Butyrate enhancement of interleukin-1β production via activation of oxidative stress pathways in lipopolysaccharide-stimulated THP-1 cells

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In inflammatory bowel diseases, interleukin-1β production is accelerated. Butyrate, a short chain fatty acid, plays an important role in inflammatory bowel diseases. We investigated the effect of butyrate on interleukin-1β production in macrophage and elucidated its underlying mechanism. We stimulated THP-1 cells, a human premonocytic cell line, by lipopolysaccharide alone and by butyrate with lipopolysaccharide. Butyrate with lipopolysaccharide increased interleukin-1β production more than lipopolysaccharide alone. Butyrate with lipopolysaccharide increased caspase-1 activity more than lipopolysaccharide alone. As for the phosphorylation pathway, PD98059 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK1/2 inhibitor) decreased caspase-1 activity and interleukin-1β production to approximately 50% of the controls. Pertussis toxin (G protein-coupled signal transduction pathway inhibitor) also reduced interleukin-1β production to approximately 50%. Butyrate with lipopolysaccharide increased reactive oxygen species levels more than lipopolysaccharide alone. The addition of N-acetyl L-cysteine reduced reactive oxygen species levels to a level similar to that of lipopolysaccharide alone. Butyrate with lipopolysaccharide increased nitric oxide production more than lipopolysaccharide alone, and the addition of N-acetyl L-cysteine reduced the elevated amount of nitric oxide. In conclusions, butyrate enhances interleukin-1β production by activating caspase-1, via reactive oxygen species, the phosphorylation of MAPK, and G protein mediated pathways in lipopolysaccharide stimulated THP-1 cells.

Key Words: butyrate, macrophage, interleukin-1β (IL-1β), caspase-1, reactive oxygen species (ROS)

Although IL-1β is regulated by p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases (ERKs) in human smooth muscle cells, the mechanism by which butyrate induces IL-1β production in monocyte-macrophages is not yet understood. In the present study, we investigated the effect of butyrate on the expression of IL-1β and caspase-1 activity in THP-1 cells, a human premonocytic cell line, and characterized the signal transduction pathways by which butyrate induces IL-1β production.

Materials and Methods

Materials. Lipopolysaccharide (LPS; Salmonella typhosa 0901) was purchased from Difco (Detroit, MI); PD98059, SB203580, SP600125, and GF109203X from Calbiochem (La Jolla, CA); Diphenyleneiodonium chloride from Sigma Chem. (St. Louis, MO); Ac-YVAD-CHO from BIOMOL (Plymouth, PA); and pertussis toxin (PTX) from Wako Pure Chem. (Osaka, Japan). Oligonucleotide primers were synthesized and purified by BEX (Tokyo, Japan). Total cell protein concentrations were determined using a DC protein assay kit (Bio-Rad Lab., Hercules, CA) with bovine serum albumin (Bio-Rad) as the standard. All other chemicals and materials (except those described below) were obtained from Nacalai Tesque (Kyoto, Japan).

Cells. THP-1 cells (ATCC, Manassas, VA) were cultured in RPMI 1640 or HAM’s F12 medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) with 292 µg/ml L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37°C and under 5% CO2. Differentiation to macrophages was induced with 100 nmol/l phorbol 12-myristate 13-acetate (PMA, Sigma) for 48 h.7 Cell viability was examined using an MTT cell viability assay kit (R&D Systems, Minneapolis, MN). The value for cell viability of the positive control cells, which were treated with 0.1 µg/ml LPS with or without 1 mmol/l butyrate in 0.5% dimethylsulfoxide (DMSO, v/v), were standardized as 100%. Incubation medium with or without substrates in all experiments did not significantly affect cell viability (data not shown).

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Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from THP-1 cells (5.0 × 10⁶) using TRIzol reagent (Invitrogen, Carlsbad, CA). Single-strand cDNA was synthesized from 1 μg of total RNA using an RT-PCR reverse transcription kit (Maxim Biotech, San Francisco, CA). Incubation was carried out at 37°C for 60 min. The temperature of the reaction was then raised to 94°C for 5 min in order to make the enzyme inactive and was then reduced to 4°C. PCR amplification was performed using a Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA). For semi-quantitative analysis, the linearity of amplification of IL-1β and GAPDH cDNA, depending on the PCR cycle number, was established in preliminary experiments. A total of 30 cycles for IL-1β and GAPDH were performed. Products were analyzed by Kodak 1-D Image Analysis software (Eastman Kodak Co. Ltd., NY). The GAPDH primers used were 5'-CCACCCATGGCAATTTCCATGGA-3' and 5'-TCTAGACGGCAGTCGGTCCACC-3'. These were designed to yield a 696-bp product. The IL-1β primers used were 5'-AAACAGATGAATGCTCCTTCCA-3' and 5'-GAGAACCACCTTGTTGCTCCA-3'. These were designed to yield a 389-bp product.

IL-1β protein production. THP-1 cells were seeded onto a 24-well plate at a density of 1.0 × 10⁶ cells/well in RPMI 1640 medium with 292 μg/ml L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS and cultured at 37°C for 48 h under a humidified atmosphere of 5% CO2. After differentiation with 100 nmol/l PMA, cells were washed with PBS and treated with PBS or 0.1 μg/ml LPS alone, 1 mmol/l butyrate with 0.1 μg/ml LPS for 3, 6, 12, and 24 h. The control cells were treated only with phosphate buffered saline (PBS), and positive control cells were treated with LPS alone. IL-1β protein concentration in the medium was examined by IL-1β Human ELISA Kit (R&D Systems). The optical density for each specimen were determined at λex 450 nm, λem 550 nm using a Molecular Devices microplate reader (Wako).

Caspase-1 activity. Caspase-1 activity was determined using a caspase-1 fluorometric assay kit (R&D Systems). Cells treated with or without chemicals for 6 h were washed in cold PBS at 3 times, then re-suspended in 400 μl of cold lysis buffer and incubated on ice for 10 min. The cell lysates were pelleted, followed by transfer of the supernatants to microcentrifuge tubes. 50 μl of 2× reaction buffer with 1 M dithiothreitol (DTT) and 5 μl of Caspase-1 fluorogenic substrate (WEHD-AFC) were added to each well, followed by 37°C for 2 h incubation. A control reaction of treated cells without WEHD-AFC was incubated. The fluorometrical density for each specimen were determined at λex 400 nm, λem 505 nm using a GENios microplate reader (Wako). The results are expressed as fold increase, with caspase-1 activity seen in 0.5% DMSO treated cells (the vehicle control) standardized as 1-fold.

Western blotting. For Western blot analysis, 5 × 10⁶ cells were lysed in M-PER Mammalian Protein Extraction reagent (Pierce, Rockford, IL). Denature proteins were separated using SDS-polyacrylamide gel electrophoresis on a 10 or 15% polyacrylamide gel and then transferred onto Immobilon-P membranes (Millipore, Billerica, MA). We used rabbit anti-human phospho-p38 MAPK, rabbit anti-human p38 MAPK, rabbit anti-human phospho-extracellular signal-regulated kinase1/2 (ERK1/2), rabbit anti-human ERK1/2, rabbit anti-human phospho-c-Jun NH2-terminal kinase1/2 (JNK1/2), rabbit anti-human JNK1/2, rabbit anti-human phospho-mitogen-activated protein kinase kinase1/2 (MEK1/2), rabbit anti-human MEK1/2 (R&D Systems, Minneapolis, MN), rabbit anti-human Caspase-1 (BIOMOL International), rabbit anti-human actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-rabbit antibody horseradish peroxidase-linked immunoglobulin G (IgG) antibodies (GE Healthcare, Bucks, UK), or nonlabeled rabbit IgG antibodies (Wako Pure Chem.). The blots were developed using an ECL (GE Healthcare) and the images were then analysed by Kodak 1-D Image Analysis software. The ratios of protein expression levels of protein were determined by dividing the band intensity of the product of interest by that of the corresponding actin band.

Effects of specific inhibitors. Differentiated cells were treated with 0.5% (v/v) DMSO alone (vehicle), 1 mmol/l butyrate with 0.1 μg/ml LPS, or 1 mmol/l butyrate with 0.1 μg/ml LPS in 50 mmol/l of PD98059 (PD), 50 mmol/l of SB203580 (SB), 10 mmol/l of SP600125 (SP), 10 mmol/l of GF109203X (GF), 10 mmol/l of diphenyleneiodonium (DPI), 20 mmol/l of N-acetyl
L-cysteine (NAC), or 100 mmol/l of Ac-YVAD-CHO (YVAD), which were dissolved in DMSO.\(^9\) The positive control cells were treated with 0.1 μg/ml LPS alone for 6 h (caspase-1 assay) or 24 h (IL-1β assay).

**Effects of PTX.** THP-1 cells were treated with 100 ng/ml PTX at 37°C for 60 min. They were then treated with 0.1 μg/ml LPS alone or 1 mmol/l butyrate with LPS in RPMI 1640 medium for 24 h.

**Detection of reactive oxygen species products.** The intracellular production of reactive oxygen species (ROS) was assessed using a 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate fluorescence probe (carboxy-H₂DCF-DA, Invitrogen, Carlsbad, CA).\(^9\) THP-1 cells were treated with 0.1 mg/ml LPS for 60 min with or without 1 mmol/l butyrate, or with or without 20 mmol/l NAC for 60 min. Then, cells were exposed to 10 mmol/l carboxy-H₂DCFDA in 1 ml PBS for 30 min at 37°C. Cells were washed twice, re-suspended in 1 ml PBS, and analyzed by fluorescence on a FACSCanto (Becton Dickson, Mountain View, CA). The intensity of DCF in the cells was analyzed by WinMDI ver 2.8 software (http://facs.scripps.edu/software.html).

**Nitrite/nitrate quantification.** THP-1 cells were seeded onto a 24-well plate at density of 1.0 × 10⁶ cells/well in HAM’s F12 medium. Differentiation cells were treated with 0.5 μg/ml LPS alone or 1 mM butyrate with LPS, and vehicle (0.5% DMSO, v/v) alone, butyrate with LPS and 20 mM NAC, and LPS with NAC for 6, 12, 24 h in HAM’s F12 medium. Nitric oxide (NO) production from incubate medium was determined by measuring the amount of nitrite/nitrate (NO₂⁻/NO₃⁻), a stable oxidation end product of NO, NO₂⁻/NO₃⁻ Assay Kit-C2 (DOJINDO Lab., Kumamoto, Japan). The optical density for each specimen were determined at 540 nm using a Molecular Devices microplate reader (Wako).

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Fig. 3. Effects of butyrate and lipopolysaccharide (LPS) on caspase-1 expression in THP-1 cells. After differentiation, cells were incubated with phosphate buffered saline (PBS); control, 1 mmol/l butyrate alone, 0.1 μg/ml LPS alone, or 1 mmol/l butyrate with 0.1 μg/ml LPS for 0, 3, 6, 12, and 24 h. Expression of caspase-1 protein and actin was determined by Western blotting. Upper panels show the representative pictures of caspase-1 and actin protein expressions. In lower panels, the values in three independent experiments are expressed as fold increase (the mean ± SD) relative to that of the controls. **p<0.01 vs the controls, by Kruskall-Wallis variance analysis and Dunnet’s multiple comparison test.
Protein determination. Total protein concentrations in lysed THP-1 cells were determined using a DC Protein Assay kit (Bio-Rad Lab.), according to the protocol of the manufacturer, with bovine serum albumin (Bio-Rad Lab.) employed as the standard.

Statistical analysis. Data are expressed as the mean ± SD. Date were assessed by Kruskall-Wallis variance analysis and Dunnet’s multiple comparison test or the Mann-Whitney U test. Statistical analysis was performed by GraphPad Prism ver 4.0 (GraphPad Software Inc., San Diego, CA). Two-tailed values of p<0.05 were considered to indicate statistical significance.

Results

Butyrate with LPS increases expression of IL-1β mRNA and protein production. Preliminary experiments using >2 mmol/l butyrate induced excessive toxicity and apoptosis after 24 h (data not shown). A 1 mmol/l butyrate concentration was therefore used in all subsequent experiments. The expression of IL-1β m-RNA was not clearly observed in either the controls or the butyrate alone-treated cells. Both LPS alone and butyrate with LPS stimulations elevated the expression of IL-1β mRNA levels (Fig. 1). After 24 h, IL-1β mRNA levels in both stimulations remained elevated to a similar level (data not shown).

LPS alone increased IL-1β protein production in a time-dependent manner compared to those in the controls or in butyrate alone-treated cells. Butyrate with LPS significantly increased IL-1β protein production compared to those in LPS alone-treated cells (Fig. 2).

Butyrate with LPS enhances caspase-1 expression. Next, we determined the caspase-1 protein expression. There was no difference of caspase-1 expression in the controls or the butyrate alone-treated cells. LPS alone significantly increased caspase-1 expression: more than 3-fold above the controls. Butyrate with LPS also increased caspase-1 expression to a similar level, which seems to match an earlier trend compared to LPS alone (Fig. 3 (a) and (b)). These results suggested that IL-1β production in THP-1 cells by LPS alone or butyrate with LPS was mediated by caspase-1 activation.

Effect of kinase inhibitors and antioxidants on caspase-1 activity and IL-1β expression. First, to determine whether butyrate affects the early activation of the MAPKs pathways to induce IL-1β release, we examined phosphorylation of p38 MAPK, c-Jun NH₂-terminal kinase1/2 (JNK1/2), mitogen-activated protein kinase kinase1/2 (MEK1/2), and extracellular signal-regulated kinase1/2 (ERK1/2) was analyzed by Western blotting as described in Materials and Methods. Actin was used as the internal control. The pictures are representative results from three independent experiments.

Fig. 4. Effects of lipopolysaccharide (LPS) and butyrate on mitogen-activated protein kinase (MAPK) cascade pathways. Cells were seeded onto a 60-mm tissue culture dish at a density of 5 x 10⁶ cells/dish and then cultured at 37°C for 48 h under a humidified atmosphere of 5% CO₂. After differentiation, cells were treated with phosphate buffered saline (PBS); control, 1 mmol/l butyrate alone, 0.1 μg/ml LPS alone, or 1 mmol/l butyrate with 0.1 μg/ml LPS for 5, 10, 20, 30, 60, 120, and 180 min. The phosphorylation of p38 MAPK, c-Jun NH₂-terminal kinase1/2 (JNK1/2), mitogen-activated protein kinase kinase1/2 (MEK1/2), and extracellular signal-regulated kinase1/2 (ERK1/2) was analyzed by Western blotting as described in Materials and Methods. Actin was used as the internal control. The pictures are representative results from three independent experiments.

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**Fig. 5.** Effects of kinase inhibitors and an antioxidant on caspase-1 activity and interleukin-1β (IL-1β) in THP-1 cells treated with butyrate and lipopolysaccharide (LPS). After differentiation, cells were seeded onto a 60-mm dish (caspase-1) or a 24-well plate (IL-1β) at a density of 1 × 10⁶ cells/well and 5 × 10⁵ cells/dish, respectively, in RPMI 1640 medium. After washing, the cells were treated with the vehicle (0.5% v/v dimethylsulfoxide (DMSO)), 100 mmol/l Ac-YVAD-CHO (YVAD), 50 mmol/l PD98059 (PD), 50 mmol/l SB203580 (SB), 10 mmol/l SP600125 (SP), 20 mmol/l N-acetyl L-cysteine (NAC), 10 mmol/l diphenyleneiodonium (DPI), and 10 mmol/l GF109203X (GF). Additional inhibitors or antioxidants cells were incubated at 37°C for 20 min, and then were treated with 0.1 μg/ml LPS alone or 1 mmol/l butyrate with 0.1 μg/ml LPS for 24 h. (a) shows the caspase-1 activity by fluorometric assay. The values in five independent experiments are expressed as fold increase (the mean ± SD) relative to that of the controls. (b) shows IL-1β concentrations measured by ELISA kit. The values in five independent experiments are expressed as the mean ± SD. *p<0.05 vs LPS alone, **p<0.01, ***p<0.001 vs butyrate with LPS, by Kruskall-Wallis variance analysis and Dunnet's multiple comparison test.

**Fig. 6.** Effects of pertussis toxin (PTX) on interleukin-1β (IL-1β) production in THP-1 cells. Cells were seeded onto a 24-well plate at a density of 1 × 10⁶ cells/well, and then cultured at 37°C for 24 h under a humidified atmosphere of 5% CO₂. After differentiation, cells were treated with the 100 ng/ml PTX, and incubating at 37°C for 1 h. They were then treated with 0.1 μg/ml lipopolysaccharide (LPS) alone or 1 mmol/l butyrate with 0.1 μg/ml LPS for 24 h. IL-1β protein concentrations in the medium were examined by ELISA kit. The values in five independent experiments are expressed as the mean ± SD. Statistical analysis was performed using the Mann-Whitney U test. **p<0.01, vs without PTX treatment.

**Fig. 7.** Effects of butyrate and N-acetyl L-cysteine (NAC) on reactive oxygen species (ROS) production in THP-1 cells. After differentiation, cells were incubated with 0.1 μg/ml lipopolysaccharide (LPS) alone or 1 mmol/l butyrate with 0.1 μg/ml LPS, or 1 mmol/l butyrate with 0.1 μg/ml LPS and 20 mmol/l NAC, for 1 h. Then, cells were stained with carboxy-H2DCFDA for 30 min. The amount of ROS was determined by FACS analysis. (a) shows the effect of LPS alone and LPS with butyrate. (b) shows the effects of butyrate with LPS, and butyrate with LPS and NAC. Each result is a representative one from three independent experiments.

Butyrate with LPS enhances nitrite/nitrate production via ROS generation. Next, to determine influence of butyrate on ROS production, we used flow cytometry to measure intracellular ROS in THP-1 cells treated with LPS alone and butyrate with LPS. As shown in Fig. 7(a), the control cells exhibited constitutive basal levels of ROS, and the LPS increased these. Butyrate with LPS increased ROS levels to an even greater extent. The addition of NAC, however, reduced ROS levels to a level similar to that seen in LPS alone-treated cells (Fig. 7(b)). We also examined the effects of nitrite/nitrate production. Butyrate with LPS significantly increased nitrite/nitrate production compared to LPS alone (Fig. 8). The addition of NAC resulted in a significant reduction in the elevated amount of nitrite/nitrate by butyrate with LPS.
1,4,5-trisphosphate, increases intracellular Ca$^{2+}$ and increased the intracellular Ca$^{2+}$ level by SCFAs and that excessive ROS stimulate MAPK pathway phosphorylation and protein kinase C (PKC), an enzyme involved in immune reactions. It has been shown that GPR41 receptors are coupled with Gi/o and that their activation induces the formation of inositol 1,4,5-trisphosphate, increases intracellular Ca$^{2+}$ concentration and ERK1/2 activation, and decreases intracellular cyclic adenosine monophosphate (cAMP). In this study, we were able to identify the association of Gi protein, although we did not examine the direct association of GPR41 in IL-1$\beta$ production by butyrate. In the future, we aim to clarify the detailed mechanisms involved in GPR41 in IL-1$\beta$ production by butyrate.

NADPH oxidase is the primary enzyme for ROS generation in phagocytic cells. Excessive ROS stimulate MAPK pathway activation and activate MAPKs promote caspase-1 and IL-1$\beta$ expression. Our results seem to be consistent with these reports. Therefore, we postulated that butyrate may lead to accelerated NADPH oxidase activity resulting in increased activation of p38 MAPK, JNK 1/2, MEK1/2, and ERK1/2 pathways, the activation of caspase-1, and finally more mature efficient IL-1$\beta$ production. However, there is also a possibility that ROS may directly activate caspase-1 through a redox-sensitive cysteine residue. Because p38 MAPK, JNK, MEK inhibitors are not fully effective and because an increase in caspase-1 expression was observed earlier in the butyrate-treated cells than in cells given LPS alone, it appears that partial intervention of the direct path needs to be considered.

SCFAs such as acetate, propionate, and butyrate are produced by anaerobic bacterial fermentation of undigested carbohydrates in the colon, and they are rapidly absorbed to provide energy for the colorectal epithelium by means of increased blood flow in rectal mucosa. SCFAs also induce apoptosis in a number of colorectal tumor cell lines. On the other hand, butyrate has anti-inflammatory effects in the colon, and it also has a prominent immunity regulation effect by modulating IL-8 inflammatory protein 2 (MIP-2) activities and suppressing the NF-κB activity, leading to the regulation of inflammatory cytokines such as TNF-α, IL-6, IL-8, and others.

However, the results of a controlled clinical trial that used enemas containing sodium butyrate showed that butyrate was not effective in the treatment of UC, suggesting that butyrate's immune control mechanisms use pathways that may lead to both suppression and aggravation. As shown in this study, butyrate may have a significant influence on the production of IL-1$\beta$. Our data can be applied to strategies for preventing anaerobic bacteria from producing butyrate, which may be an effective treatment of IBD. Pharmacological inhibition of p38 MAPK, JNK 1/2, MEK1/2, and ERK1/2 seems to be beneficial effects for the treatment of inflammatory responses based on experiments with animal models and IL-1$\beta$ has been reduced by the inhibition of p38 MAPK phosphorylation in the rat dentate gyrus in vitro.

This study has some limitations. For example, we found that
butyrate-mediated induction of activated p38 MAPK occurred within 10 min, nitrite detection outside the cell occurred after 6 h, and intracellular detection of ROS occurred after 60 min. These time lags may be due to the reaction time for NO detection in the medium and the reagent sensitivity of the DCFH-DA fluorescent probe for intracellular ROS detection. Moreover, the LPS concentration (0.1 μg/ml) used to alter NO production in the culture medium was below the lower detection limit. A higher concentration (0.5 μg/ml) should be used to examine these problems.

In conclusion, butyrate enhances IL-1β production by activating caspase-1 via ROS and the phosphorylation of MAPK pathways and the Gi protein mediated pathway in LPS stimulated THP-1 cells.

Acknowledgments

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Abbreviations

carboxy-H2DCF-DA 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate
DMSO Dimethylsulfoxide
DPI Diphenyleneiodonium
ERKs Extracellular signal-regulated kinases

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