Acid sphingomyelinase is induced by butyrate but does not initiate the anticancer effect of butyrate in HT29 and HepG2 cells

Jun Wu,* Yajun Cheng,* Bo A. G. Jönsson,† Åke Nilsson,* and Rui-Dong Duan[1],*

Gastroenterology Laboratory,* Biomedical Center B11, Lund University, S-221 84 Lund, Sweden; and Department of Occupational and Environmental Medicine,† Institute of Laboratory Medicine, University Hospital, S-221 85 Lund, Sweden

Abstract **Butyric acid and sphingomyelin (SM) affect colonic tumorigenesis. We examined the potential link between butyrate stimulation and SM metabolism in colonic and hepatic cancer cell lines. After incubating HT29 and HepG2 cells with butyrate and other short-chain fatty acids, we found that butyrate increased acid but not neutral or alkaline sphingomyelinase (SMase) activity by 10- to 20-fold. The effects occurred after 16 h of incubation and were associated with reduced SM and phosphatidylcholine contents and increased ceramide levels. Northern blotting showed increased acid SMase mRNA levels in these cells after butyrate stimulation. Propionate was less potent, and acetate had no effect. No similar changes of acid phosphatase could be identified. At concentrations that increased acid SMase expression, butyrate inhibited cell proliferation, activated caspase 3, and induced apoptosis. However, the antiproliferative and apoptotic effects of butyrate preceded the changes of acid SMase and were not affected by knocking down acid SMase expression by small, interfering RNA. In addition, butyrate-induced acid SMase expression was not affected by blocking the caspase pathway. In conclusion, butyrate regulates SM metabolism by stimulating acid SMase expression in colon and liver cancer cells, but the increased acid SMase is not a critical mechanism for initiating the anticancer effects of butyrate.**—Wu, J., Y. Cheng, B. A. G. Jönsson, Å. Nilsson, and R-D. Duan. **Acid sphingomyelinase is induced by butyrate but does not initiate the anticancer effect of butyrate in HT29 and HepG2 cells. *J. Lipid Res.* 2005.** 46: **1944–1952.**

Supplementary key words short-chain fatty acid • colon • liver • sphingomyelin • apoptosis • caspase • small, interfering RNA

It is well known that colonic short-chain fatty acids derived from fermentable dietary fiber under the action of colonic microflora have biological effects on colonic cells. Among these short-chain fatty acids, butyrate is the most effective, having been found to stimulate cell differentiation, induce apoptosis, and modify the responses of the cells to mitogenic factors (1–3). Although the anticancer effects are clear, the mechanism by which butyrate exerts these effects is complex. The functions of various signaling molecules have been found to be affected by butyrate, including protein kinase C, p38MAP kinase, bcl-2, cyclin D1 and D3, p53, caspases, and c-*myc* (4–7). Butyrate also inhibits cell proliferation and induces apoptosis in liver cells (8) and may enhance the efficiency of anticancer drugs on liver cancer (9). In a recent study in human hepatoma HuH6 and HepG2 cells (10), butyrate was found to induce apoptosis by reducing mitochondrial membrane potential, releasing cytochrome *c*, and activating the caspase 9 and 3 pathways, which causes a secondary decrease in antiapoptotic factors such as β-catenin, pRb, cyclins and Bcl-X_l.

Sphingomyelin (SM) hydrolysis triggered by sphingomyelinase (SMase) generates both antiproliferative molecules such as ceramide and sphingosine and proliferative molecules such as sphingosine-1-phosphate (11, 12). Studies indicate that digestion of SM in the intestinal tract may have implications for tumorigenesis in the colon (13). Animal studies found that supplementary SM and ceramide analogs in the diet inhibited the formation of aberrant crypt foci and the development of carcinomas induced by chemical carcinogens (14). In the intestinal tract, we previously reported that the development of human colonic carcinoma was associated with reductions of SMase activities, with the reduction of alkaline SMase being predominant (15). Therefore, an interesting question is whether butyrate can affect SM metabolism and change its anticancer effects. Our study, for the first time, links the effects of butyrate to SM metabolism via acid SMase, but the increased acid SMase is not involved directly in mediating the antiproliferative and apoptotic effects of butyrate in these cells.

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[1] To whom correspondence should be addressed.
e-mail: rui-dong.duan@med.lu.se

MATERIALS AND METHODS

Materials

HT29 and HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD). Butyrate, propionate, acetate, choline chloride, choline phosphate, Dragendorff's reagent, *N*-stearoyl-sphingosine, and *N*-palmitoyl-sphingosine were purchased from Sigma Chemical Co. (Stockholm, Sweden). SM was purified from bovine milk and labeled with [^{14}C-CH$_3$]choline at Astra Zeneca (Stockholm Sweden) as described (16). [^{3}H]choline chloride, Quickprep™ total RNA extraction kit, Gene Images Random Prime Labeling module, and Gene Images CDP-Star detection module for Northern blot were obtained from Amersham Biosciences (Uppsala Sweden). The cell death detection kit and 4-[3-(iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazoliol]1,3-benzene disulfonate (WST-1) for the cell proliferation assay were purchased from Roche Applied Science (Bromma, Sweden). The substrate of caspase 3 Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA) was obtained from VWR International AB (Stockholm, Sweden), and the general caspase inhibitor Z-VAD-FMK was from R&D Systems (Abingdon, UK). Primers used were purchased from Invitrogen (Paisley, UK). The acid SMase small, interfering RNA (siRNA) and silenced negative control siRNA were purchased from Ambion, Inc. (Austin, TX). The sense sequence of acid SMase siRNA is 5′-GGUUACAUCGCAUAGUGCCTT-3′, and the antisense sequence is 5′-GGCACUAUGCGAUGUAACCTG-3′.

Cell culture, cell stimulation, and cell-free extraction

HT29 and HepG2 cells were cultured in RPMI 1640 medium containing 1.5 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) heat-inactivated fetal calf serum. Unless specified elsewhere, the cells at ~75% confluence were stimulated with the short-chain fatty acids tested for 24 h. The cells were scraped and centrifuged at 1,000 *g* at 4°C for 10 min. The pellets were rinsed with PBS once and suspended in 100 μl of lysis buffer containing 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.5 mM DTT, and 10 mM taurocholate. The suspension was kept on ice for 15 min, followed by sonication for 5 s. The samples were then centrifuged at 15,000 *g* for 10 min at 4°C, and the supernatant was used for biochemical determinations.

SMase assay

The activities of different types of SMase were analyzed as described, with different buffers (17). The buffers used were 50 mM Tris-maleate buffer, pH 5.0, containing 0.15 M NaCl and 0.12% Triton X-100 for acid SMase, 50 mM Tris-HCl buffer, pH 7.5, containing 4 mM Mg^{2+} and 0.12% Triton X-100 for neutral SMase, and 50 mM Tris-HCl, pH 9.0, containing 2 mM EDTA, 0.15 M NaCl, and 6 mM taurocholate for alkaline SMase. For each determination, a 10 μl sample was mixed with 90 μl of the corresponding buffer containing 80 pmol of [^{14}C]SM (~8,000 dpm) and incubated at 37°C for 30 min. The reaction was terminated by adding 0.4 ml of chloroform-methanol (2:1, v/v) followed by centrifugation at 10,000 *g* for 5 s. An aliquot of the upper phase was taken, and the production of [^{14}C]phosphocholine was determined by liquid scintillation.

Identification of the cleaved products of SM

To identify hydrolytic products of SM, 10 μl of the lysate of the butyrate-treated cells was incubated with 160 pmol of [^{14}C]choline-labeled SM in acid SMase assay buffer for 1 h at 37°C. The reaction was terminated as described, and the upper phase was saved, dried under nitrogen, and dissolved in methanol. The samples were then spotted on a 60 F254 high-performance TLC plate (Merck) that was developed in chloroform-methanol-3 M trichloroacetic acid-water (20:30:10:6). Standard choline and phosphocholine were used as authentic markers, and the bands were visualized by spraying the plate with Dragendorff's reagent. The bands corresponding to choline and phosphocholine standards were scraped, and the radioactivities in the bands were counted by liquid scintillation.

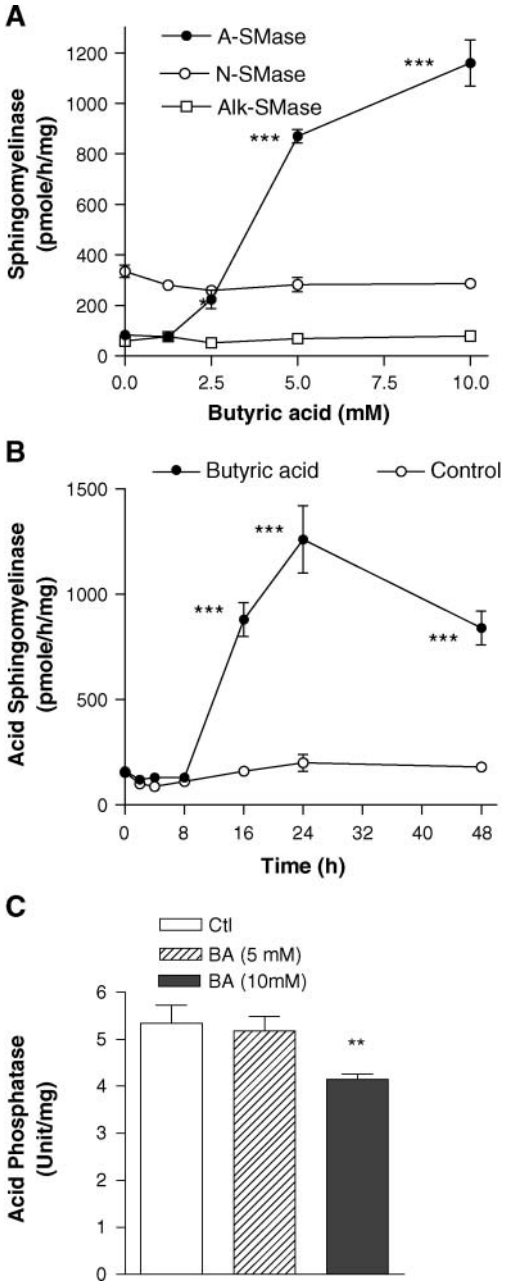

Fig. 1. Effects of butyrate on acid sphingomyelinase (SMase) activity in HT29 cells. The cells were treated with butyrate at different concentrations for 24 h, and the activities of acid (A), neutral (N), and alkaline (Alk) SMases were determined in the cell-free extracts (top panel). In the middle panel, the cells were treated with 5 mM butyrate for different times. In the bottom panel, the cells were treated with butyrate at 5 and 10 mM for 24 h, and the activity of acid phosphatase was determined. All results are means ± SEM obtained from triplicate determinations in three separate experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with controls.

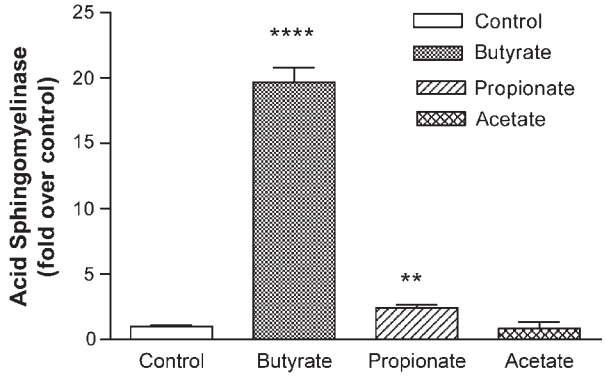

Fig. 2. Comparison of the effects of short-chain fatty acids. The cells were incubated with different short-chain fatty acids (5 mM) for 24 h. The cell-free extracts were prepared, and acid SMase activity was determined. Results are means ± SEM obtained from triplicate determinations in three separate experiments. ** $P < 0.01$, **** $P < 0.0001$ compared with controls.

Changes of SM, phosphatidylcholine, and ceramide in the cells

The cellular levels of SM and phosphatidylcholine (PC) were determined as described (18). In brief, the cells were labeled with [^{3}H]choline chloride (0.5 μCi/ml) for 48 h and then stimulated with 5 mM butyrate for 24 h. Total lipids in the cells were extracted according to Bligh and Dyer (19) and applied on 60 F Silica Gel plates (0.25 mm; Merck) for TLC. The plates were developed in chloroform-methanol-25% ammonium hydroxide (65:25:4) and stained with iodine vapor. The bands of SM and PC were scraped according to the authentic standards, and the radioactivities in the bands were measured by liquid scintillation.

Ceramide in the cells was determined by liquid chromatography-tandem mass spectrometry. The cells were first treated with 5 mM butyrate, and total lipids were extracted as described above. The lipid extracts were injected into a Perkin-Elmer series 200 liquid chromatography system with an autosampler (Applied Biosystems, Norfolk, CT) coupled to an API 3000 tandem mass spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, Canada) with a Turbo electrospray ion source operated in the positive ion mode at 350°C. The mobile phases were water containing 0.5% acetic acid (A) and methanol containing 0.5% acetic acid and 2.5% tetrahydrofuran (B). A gradient from 85% A to 100% B was applied in 3 min and then was kept for another 3 min. The declustering potential was 35 V. The fragments analyzed were *m/z* 566.5/264.4 at a collision energy of 40 V for *N*-stearoyl-sphingosine and *m/z* 538.5/264.5 at a collision energy of 39 V for *N*-palmitoyl-sphingosine. Weighed standards *N*-stearoyl-sphingo-

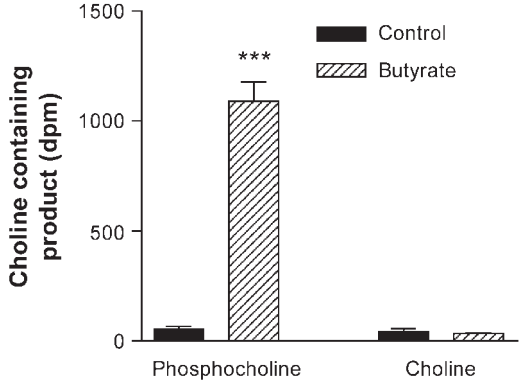

Fig. 3. Identification of the hydrolytic products. HT29 cells were treated with 5 mM butyrate for 24 h. The cell-free extracts were incubated with [^{14}C]sphingomyelin (SM) for 1 h. The cleaved choline and phosphocholine were separated by TLC and visualized with Dragendorff's reagent. The bands were scraped and radioactivities counted by liquid scintillation. Results are means ± SEM from duplicate determinations in three separate experiments. *** $P < 0.001$ compared with controls.

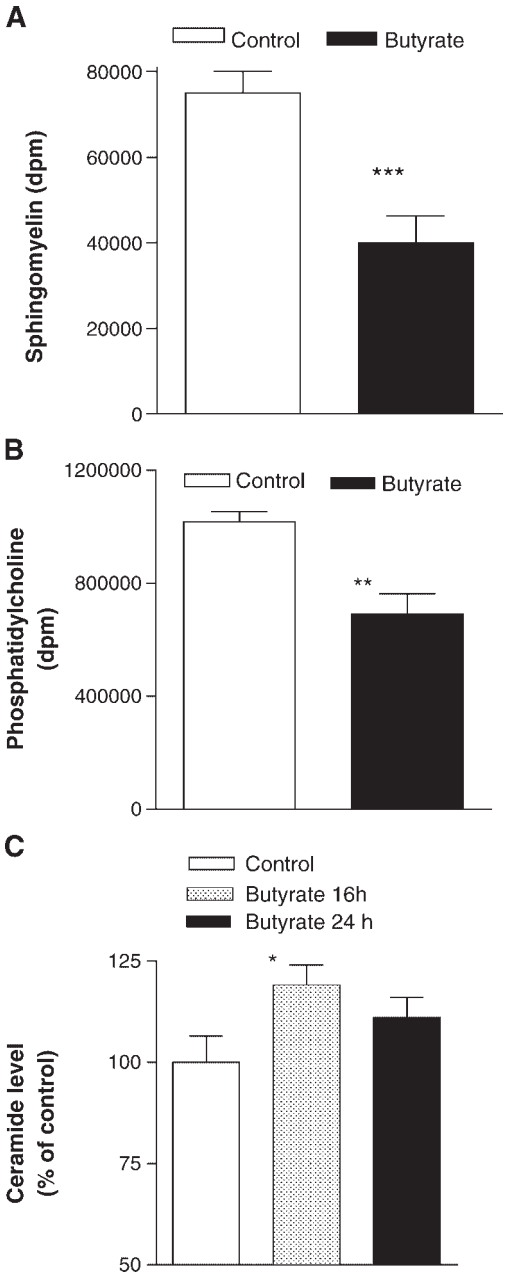

Fig. 4. Changes of SM (top panel), phosphatidylcholine (PC; middle panel), and ceramide (bottom panel) in HT29 cells after butyrate stimulation. The cells were prelabeled with [^{3}H]choline chloride and then treated with 5 mM butyrate for 24 h. Total lipids were extracted and resolved by TLC, and the radioactivities of the SM and PC bands were determined. Ceramide was analyzed by liquid chromatography-tandem mass spectrometry. Results are means ± SEM obtained from duplicate determinations in three separate experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with controls.

sine and *N*-palmitoyl-sphingosine were used for the preparation of external calibration curves.

Northern blotting of acid SMase mRNA

A segment of 318 bp from human acid SMase cDNA was first amplified by RT-PCR using sense primer 5′-ACTCTAGAATGCCCCGCTACGGAGCGTCAC-3′ and antisense primer 5′-ATGGTACCCTTCAGCCCGAGGTTGATGGC-3′ and labeled with fluorescein. Northern blotting was carried out using a commercial kit as described above. In brief, total RNA was extracted from the cells treated with 5 mM butyrate for 24 h and purified. Each sample with 20 μg of RNA was subjected to agarose gel electrophoresis, and the RNA was transferred to a GT nylon membrane and hybridized with the labeled probes overnight. The probes were detected with a specific anti-fluorescein antibody conjugated with alkaline phosphatase in 1:5,000 dilutions. The membranes were then incubated with dioxetane CDP-Star, and the remitted light was recorded on Kodak X-films. The membranes were then stripped with boiling water containing 0.1% SDS for 10 min and rehybridized with β-actin probe as a control.

Assay of cell proliferation and apoptosis

Cell proliferation and apoptosis were determined as described (20). For the proliferation assay, 2×10^4 cells were incubated with butyrate for 24 h on a 96-well microplate. WST-1 reagent was then added in each well followed by incubation for 1 h. The production of formazan was measured photometrically in a microreader. Apoptotic effects were analyzed by the enrichment of DNA-histone complex in the cytoplasm and by caspase 3 determination. The cells were incubated with butyrate at different concentrations for 24 h or with one concentration (5 mM) for different times. The amount of cytoplasmic histone-associated DNA fragments in the cell lysate was determined by the Cell Death Detection ELISA kit using a mixture of anti-histone and anti-DNA antibodies. Caspase 3 activity was determined spectrophotometrically using DEVD-pNA as a substrate, as described previously (20).

To study the effect of caspase inhibition, the cells were preincubated with 60 μM Z-VAD-FMK for 2 h and then stimulated with butyrate for 24 h. The cell-free extracts were prepared as described above, and caspase 3 and acid SMase activities were determined. Our pilot study showed that Z-VAD-FMK at this concentration could effectively block the apoptosis caused by butyrate.

Knockdown of acid SMase by siRNA

To silence acid SMase expression, HT29 and HepG2 cultured cells were detached with trypsin, washed, and suspended in serum-free DMEM. A total of 2×10^6 cells in 200 μl were incubated with 200 pmol of acid SMase siRNA or control siRNA for 10 min at room temperature. The HT29 and HepG2 cells were then electroporated with 500 and 260 V, respectively, for 220 μs, which was repeated once after 1 min. The electroporated cells were transferred in DMEM with 10% fetal calf serum and incubated at 37°C for 48 h, followed by transfection by electroporation as above to maximize the efficiency of siRNA transfection. The cells were then cultured with butyrate at different concentrations for 24 h. Cell-free extracts were prepared, and the changes of acid SMase activity, cell proliferation, apoptosis, and caspase 3 activity after butyrate stimulation were determined.

Other biochemical determinations and statistical analysis

Acid phosphatase was determined as described previously using *p*-nitrophenyl phosphate as substrate (21). The proteins were analyzed using the DC protein assay kit from Bio-Rad. The results are presented as means ± SEM, and the differences were determined by unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant.

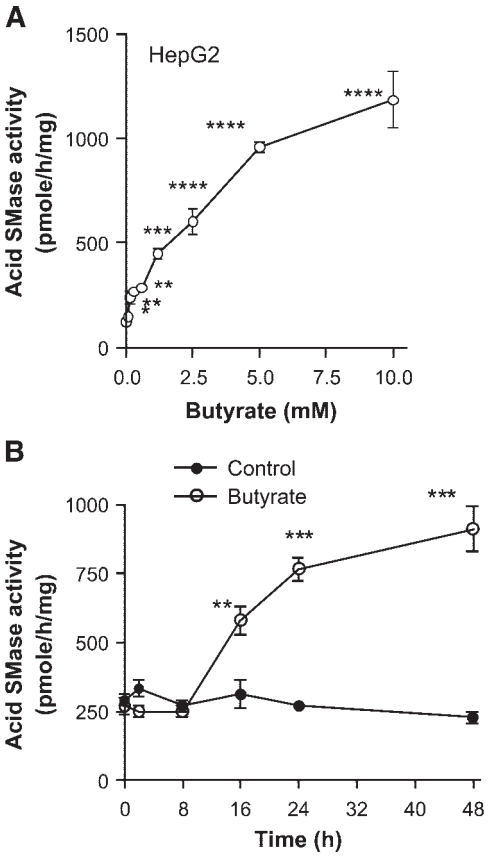

Fig. 5. Changes of acid SMase activity in HepG2 cells. The cells were treated with butyrate at different concentrations for 24 h, and the acid SMase activities in cell-free extracts were determined (top panel). In the bottom panel, the cells were treated with 5 mM butyrate for different times. Results are means ± SEM from triplicate determinations in three separate experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

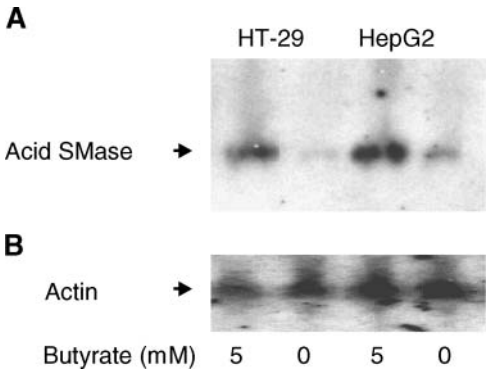

Fig. 6. Northern blotting of acid SMase mRNA. A segment of acid SMase cDNA was amplified by RT-PCR and labeled with fluorescein. The cells were treated with or without 5 mM butyrate for 24 h. Total RNA was extracted and blotted with the labeled probe, followed by reacting with anti-fluorescein antibody conjugated with alkaline phosphatase. In the bottom panel, the membrane was stripped and reblotted with β-actin probe. Similar results were obtained from two other experiments.

RESULTS

Changes of acid SMase activity by butyrate in HT29 cells

After incubating HT29 cells with butyrate for 24 h, the acid SMase activity was increased in a dose-dependent manner (**Fig. 1**, top panel). The threshold concentration of butyrate was ~2.5 mM, and 5 mM butyrate increased the acid SMase activity by ~20-fold. With the concentrations tested, butyrate had no effect on either neutral or alkaline SMase activity in the cells. Time course study showed that the effect of butyrate was not detectable at 8 h of incubation, was seen at 16 h, peaked at 24 h, and declined at 48 h (Fig. 1, middle panel). To further assess the specificity of the effect, the changes of another lysosomal enzyme, acid phosphatase, were determined. As shown, butyrate had no effect on acid phosphatase activity at 5 mM (Fig. 1, bottom panel); 10 mM butyrate even reduced acid phosphatase activity by ~20%. Whether other short-chain fatty acids have similar effects is shown in **Fig. 2**. At the same concentration (5 mM), propionate also increased acid SMase in HT29 cells to a much smaller extent than that induced by butyrate (2.4-fold vs. 20-fold). Acetate at this concentration had no detectable effect.

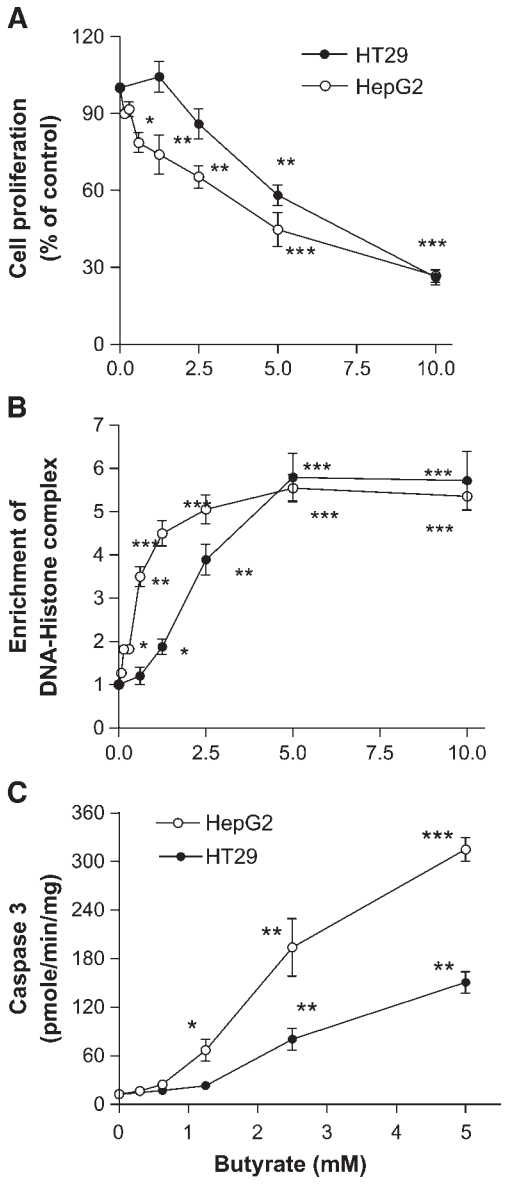

Fig. 7. Changes of cell proliferation, apoptosis, and caspase 3 activity induced by butyrate in HT29 and HepG2 cells. The cells were treated with butyrate at different concentrations for 24 h. The cell proliferation rate (top panel), enrichment of the cytosol DNA-histone complex (middle panel), and activity of caspase 3 (bottom panel) were determined. Results are means ± SEM of at least three separate experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with 0 concentration.

Identification of hydrolytic products

Because the activity of acid SMase was determined using choline-labeled SM, it is necessary to examine whether the cleaved head group is phosphocholine or choline (i.e., whether it is cleaved by a type of phospholipase D or a type of phospholipase C). The question was addressed by incubating the choline-labeled SM with the lysate of the cells treated with butyrate and analyzing the hydrolytic products by TLC. The results are shown in **Fig. 3**. Treating the cells with butyrate sharply increased the ability of the cells to generate phosphocholine but not choline from SM, which verified that the increased activity was a type of phospholipase C and not phospholipase D.

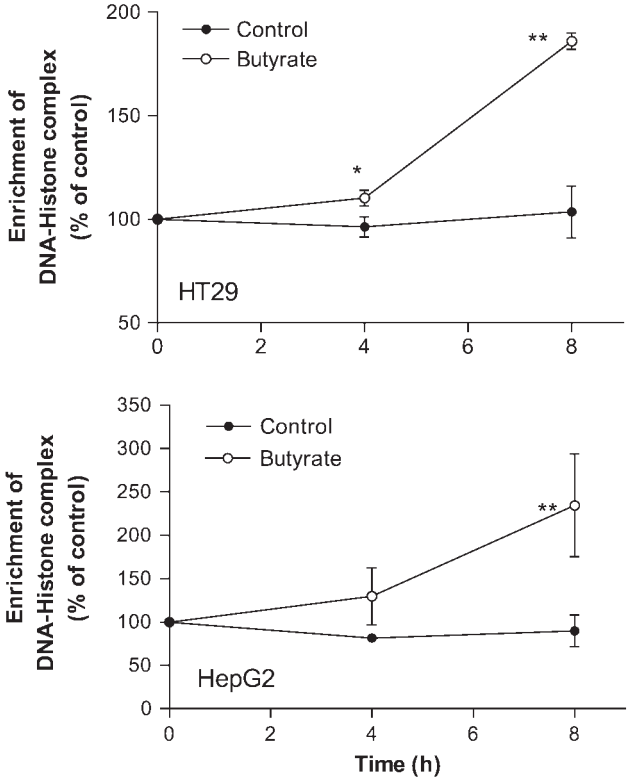

Fig. 8. Time course of apoptosis caused by butyrate. HT29 and HepG2 cells were treated with 5 mM butyrate for 4 and 8 h. The enrichment of the DNA-histone complex in the cell extract was determined. Results are means ± SEM from duplicate determinations in three separate experiments. * $P < 0.05$, ** $P < 0.001$ compared with controls.

Cellular content of acid SMase substrates and sphingolipid metabolites

It is known that acid SMase can hydrolyze both SM and PC (22). Therefore, changes of SM and PC in the cells after butyrate stimulation were determined. As shown in **Fig. 4**, stimulating the cells with 5 mM butyrate for 24 h decreased SM by 45% (top panel) and PC by 30% (middle panel). The cellular ceramide content was increased by 19.7% at 16 h of stimulation, and this increase declined to 11% after 24 h of incubation (bottom panel).

Effects of butyrate on HepG2 cells

We also examined the effects of butyrate on HepG2 cells (**Fig. 5**). Basal acid SMase activity was higher in HepG2 cells than in HT29 cells. Butyrate sharply increased acid SMase activity, and the threshold concentration was as low as 0.125 mM. At 5 mM, butyrate increased acid SMase in the cells by 10-fold (top panel). No change of neutral or alkaline SMase induced by butyrate was found (data not shown). The time course of the cell response to butyrate is shown in the bottom panel. As in the experiment with HT29 cells, the increase in acid SMase activity was not detected at 8 h of incubation but was clearly demonstrated at 16 h of incubation.

Changes of acid SMase mRNA induced by butyrate

The changes of acid SMase mRNA in HT29 and HepG2 cells after incubation with 5 mM butyrate for 24 h are shown in **Fig. 6**. Butyrate significantly increased mRNA levels of acid SMase in both cell lines. The equal amounts

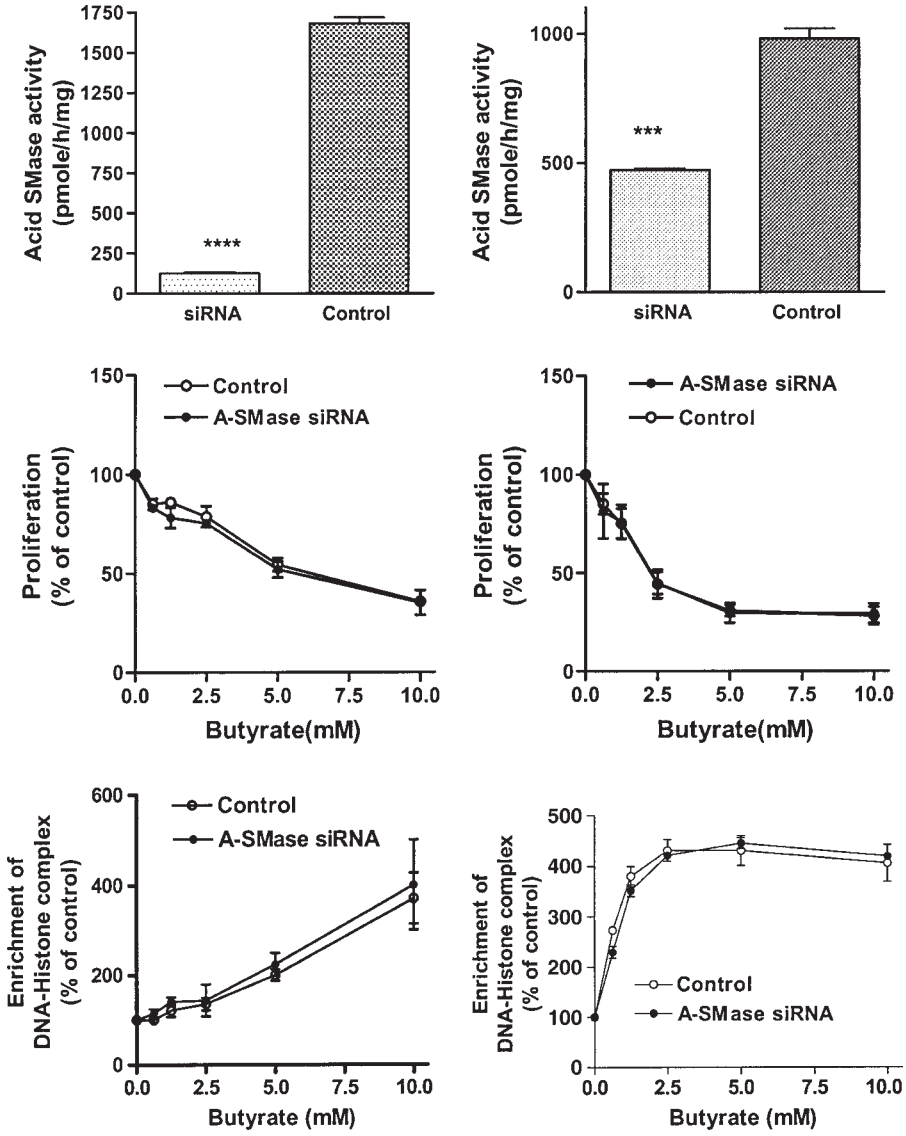

Fig. 9. Effects of knocking down acid SMase on butyrate-induced cell proliferation and apoptosis. The cells were transfected with acid SMase small, interfering RNA (siRNA) and then stimulated with butyrate at different concentrations. Acid SMase activity and cell proliferation and apoptosis were determined compared with negative controls. The top panels show the changes of butyrate-induced acid SMase activities after the RNA interference. The middle panels show the changes of cell proliferation, and the bottom panels show the changes of apoptosis, after siRNA transfection. The results for HT29 cells are at left, and those for HepG2 cells are at right. Data are means ± SEM from duplicate determinations in three separate experiments. *** $P < 0.001$, **** $P < 0.0001$ compared with controls.

of β-actin in the samples indicate that the changes of acid SMase mRNA were not caused by different levels of RNA loaded in the samples.

Changes of cell proliferation and apoptosis by butyrate

When cell proliferation and apoptosis were determined, we found that butyrate at concentrations that stimulated acid SMase expression significantly inhibited cell proliferation (**Fig. 7**, top panel), induced apoptosis (middle panel), and increased caspase 3 activity (bottom panel) dose-dependently. The concentrations required for such anticancer effects were lower for HepG2 cells than for HT29 cells, indicating that HepG2 cells are more sensitive to butyrate stimulation.

Does the increased acid SMase mediate the anticancer effects of butyrate?

Because some studies indicate that acid SMase may play an important role in the mediation of apoptosis (23–25), we addressed the question of whether the anticancer effects of butyrate were mediated by increased acid SMase. We first determined the time course of butyrate-induced apoptosis compared with the changes of acid SMase and found that the apoptotic activity was already detectable at 4 h of stimulation and was significantly increased by 80% in HT29 cells and by 135% in HepG2 cells at 8 h of incubation (**Fig. 8**). At these time points, no increase of acid SMase was detected (Figs. 1, 5). Second, we examined changes in the anticancer effects of butyrate after knocking down acid SMase expression by siRNA. As shown in the two top panels of **Fig. 9**, siRNA transfection significantly reduced butyrate-stimulated acid SMase activity by 91% in HT29 cells (left) and by 51% in HepG2 cells (right). However, such inhibitions of acid SMase expression did not change the response of the cells to butyrate in cell proliferation and apoptosis. We also found that knockdown of acid SMase did not change the increase of caspase 3 activity by butyrate (data not shown). Finally, we addressed the question of whether the increased acid SMase is a downstream event triggered by apoptosis. We used a caspase inhibitor to block apoptosis and study the possible changes of acid SMase activity caused by butyrate. As shown in **Fig. 10**, caspase inhibitor significantly reduced caspase 3 activity by 80% in HT29 cells and by 95% in HepG2 cells. Blocking of caspases, however, did not show any effects on the response of acid SMase to butyrate.

DISCUSSION

The present study demonstrates that butyrate increases acid SMase, but not neutral or alkaline SMase, activity in human colon cancer HT29 cells and liver cancer HepG2 cells. The identity of acid SMase was confirmed by the reduction of the substrate SM, the increase of the products ceramide and phosphocholine, and the changes of acid SMase mRNA. The increase in acid SMase mRNA indicates that butyrate increased either the transcription of the acid SMase gene or the stability of the enzyme mRNA. Because the increase was ~10- to 20-fold, butyrate could be considered a strong regulator for SM metabolism through acid SMase expression. The regulatory effect is of physiological relevance, as butyrate is produced mainly in

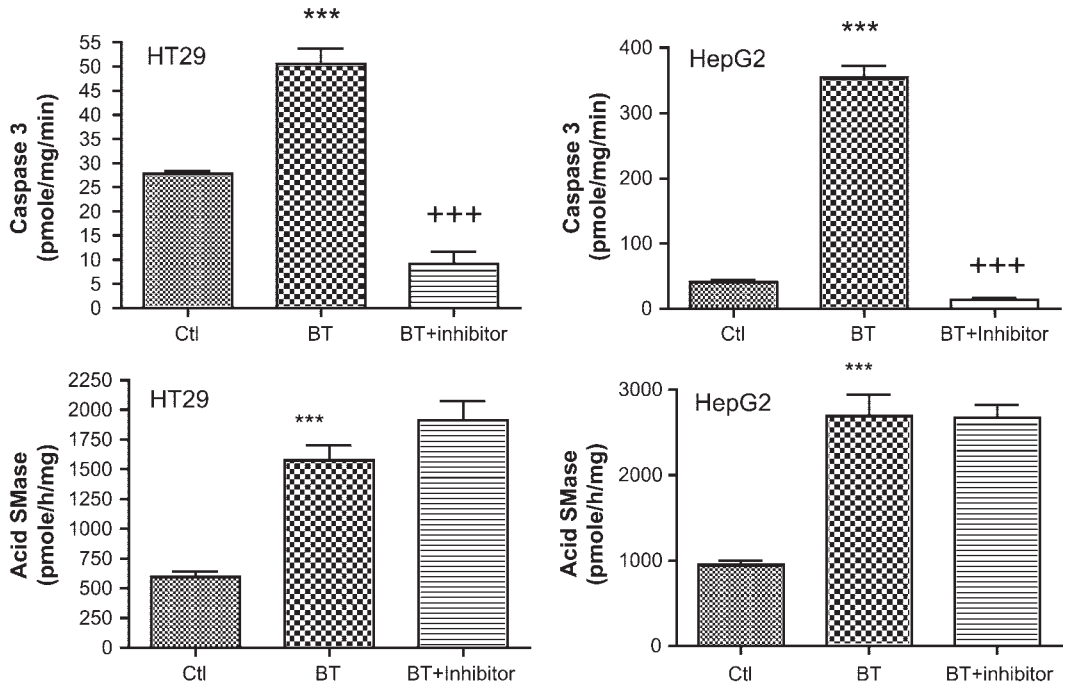

Fig. 10. Effects of caspase inhibition on butyrate-induced increases of acid SMase. The cells were pretreated with 60 μM Z-VAD-FMK for 2 h followed by stimulation with 5 mM butyrate for 24 h. The activities of caspase 3 and acid SMase were determined in cell-free extracts. Results are means ± SEM from duplicate determinations in three separate experiments. *** $P < 0.0001$ compared with controls (Ctl); $^{+++}$ $P < 0.0001$ compared with butyrate (BT) stimulation.

the colon, where it is absorbed, used, or transported via the portal vein to the liver. In healthy humans, the concentration of butyrate in the colon was reported to be 15–60 mM, varying with the location of the colon (26), the intake of fermentable fiber (27), and the movement of the bowel (28). In the portal vein of humans without fasting, the concentration of total short-chain fatty acids is ~375 μM, which is decreased to 140 μM in hepatic blood (29). In the present study, butyrate increased acid SMase activity in both colon and liver cells in concentrations that can be reached under physiological conditions, implying that short-chain fatty acids, particularly butyrate, are stimulators for acid SMase expression and may maintain a certain level of expression under physiological conditions.

Butyrate has been reported to induce apoptosis in several cell types, including colon carcinoma and hepatoma cell lines (1–7, 10). Such anticancer effects were confirmed in the present study. Because acid SMase may mediate apoptosis caused by stress and cytokine in some cell types (23, 24), the question is raised whether the butyrate-induced apoptosis seen in this study is mediated by the increased acid SMase. Our studies provided two pieces of evidence that argue against a critical role of acid SMase in the initiation of butyrate-induced apoptosis. First, the increase in acid SMase was a late event, seen at 16 h, whereas the apoptotic response was already detected at 4 h and became distinctive at 8 h of incubation. Second, RNAi inhibited butyrate-induced acid SMase expression by 90% in HT29 cells and by 50% in HepG2 cells, but such inhibitions did not induce any proliferative and apoptotic changes of the cells to butyrate. This finding indicates that the effects of acid SMase on cell fate vary with the types of the cells and the stimuli. Retinoids were reported to stimulate acid SMase expression in leukemia cells (30) and neuroblastoma cells (31). Reduction of acid SMase expression by 50–60% by RNAi can sharply inhibit retinoid-induced apoptosis by 70% (31). However, in other cells, such as fibroblasts and B-cells isolated from both types A and B Niemann-Pick disease, and in macrophages isolated from acid SMase knockout mice, the apoptotic responses of these cells to cytokines are not different from those of normal cells (32–34). The hepatocytes from acid SMase knockout mice were originally found to be resistant to Fas-mediated cell death (35), but this resistance was not attributable to the lack of acid SMase but to the failure to generate ceramide (36). Our results in colon and liver cells are an additional piece of evidence against an initiative role of acid SMase in apoptosis in these cells. The inability of acid SMase to induce apoptosis may be related to the fact that the ceramide formed by acid SMase in lysosomes is rapidly metabolized. As demonstrated here, the increase of ceramide found after 16 h of stimulation with butyrate was only 20%, which was much smaller compared with the 10- to 20-fold increase of acid SMase activity. Alternatively, the increased acid SMase may not be translocated to the membrane of the cells to hydrolyze the membrane-bound SM, which is a critical event for acid SMase to elicit apoptosis (25). Our findings add to the general view that the relation between SMase levels, ceramide formation, and biological function is complex and situation-dependent, as discussed in several reviews (37–39).

The function of this 10- to 20-fold increase of acid SMase induced by butyrate remains unknown. We hypothesized that the great increase of acid SMase may play a role in clearance of the apoptotic cells, a field that has recently gained increasing interest (40). As has been described (41), apoptosis is a programmed process that includes the separation of the apoptotic cells from neighboring cells, the condensation of the cytoplasm and nuclei, and the formation of apoptotic bodies. During these steps, the integrity of the cell membrane is well maintained and the lysosomal contents remain intact (42). The apoptotic bodies are engulfed by macrophages or neighboring normal cells and finally lysed by lysosomal digestion. Therefore, the lysosomal digestion of the apoptotic bodies is the last phase of apoptosis. Because the apoptotic bodies are surrounded by a membrane whose integrity is well maintained, destruction of the membrane integrity is a key factor for clearing up the apoptotic bodies. SM has long been considered an important membrane constituent for the maintenance of membrane integrity. The increased acid SMase could readily hydrolyze SM in the membrane of the engulfed apoptotic bodies during the completion of the suicide program. This hypothesis should be a target for further study, but it may not be suitable to test in conventional cell culture systems, because the apoptotic cells are normally detached from the monolayer and are not readily engulfed by the surrounding normal cells.

About the mechanism by which butyrate upregulates acid SMase expression, we examined whether the increase in acid SMase is a secondary reaction after apoptosis, because ceramide formation caused by acid SMase may be induced by caspase activation (43, 44). Our results showed that it was not the case for this study, because blocking all caspase pathways and apoptosis had no influence on the changes of acid SMase activity.

In summary, this study identified a strong and novel biochemical function of butyrate in regulating acid SMase expression. Although the function of the increased acid SMase is still unresolved, it is not involved in mediating the anticancer effects of butyrate in these cells. The potential physiological and pathological implications of such responses could be an interesting topic for further investigation.

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