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Butyrate suppresses murine mast cell proliferation and cytokine production through inhibiting histone deacetylase

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Abstract

Beyond their nutritional impact to colonic epithelial cells, the intestinal microbiota metabolite butyrate has pleotropic effects to host cells and is known for its beneficial effects on intestinal homeostasis and metabolism. However, it remains unclear how it modulates mast cell function. Here, we demonstrate that butyrate profoundly inhibited proliferation of mouse mastocytoma P815 cells through inducing cell cycle arrest and apoptosis, as well as decreasing c-Kit activation. In addition, butyrate increased early- and late-stage apoptotic P815 cells. In murine bone marrow-derived mast cells (BMMC), butyrate-suppressed FcεRI-dependent tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) release without affecting β-Hexosaminidase, but that was associated with decreased mitogen-activated protein kinase extracellular signal-regulated kinase 1/2, p38 and c-Jun N-terminal kinases activation. Butyrate treatment substantially enhanced histone 3 acetylation in both P815 and BMMC and decreased FcεRI-dependent mRNA expression of *tnf-α* and *il-6* in BMMC, mimicking the effect of Trichostatin A, a known histone deacetylase inhibitor. Chromatin immunoprecipitation revealed that butyrate enhanced acetylation of the *tnf-α* and *il-6* promoter regions but blocked RNA polymerase II binding to the promoters of *tnf-α* and *il-6* genes, indicating suppressed transcription initiation. These phenotypes mimicked those of Trichostatin A treatment. In conclusion, butyrate inhibits cell proliferation and increases cell apoptosis in mastocytoma P815 cells and suppresses FcεRI-dependent cytokine production in murine primary BMMC, which are likely mediated by HDAC inhibition.
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Keywords: Butyrate; Histone deacetylase; Mast cells; Cytokines; Proliferation

1. Introduction

Mast cells play a central role in inflammation and anaphylaxis and are necessary in both innate and adaptive immunity [1]. During IgE-mediated allergic reactions, mast cells are recruited in large numbers and migrate into sites of inflammation, where they are activated and release multiple mediators [2]. Some of the mediators such as histamine, proteases and proteoglycans are stored in granules that can be released within seconds, while others such as lipid mediators and most cytokines are *de novo* synthesized within minutes to hours upon activation [3]. C-Kit, a tyrosine kinase receptor expressed on the surface of mast cells, plays pivotal roles in the maturation, proliferation and survival of mast cells [4]. It is activated by ligation of stem cell factor (SCF), and c-Kit constitutive activation mutations are quite common in malignant mast cells, such as the catalytic domain point mutation (D814V/D816V) of c-Kit proto-oncogene in mouse and human mastocytoma [5]. Therefore, inhibition of c-Kit signaling is a target of therapeutic intervention for mast cell-related diseases.

Short-chain fatty acids (SCFA), mainly including acetate, propionate and butyrate, are the products of microflora fermentation in the colon. They have beneficial effects on maintaining intestinal homeostasis and suppressing intestinal inflammation [6–9]. SCFA recruit neutrophils [10], regulate differentiation and function of colonic regulatory T cells (Treg) and ameliorate colitis through SCFA receptors, G-protein-coupled receptor (GPR) 41, GPR43 or GPR109A [8,9]. On the other hand, butyrate and propionate act as histone deacetylase inhibitor (HDACi) in various types of cells. Through inhibiting HDAC activity, they suppress the expression of proinflammatory cytokines in dendritic cells to facilitate the generation of peripheral Treg cells [6]. In neutrophils, they diminish production of proinflammatory mediators [11], inhibit cell differentiation [12] and induce apoptosis through inhibiting HDAC without activating GPR41/GPR43 signaling [13]. In macrophages, butyrate decreases lipopolysaccharide-induced release of proinflammatory mediators through increasing histone 3 lysine 9 (H3K9) acetylation [7]. However, it remains to be determined whether and how they affect mast cells. In current study, we evaluated the effect of butyrate on the function of murine mast cells using two types

Abbreviations: HDACi, histone deacetylase inhibitor; GPR, G-protein coupled receptor; BMMC, bone marrow derived mast cells; SCFA, short chain fatty acid; Ac-H3K9, acetylated histone 3 lysine 9; TSA, trichostatin A; BSA, bovine serum albumin; PARP, poly (ADP-ribose) polymerase.

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of murine mast cells, P815 mastocytoma cells and primary bone marrow-derived mast cells (BMMCs). Given the well-known anticancer effect of HDACi on malignant cells, we explored the effect of butyrate on cell proliferation and apoptosis using P815 cells. Because P815 cells do not express IgE receptor FcεRI, we further used BMMCs to study biological effects of butyrate on mast cell functions.

2. Materials and methods

2.1. Reagents and antibodies

Sodium acetate, sodium butyrate, sodium propionate, Trichostatin A (TSA), Hoechst 33342, bovine serum albumin (BSA) and β-Actin antibody were purchased from Sigma (St. Louis, MO, USA). Antibodies against caspase-3, poly (ADP-ribose) polymerase (PARP), extracellular signal-regulated kinase 1/2 (ERK1/2), phos-ERK1/2, p38, phos-p38, c-Jun N-terminal kinases (JNK), phos-JNK, acetylated histone 3 lysine 9 (Ac-H3K9), RNA polymerase II (Rpb 1), phos-c-Kit and c-Kit were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against acetylated α-tubulin (Ac-α-tubulin) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Cell line and BMMC culture

Murine mast cell line P815 was obtained from ATCC (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100-U/ml penicillin and 100-μg/ml streptomycin (Sigma). To prepare BMMC, bone marrow was flushed from the femurs of 6 to 8 weeks old C57BL/6J male mice (The Jackson Laboratory, Bar Harbor, ME, USA) and cultured in RPMI 1640 medium (Sigma) containing 10% FBS, nonessential amino acids (Sigma), 100-U/ml penicillin, 100-μg/ml streptomycin, 25-mM HEPES, 50-μM β-mercaptoethanol, 10-ng/ml murine SCF (Peprotech, Rocky Hill, NJ, USA) and 10-ng/ml murine IL-3 (Peprotech). Nonadherent cells are harvested and plated into new petri dishes each week consecutively for 4–6 weeks, when the BMMC population was greater than 95%, as determined by toluidine blue staining [14]. All cells were cultured in a 37°C humidified incubator with 5% CO_2.

2.3. P815 cell proliferation assay

P815 cells were plated at 5×10^4 cells/ml and grew for 12 h in 96-well plates, then they were treated with with/without SCFA (10-mM acetate, 2-mM propionate, 2-mM butyrate) or different concentrations (0, 1, 2, 4 mM) of butyrate for 24 h and 48 h. After that, cells were washed with PBS and stained with 5-μg/ml Hoechst 33342 in DMEM for 30 min at 37°C [15]. The relative fluorescence unit value was obtained at excitation 355 nm and emission 460 nm in a microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA). Based on cell proliferation data, 2-mM butyrate was used in all other assays.

2.4. Cell cycle and apoptosis analysis

P815 cell cycle was measured by propidium iodide (PI) staining as previously described [16]. Briefly, 2×10^5 cells were treated with 2 mM of butyrate or 50 nM of TSA for 24 h in 96-well plates. After washed in 0.1% glucose/PBS, cells were fixed with ice-cold 70% ethanol at 4°C overnight, washed in 0.1% glucose/PBS and stained with 5-μl PI solution (eBioscience, Hatfield, AL, USA) in the presence of 100-μg/ml RNase A in 37°C for 10 min. Stained cells were analyzed by the flow cytometer (Millipore Guava easycyte 5 HT, Billerica, MA, USA). Cell debris is gated out constantly for all samples using FlowJo.

Apoptosis assessment was conducted using the Annexin V and PI apoptosis detection kit (eBioscience, Hatfield, AL, USA) according to the manufacture procedures. Briefly, 1 ml of 2×10^5 cells were seeded and treated with butyrate or TSA for 24 h in 24-well plates. After washing with PBS and binding buffer sequentially, cells were stained with 5-μl FITC-Annexin V for 15 min. Then, cells were washed and subjected to PI staining before measurement by flow cytometry. During early-stage apoptosis, plasma membrane loses its asymmetric distribution of the phosphatidylserine and binds to Annexins V but excludes viability dyes PI.

2.5. BMMC chemotaxis

Migration of BMMC was assayed in fibronectin-coated 5-μm 24-well transwell inserts (Corning) as previously described [17]. Briefly, BMMC (1×10^6) were pretreated with/without butyrate for 12 h in cytokine-free RPMI 1640 medium, washed, resuspended in 200-μl RPMI 1640/1% BSA medium with/without butyrate and then transferred into the upper chamber. The lower chamber contains 600 μl of the same medium with 100-ng/ml murine SCF. Cells migrated into the lower chamber were counted 8 h postincubation at 37°C.

2.6. BMMC degranulation and cytokine production

For degranulation, BMMC were sensitized overnight with 100-ng/ml TNP (trintrophenol)-specific IgE (eBioscience) with/without butyrate in cytokine-free RPMI 1640 medium and then washed and resuspended with Tyrode's buffer (10-mM HEPES pH 7.4, 130-mM NaCl, 5-mM KCl, 5.6-mM glucose, 1.4-mM $CaCl_2\cdot2H_2O$, 1-mM $MgCl_2\cdot6H_2O$) containing 0.1% BSA as described [18]. Cell aliquots (5×10^4 cells) were transferred to individual well of a 96-well V-bottom 96-well plate (Thermo Scientific, Waltham, MA, USA) and stimulated with 10-ng/ml TNP-BSA (Biosearch Technologies, Petaluma, CA, USA) for 30 min. β-Hexosaminidase released into the supernatants was quantified by reacting with 4-Nitrophenyl N-acetyl-β-D-glucosaminide (Sigma) and expressed as percentage of the total content.

For cytokine production, BMMC were sensitized and treated as above in 24-well plates, washed with cytokine-free RPMI 1640 medium and stimulated with 10-ng/ml TNP-BSA±butyrate for 6 h [18]. Cell supernatant was collected and measured for TNF-α and IL-6 levels by ELISA per manual instructions (eBioscience).

2.7. Immunoblotting analysis

P815 cells and BMMC (2×10^6) were treated with/without butyrate or TSA for 12 or 24 h, and then cells were collected for immunoblotting analysis as previously described [19]. β-actin was used as the housekeeping gene. Density of bands was quantified by Image J.

2.8. Quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was extracted using Trizol®Reagent (Sigma), treated with DNase I (Thermo Scientific). The cDNA was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). qRT-PCR was conducted on a Bio-Rad CXF96 thermocycler using SYBR Green Master Mix (Bio-Rad Laboratories). β-Actin was used as the housekeeping gene. The primer sequences are listed in Table 1. The amplification efficiency was 0.90 to 0.99. The qRT-PCR conditions were 95°C, 3 min; 35 cycles of 95°C for 15 s, 54°C for 30 s and 72°C for 30 s [19].

2.9. Chromatin immunoprecipitation (ChIP)

BMMC were sensitized as described above and stimulated with 10-ng/ml TNP-BSA± butyrate or TSA for 0.5 h. ChIP assay was conducted as previously described [7] with modifications. Briefly, cells were cross-linked with 1% formaldehyde for 10 min. After washing, cells were lysed in lysis buffer (1% SDS, 10-mM EDTA, 50-mM Tris–HCl, pH 8.1) containing 1% protease inhibitor (Sigma). Cell lysates were sonicated, precleared by centrifuging at 12,000 *g* for 10 min. A small aliquot of the resulting supernatant was transferred into a clean tube, which was used as "input" to normalize ChIP PCR. The remaining supernatant was 1:10 diluted with ChIP dilution buffer, blocked and incubated with the primary antibody overnight. Then, protein G agarose beads (Cell Signaling) were added to pull down antibody–chromatin complexes. Recovered DNA was purified by ChIP DNA Clean & Concentrator kit (Zymo Research, Irvine, CA, USA) and quantified by qPCR with primers specific to *tnf*-α or *il-6* promoter region (Table 2). Each sample is normalized by its own input. The PCR amplicons for Ac-H3K9 ChIP locates between −167 and −38 bp in the *tnf*-α promoter and −975 and −815 bp in the *il-6* promoter. For RNA polymerase II ChIP, PCR amplicons locates between −152 and +3 bp in the *tnf*-α promoter and −127 and −35 bp in the *il-6* promoter, both covering transcription starting sites.

2.10. Statistical analyses

Statistical analyses were conducted as previously described [19]. Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System, SAS, 2000). Mean±standard error of the mean (S.E.M.) are reported. Mean difference was separated by paired *t* test. Statistical significance is considered as $P<0.05$.

3. Results

3.1. Butyrate induces apoptosis of P815 cells

To determine the biologic effects of SCFA on malignant mast cells, P815 cells were treated with acetate, propionate or butyrate for 24 h and 48 h, and then the cell proliferation was assessed by H33342 staining. Acetate, propionate and butyrate all inhibited P815 cell proliferation, with butyrate having the most potent effects (Fig. 1A). Butyrate inhibited P815 cell proliferation in a dose-dependent manner, with 2-mM butyrate effectively inhibited proliferation (Fig. 1B). Thus, 2-mM butyrate was used in the subsequent experiments. Based on flow cytometry analysis, butyrate treatment arrested P815 cell-cycle at G1, reminiscent of that induced by a well-known HDACi, TSA (50 nM) (Fig. 1C). Furthermore, butyrate or TSA treatment profoundly increased the ratio of both early- and late-stage apoptotic P815 cells as demonstrated by Annexin V/PI staining (Fig. 1D).