-colitic effect of butyrate was IL-10 independent. Sodium butyrate could therefore be a therapeutic agent for treating colitis.
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국문 초록

서론: 부틸레이트는 대장 내 미생물에 의해 식이섬유가 소화되면서 발생하는 단쇄지방산의 하나로 항염증 효과가 알려져 있다. 하지만, 아직까지 대장염에서 부틸레이트 경구요법의 치료효과는 알려진 바 없다. 본 연구에서는 부틸레이트 경구 요법이 마우스 대장염에 미치는 효과 및 기전을 연구하였다.

방법: 사람 대장세포주인 COLO 205, 마우스 대식세포주인 RAW 264.7 그리고, IL-10 결핍 마우스의 복강 대식세포를 활용하였다. 각 세포주에 부틸레이트 전처치 후 LPS자극으로 증가되는 사이토카인의 분비를 ELISA를 이용해 측정하였다. 부틸레이트의 항염증 기전을 파악하기 위해 IkBa의 인산화 및 분해, 그리고 히스톤 H3의 아세틸화를 western blot을 이용하여 평가하고 EMSA (electrophoretic mobility shift assay)를 이용해 NF-κB의 DNA 결합을 측정하였다. 이러한 항염증 효과가 생체 내에서도 일어나는지 확인하기 위해 DSS 유도 급성 대장염 모델과 IL-10 결핍 마우스의 만성 대장염 모델에 부틸레이트를 20 mg/kg와 100 mg/kg의 농도로 경구 투여하였다. 이후 조직학적 분석을 통해 대장염을 평가하고, 면역염색을 통해 대장조직의 인산화 IkBa 및 아세틸화 히스톤 H3를 측정했다.

결과: 부틸레이트는 LPS에 의해 유도되는 염증성 사이토카인, 즉 COLO 205의 IL-8, RAW 264.7의 TNF-α 및 IL-6, IL-12의 분비를 억제하였고, 항염증 사이토카인인 IL-10의 분비를 촉진하였다. 또한, 각 세포내에서 IkBa의 인산화 및 분해를 억제하고 NF-κB의 DNA 결합을 방해하며, 히스톤 H3의 아세틸화를 회복시켰다. 이러한 효과는 IL-10 결핍 마우스의 복강세포에서도 마찬가지로 확인되었다. 부
틸레이트 100 mg/kg의 경구 투여는 IL-10 결핍 마우스 및 DSS 대장염 모델에서 대장염을 완화시키고, 대장점막의 IkBa 인산화를 억제하며, 히스톤 H3의 아세틸화를 회복시켰다.

결론: 부틸레이트 경구투여는 IL-10 분비와는 독립적으로 NF-κB 신호전달 경로를 억제하고, 히스톤의 탈아세틸화를 억제하며 대장염을 완화시켰다. 결론적으로 부틸레이트는 염증성 장질환 치료 후보 물질로 활용될 수 있을 것이다.

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주요어 : 부틸레이트; 히스톤탈아세틸화 효소 억제제; 엔에프-카파비; 염증성장질환; 마우스 모델
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