Akita J Med **38** : 111-119, 2011

BUTYRATE REDUCES FREE CHOLESTEROL ACCUMULATION IN NIEMANN-PICK DISEASE TYPE C1 CELLS (NOVA SCOTIA FORM) THROUGH THE INDUCTION OF ACID SPHINGOMYELINASE

Chikako Oyama[1)], Masashi Hirayama[2)], Atsuko Noguchi[1)], Hirokazu Arai[1)] and Tsutomu Takahashi[1)]

(received 14 December 2011, accepted 6 January 2012)

[1)]Department of Pediatrics, Akita University Graduate School of Medicine, Akita 010-8543, Japan
[2)]Department of Pediatrics, Nakadori General Hospital, Akita 010-8577, Japan

Abstract
Niemann-Pick disease type C (NPC) is an inherited disorder caused by mutations in either the *NPC1* or the *NPC2* gene that affect intracellular free cholesterol trafficking. Most cases of NPC have mutations in the *NPC1* gene. Acid sphingomyelinase (ASM) is a lysosomal enzyme in which an inherited deficiency leads to Niemann-Pick disease types A and B (NPDA/NPDB). Despite having a normal *ASM* gene, NPC cells have a secondary defect in ASM activity ; the mechanism remains to be fully elucidated. Butyrate, one of the short chain fatty acids, is known as an inducer of ASM. We investigated the effects of butyrate on ASM activity and mRNA expression in normal and NPC1 mutant (Nova Scotia form ; $NPC1^{Nova\ Scotia}$) lymphoblasts. After incubation with 10 mM butyric acid for 24 h, ASM activity was significantly increased by 3.3- and 4.6-fold in normal and $NPC1^{Nova\ Scotia}$ cells, respectively ($p<0.01$). The secondary defect of ASM activity in $NPC1^{Nova\ Scotia}$ cells was restored to normal levels by treatment of 10 mM butyric acid. In quantitative RT-PCR analysis, butyric acid significantly increased ASM mRNA levels in both types of cells ($p<0.01$). In addition, we investigated the effects of butyrate on intracellular free cholesterol levels in $NPC1^{Nova\ Scotia}$ lymphoblasts. Free cholesterol levels in cells treated with or without 10 mM butyric acid were 0.019 ± 0.002 and 0.026 ± 0.006 μg/μg protein, respectively, demonstrating that butyric acid significantly decreased free cholesterol levels in $NPC1^{Nova\ Scotia}$ cells ($p<0.05$). To determine whether ASM induced by butyric acid is directly associated with the reduction of free cholesterol accumulation in $NPC1^{Nova\ Scotia}$ cells, NPDB and $NPC1^{Nova\ Scotia}$ fibroblasts were treated with 10 mM butyric acid and stained with filipin. We found that butyric acid dramatically reduced the accumulation of intracellular free cholesterol in $NPC1^{Nova\ Scotia}$ cells, but it did not affect NPDB cells. These data suggest that ASM induced by butyrate is related to intracellular cholesterol trafficking and metabolism in $NPC1^{Nova\ Scotia}$ cells. ASM inducers, such as butyrate, may reduce the accumulation of intracellular free cholesterol in $NPC1^{Nova\ Scotia}$ cells.

Key words : butyrate, acid sphingomyelinase, cholesterol, Niemann-Pick disease type C

Correspondence : Tsutomu Takahashi, M.D., Ph.D.
Department of Pediatrics, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan
Tel : 81-18-884-6159
Fax : 81-18-836-2620
E-mail : tomy@med.akita-u.ac.jp

Background

Niemann-Pick disease type C (NPC) is a lipid storage disorder caused by mutations in either the *NPC1* or the *NPC2* gene that lead to a defect of either NPC1 or NPC2 protein and it affects intracellular free cholesterol traf-

ficking. NPC1 is a 13 transmembrane domain protein residing in the limiting membrane of the late endosome/lysosome[1], whereas NPC2 is a soluble lysosomal protein that can bind cholesterol[2]. NPC2 has been shown to shuttle free cholesterol to and from membranes in vitro and to the N-terminal cholesterol-binding domain of NPC1[3,4]. Most cases of NPC result from mutations in the *NPC1* gene[5]. With regard to clinical manifestations and biochemical features, NPC shows hepatosplenomegaly, progressive neurological disease[5,6] and an accumulation of free cholesterol, sphingomyelin, and other lipids in the late endosome/lysosome[7-9]. A mutation in the *NPC1* gene, p.G992W, is responsible for Niemann-Pick disease type D (NPD), which is known as an allelic variant of NPC[10]. Patients with NPD have been traced to a single Acadian ancestor in Nova Scotia (Canada) and are characterized by mild to moderate hepatosplenomegaly, supranuclear gaze paresis in the vertical plane, slowly progressing ataxia, and mental retardation[5,11].

NPC cells have a secondary defect in acid sphingomyelinase (ASM) activity despite having a normal *ASM* gene. ASM is the lysosomal enzyme responsible for the hydrolysis of sphingomyelin to ceramide and phosphorylcholine. A defect in ASM activity caused by mutations in the *ASM* gene leads to Niemann-Pick disease types A and B (NPDA/NPDB), which are characterized by the accumulation of sphingomyelin, cholesterol, and other lipids in lysosomes[6]. ASM activity in NPC fibroblasts is reduced by as much as 80% when cells are grown in medium containing 10% fetal bovine serum or low density lipoprotein (LDL) cholesterol[12-14]. However, removal of lysosomal cholesterol by growth in medium containing lipoprotein-deficient serum restores ASM activity to normal levels[15]. In Chinese hamster ovary mutant cells with a defective NPC1 protein, it has been shown that ASM activity is suppressed to 5-10% of that in wild type cells[16]. In addition, NPC fibroblasts displayed normal ASM protein but negligible levels of ASM activity[16]. Although it remains to be fully determined why NPC cells have a secondary defect in ASM activity despite having a normal *ASM* gene, these previous studies have suggested that ASM activity in NPC cells is affected or regulated by the concentration of lysosomal cholesterol at the post-translational level. On the other hand, in ASM-deficient macrophages, it has been reported that intracellular accumulation of sphingomyelin due to ASM deficiency or to internalization of excess sphingomyelin leads to defective cholesterol trafficking and efflux[17]. Sphingomyelin has a high affinity for cholesterol, leading to the identical subcellular distribution of these two lipids, which are concentrated in the plasma membrane[18]. Therefore, ASM plays an important role in the intracellular relationship between sphingomyelin and cholesterol metabolism.

A recent study showed that restoration of ASM activity levels via either normal ASM gene transfection or exogenous addition of recombinant ASM in NPC cells results in a dramatic reduction in lysosomal free cholesterol and bis-(monoacylglycerol) phosphate[19]. Additionally, it was found that increased ASM activity improves defective transferrin receptor recycling, which is a secondary abnormality observed in NPC cells[19]. There is a limit to treatment options for NPC, for which the only approved drug is Zavesca (Miglustat)[20]. Therefore, correction of a secondary defect in ASM activity may offer new therapeutic approaches for NPC.

Butyrate, one of the short chain fatty acids, is known as an inducer of ASM. It has previously been reported that butyrate increased ASM activity by 10- to 20-fold and increased mRNA levels of ASM in human colon cancer HT29 cells and liver cancer HepG2 cells[21]. Butyrate is a natural substance present in the gastrointestinal tract, in milk, as well as in the sweat and feces of most mammals. It is derived from fermentable dietary fiber under the action of colonic microflora. The multiple beneficial effects of butyrate on human health and disease as represented by colorectal cancer are well documented[22,23]. However, it is still unknown whether butyrate induces ASM activity or affects intracellular cholesterol trafficking in NPC cells.

In this study, we investigated the effects of butyrate on ASM activity and ASM mRNA expression in normal and $NPC1^{Nova\ Scotia}$ lymphoblasts and on intracellular free cholesterol in $NPC1^{Nova\ Scotia}$ lymphoblasts and fibroblasts.

Materials and methods

Cell lines and cell culture

One patient with NPC and two patients with NPDB

were enrolled in this study. In the patient with NPC, mutations in the *NPC1* gene were p.G992W/p.G992W ($NPC1^{Nova\ Scotia}$)[24]. In the patients with NPDB, mutations in the *ASM* (*SMPD1*) gene were p.S436R/p.S436R[25-27] in one patient and p.S231P/p.P189fs in the other patient. Epstein-Barr virus (EBV)-transformed human lymphoblasts were established by standard techniques from a normal subject and the patient with $NPC1^{Nova\ Scotia}$. Skin fibroblasts were established from the patients with $NPC1^{Nova\ Scotia}$ and NPDB. All samples were collected after obtaining written informed consent. All cell lines were maintained in BD Falcon™ 25 cm^2 cell culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA). Lymphoblasts were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (HI-FBS ; Moregate Biotech, Bulimba, Australia) and used at full confluency. Fibroblasts were also cultured in Dulbecco's modified Eagle's medium supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% HI-FBS. All cells were grown in 5% CO_2 at 37°C in a humidified incubator.

Assay of ASM enzyme activity

Normal and $NPC1^{Nova\ Scotia}$ lymphoblasts were incubated with culture medium including butyric acid (Aldrich® Chemistry, USA) at different concentrations for 24 h at 37°C. The cells were washed two times with 1 × PBS to remove residual medium and pelleted by centrifugation at 800 g for 5 min at 4°C. The cells were disrupted by sonication on ice using three 15-s bursts, and cellular homogenates were assayed for protein content by the method of Lowry *et al*.[28] and for ASM activity as described below. Assays for ASM activity were carried out as follows. ^{14}C-labeled sphingomyelin was purchased from NEN Life Science Products. The standard 200 μl assay mixture consisted of 100 μl of sample and 50 μl of assay buffer containing 4% Triton X-100 (1.0 M sodium acetate, pH 5.0 ; final concentration of Triton X-100 in the 200 μl assay mix, 1%). The reaction was initiated by addition of 50 μl of substrate (20 nmol, ^{14}C-labeled sphingomyelin, 0.08 μCi/20 nmol) in 0.2% taurodeoxycholic acid. EDTA was added to the assay mixture at a final concentration of 0.02 mM. The assay mixtures were incubated at 37°C for 1 h and quantified by the method of Chatterjee and Ghosh[29].

Amplex® Red cholesterol quantification

$NPC1^{Nova\ Scotia}$ lymphoblasts were incubated in culture medium with or without 10 mM butyric acid for 24 h at 37°C. The cells were processed as described above. Total and free cholesterol levels were quantified using an Amplex® Red cholesterol assay kit (Molecular Probes Invitrogen Detection Technologies, Paisley, UK). In brief, samples were diluted in reaction buffer, after which an equivalent volume of Amplex® Red working solution (300 μM Amplex® Red, 2 U/ml cholesterol oxidase, and 2 U/ml horseradish peroxidase ; in addition to these reagents, 0.2 U/ml cholesterol esterase was used in the measurement of total cholesterol levels) was added. The samples were incubated at 37°C for 30 min, and the fluorescence intensity was measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using the Fluoroscan Ascent system (Thermo Labsystems). Total and free cholesterol levels were calculated using known cholesterol solutions and normalized to protein content.

RNA isolation and quantitative RT-PCR

Normal and $NPC1^{Nova\ Scotia}$ lymphoblasts were incubated in culture medium with or without 10 mM butyric acid for 24 h at 37°C. The cells were washed two times with 1 × PBS to remove residual medium and pelleted by centrifugation at 800 g for 5 min at 4°C. Total RNA was isolated with TRIzol® reagent (Invitrogen). The mRNA was transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland), and real-time PCR was performed with LightCycler®480 SYBR Green I Master (Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions. The primers for human ACTB (GenBank Accession# NM_001101) were purchased from Nihon Gene Research Laboratories (Cat# 5026385). The primer sequences of human ASM (GenBank Accession# NM_000543) were Forward 5′-ACC GAA TTG TAG CCA GGT AT-3′ and Reverse 5′-GGC TCA GAG TCT CTT CAT CA-3′. Real-time PCR was performed using the LightCycler®480 (Roche Diagnostics, Basel, Switzerland) for 40 cycles.

All samples were normalized to ACTB.

Filipin staining

NPC1$^{Nova\ Scotia}$ fibroblasts were grown to 50% confluence on glass coverslips in growth medium. After 1 day of growth, the medium was changed and the cells were incubated in growth medium with or without 10 mM butyric acid for 24 h at 37°C. NPDB fibroblasts were also incubated for 24 h in the same manner, but Lipoproteins Low Density Human Plasma (EMD Chemicals, Inc., San Diego, CA) was added to the medium (50 μg/ml) to induce more accumulation of free cholesterol in lysosomes. Both types of cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed in 1×PBS (three times for 5 min), and permeabilized with 0.1% Triton X-100 in 1×PBS for 10 min. To detect intracellular free cholesterol, filipin complex (Sigma-Aldrich Corporation) was added to the fixed cells (300 μg/ml in 1×PBS) for 45 min at room temperature. Finally, the cells were washed three times with 1×PBS, and analyzed using a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Thornwood, NY, USA) equipped with a UV laser.

Statistical analysis

Measurements of ASM activity and quantitative RT-PCR were performed in triplicate. Measurements of cholesterol levels were performed in quintuplicate. All the experiments were repeated at least twice. Data are presented as means±SD. Statistical analysis was performed by an unpaired two-tailed t-test using Stat View-J4.5 (Abacus Concepts, Berkeley, CA, USA). Differences of $p<0.05$ were considered to be statistically significant.

Results

Effect of butyrate on ASM activity in normal and NPC1$^{Nova\ Scotia}$ lymphoblasts

After incubating normal lymphoblasts with butyric acid at different concentrations for 24 h, ASM activity was increased in a dose-dependent manner (Fig. 1). The concentration of 10 mM butyric acid significantly increased ASM activity by 3.3-fold compared with the control ($p<0.01$).

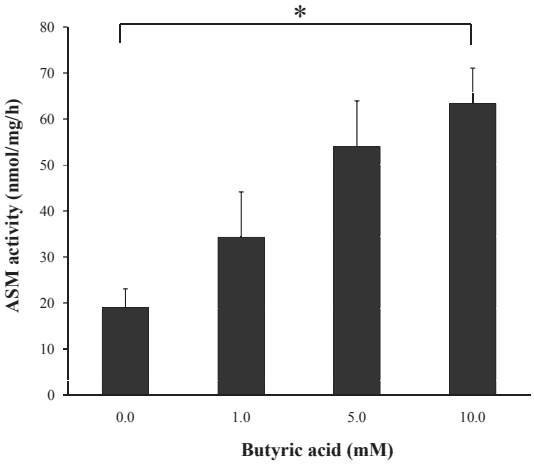

Fig. 1. Butyric acid induces ASM activity in normal lymphoblasts. Normal lymphoblasts were incubated with culture medium including butyric acid at different concentrations for 24 h at 37°C. The extracts were assayed for ASM activity. ASM activity in the cells was increased in a dose-dependent manner. The concentration of 10 mM butyric acid significantly increased ASM activity by 3.3-fold compared with the control ($^*p<0.01$). Data are expressed as means±SD.

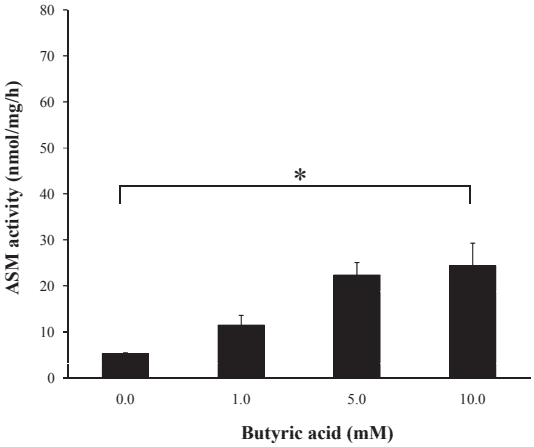

Fig. 2. Butyric acid restores ASM activity in NPC1$^{Nova\ Scotia}$ lymphoblasts. NPC1$^{Nova\ Scotia}$ lymphoblasts were incubated with culture medium including butyric acid at different concentrations for 24 h at 37°C. The extracts were assayed for ASM activity. Although basal ASM activity in NPC1$^{Nova\ Scotia}$ cells was suppressed by 28% of that in normal cells, it was increased in a dose-dependent manner. The concentration of 10 mM butyric acid significantly increased ASM activity by 4.6-fold compared with the control ($^*p<0.01$). Data are expressed as means±SD.

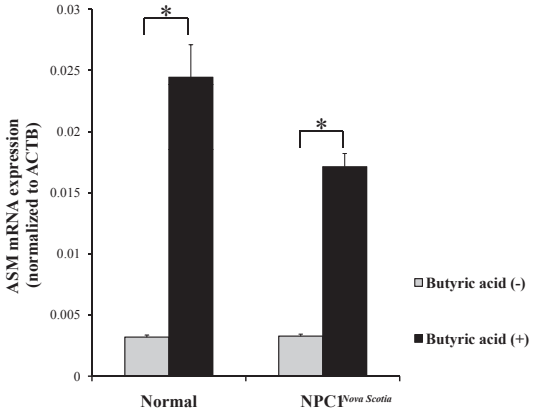

Fig. 3. Butyric acid increases ASM mRNA levels in normal and NPC1$^{\text{Nova Scotia}}$ lymphoblasts. Normal or NPC1$^{\text{Nova Scotia}}$ lymphoblasts were incubated in culture medium with or without 10 mM butyric acid for 24 h at 37°C. RNA isolation and quantitative RT-PCR were performed as described in the Materials and Methods. All samples were normalized to ACTB. Butyric acid significantly increased ASM mRNA levels in normal and NPC1$^{\text{Nova Scotia}}$ lymphoblasts by 7.7 and 5.2-fold, respectively ($^{*}p<0.01$). Data are expressed as means ± SD.

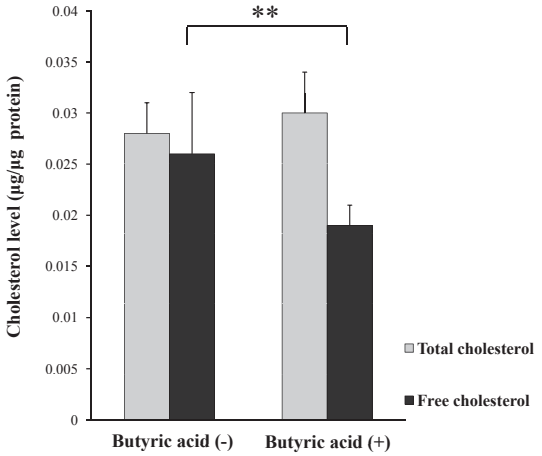

Fig. 4. Treatment of butyric acid decreases intracellular free cholesterol levels in NPC1$^{\text{Nova Scotia}}$ lymphoblasts. NPC1$^{\text{Nova Scotia}}$ lymphoblasts were incubated in culture medium with or without 10 mM butyric acid for 24 h at 37°C. The extracts were assayed for Amplex® Red cholesterol quantification. Butyric acid significantly decreased free cholesterol levels in the cells ($^{**}p<0.05$). There was no significant difference in total cholesterol levels. Data are expressed as means ± SD.

In NPC1$^{\text{Nova Scotia}}$ lymphoblasts, although basal ASM activity was suppressed by 28% of that in normal cells, it was also increased in a dose-dependent manner. The concentration of 10 mM butyric acid significantly increased ASM activity by 4.6-fold compared with the control (Fig. 2, $p<0.01$).

Effect of butyrate on ASM mRNA expression in normal and NPC1$^{\text{Nova Scotia}}$ lymphoblasts

Butyric acid significantly increased ASM mRNA levels in normal and NPC1$^{\text{Nova Scotia}}$ lymphoblasts by 7.7 and 5.2-fold, respectively (Fig. 3, $p<0.01$). There was no significant difference in basal ASM mRNA levels between normal and NPC1$^{\text{Nova Scotia}}$ cells.

Effect of butyrate on intracellular free cholesterol in NPC1$^{\text{Nova Scotia}}$ lymphoblasts

Free cholesterol levels in NPC1$^{\text{Nova Scotia}}$ lymphoblasts treated with butyric acid were significantly lower (0.019 ± 0.002 μg/μg protein) compared with those that were not treated with butyric acid (0.026 ± 0.006 μg/μg protein, Fig. 4, $p<0.05$). There was no significant difference in total cholesterol levels.

Filipin staining in NPC1$^{\text{Nova Scotia}}$ and NPDB fibroblasts by treatment with butyrate

The accumulation of intracellular free cholesterol was observed by filipin staining. In NPC1$^{\text{Nova Scotia}}$ fibroblasts, butyric acid dramatically reduced the accumulation of intracellular free cholesterol (Fig. 5), but it did not affect NPDB cells (Fig. 6).

Discussion

Butyrate has been reported to increase ASM activity and ASM mRNA levels in human colon cancer HT29 cells and liver cancer HepG2 cells[21]. In the current study, we showed that butyric acid increased ASM activity in a dose-dependent manner in both normal and NPC-1$^{\text{Nova Scotia}}$ lymphoblasts (Figs. 1, 2). In addition, the secondary defect of ASM activity in NPC1$^{\text{Nova Scotia}}$ cells was restored to normal levels by treatment of 10 mM butyric acid (Fig. 2). We also showed that butyric acid signifi-

Fig. 5. Butyric acid reduces the accumulation of free cholesterol in NPC1$^{Nova\ Scotia}$ fibroblasts. NPC1$^{Nova\ Scotia}$ fibroblasts were incubated in growth medium alone (A) or containing 10 mM butyric acid (B) for 24 h at 37°C. The cells were washed with PBS, fixed, and stained with filipin. The cells were visualized by confocal microscopy with a UV laser. The images are displayed with the same grayscale range. Treatment with butyric acid resulted in a dramatically reduced accumulation of free cholesterol in NPC1$^{Nova\ Scotia}$ cells. Scale bar, 20 μm.

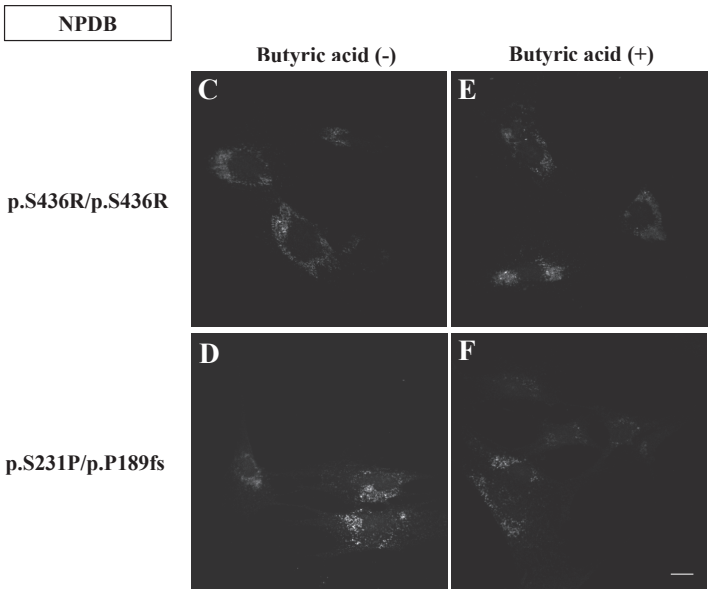

Fig. 6. Butyric acid does not reduce the accumulation of free cholesterol in NPDB fibroblasts. Fibroblasts from two patients with NPDB (mutations in the *ASM* gene : p.S436R/p.S436R and p.S231P/p.P189fs) were incubated in growth medium containing LDL in the absence (C, D) or presence of 10 mM butyric acid (E, F) for 24 h at 37°C. The cells were washed with PBS, fixed, and stained with filipin. The cells were visualized by confocal microscopy with a UV laser. The images are displayed with the same grayscale range. In NPDB cells, butyric acid did not affect the accumulation of free cholesterol. Scale bar, 20 μm.

cantly increased ASM mRNA levels in both normal and NPC1$^{Nova\ Scotia}$ lymphoblasts (Fig. 3). These data indicate that butyric acid increases transcription of the *ASM* gene.

Recent studies have shown that several drug treatments such as ASM gene transfection, recombinant ASM, curcumin, and thapsigargin in NPC cells lead to the improvement of abnormal cholesterol or sphingolipid trafficking[8,19]. In our study, the accumulation of intracellular free cholesterol was reduced by treatment of butyric acid in NPC1$^{Nova\ Scotia}$ lymphoblasts and fibroblasts (Figs. 4, 5).

Butyrate is also known as a histone deacetylase (HDAC) inhibitor. Histone acetyl transferases and HDAC have attracted considerable attention because of their regulatory role in chromatin remodeling and gene transcription. Butyrate exerts potentially useful effects on many conditions and diseases ; the mechanisms of butyrate action are mostly related to its potent regulatory effects on gene expression[22,23]. It has recently been shown that cholesterol homeostasis in NPC1$^{-/-}$ mice is improved by treatment with valproic acid, a very weak HDAC inhibitor[30]. In another study, HDAC inhibitor treatment dramatically reduced free cholesterol accumulation in NPC1 fibroblasts[31]. This previous study also demonstrated that the correction of free cholesterol accumulation is partially associated with increased expression of NPC1 protein by an HDAC inhibitor. In our study, butyrate induced ASM activity and reduced the accumulation of free cholesterol in NPC1$^{Nova\ Scotia}$ cells. To determine whether ASM induced by butyrate is directly associated with the reduction of free cholesterol accumulation in NPC1$^{Nova\ Scotia}$ cells, we investigated the effect of butyrate on free cholesterol accumulation in NPDB fibroblasts, in which ASM activity is not inducible by butyrate. We found that butyrate did not reduce the accumulation of free cholesterol in NPDB cells (Fig. 6). These data suggest that ASM induced by butyrate is related to intracellular cholesterol trafficking and metabolism in NPC1$^{Nova\ Scotia}$ cells. ASM inducers, such as butyrate, may reduce the accumulation of intracellular free cholesterol in NPC1$^{Nova\ Scotia}$ cells.

Conclusions

This study suggests that ASM inducers, such as butyrate, may reduce the accumulation of intracellular free cholesterol in NPC1$^{Nova\ Scotia}$ cells.

Acknowledgements

The authors thank Harumi Sugawara and Maiko Ito at Akita University Graduate School of Medicine for their technical assistance.

References

1) Kwon, H.J., Abi-Mosleh, L., Wang, M.L., Deisenhofer, J., Goldstein, J.L., Brown, M.S. and Infante, R.E. (2009) Structure of N-terminal domain of NPC1 reveals distinct subdomains for binding and transfer of cholesterol. *Cell*, **137**, 1213-1224.

2) Friedland, N., Liou, H.L., Lobel, P. and Stock, A.M. (2003) Structure of a cholesterol-binding protein deficient in Niemann-Pick type C2 disease. *Proc. Natl. Acad. Sci. USA*, **100**, 2512-2517.

3) Infante, R.E., Wang, M.L., Radhakrishnan, A., Kwon, H.J., Brown, M.S. and Goldstein, J.L. (2008) NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosomes. *Proc. Natl. Acad. Sci. USA*, **105**, 15287-15292.

4) Xu, Z., Farver, W., Kodukula, S. and Storch, J. (2008) Regulation of sterol transport between membranes and NPC2. *Biochemistry*, **47**, 11134-11143.

5) Patterson, M.C., Vanier, M.T., Suzuki, K., Morris, J.A., Carstea, E., Neufeld, E.B., Blanchette-Mackie, E.J. and Pentchev, P.G. (2001) Niemann-Pick disease type C : a lipid trafficking disorder. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds.) *The Metabolic and Molecular Bases of Inherited Disease.* McGraw-Hill, New York, pp. 3611-3634.

6) Schuchman, E.H. and Desnick, R.J. (2001) Niemann-Pick disease type A and B : acid sphingomyelinase deficiencies. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds.) *The Metabolic and Molecular Bases of Inherited Disease.* McGraw-Hill, New York, pp. 3589-3610.

7) Puri, V., Watanabe, R., Dominguez, M., Sun, X.,

Wheatley, C.L., Marks, D.L. and Pagano, R.E. (1999) Cholesterol modulates membrane traffic along the endocytic pathway in sphingolipid-storage diseases. *Nat. Cell. Biol.*, **1**, 386-388.

8) Lloyd-Evans, E., Morgan, A.J., He, X., Smith, D.A., Elliot-Smith, E., Sillence, D.J., Churchill, G.C., Schuchman, E.H., Galione, A. and Platt, F.M. (2008) Niemann-Pick disease type C1 is a sphingosine storage disease that causes deregulation of lysosomal calcium. *Nat. Med.*, **14**, 1247-1255.

9) Chevallier, J., Chamoun, Z., Jiang, G., Prestwich, G., Sakai, N., Matile, S., Parton, R.G. and Gruenberg, J. (2008) Lysobisphosphatidic acid controls endosomal cholesterol levels. *J. Biol. Chem.*, **283**, 27871-27880.

10) Greer, W.L., Riddell, D.C., Gillan, T.L., Girouard, G.S., Sparrow, S.M., Byers, D.M., Dobson, M.J. and Neumann, P.E. (1998) The Nova Scotia (type D) form of Niemann-Pick disease is caused by a G3097-->T transversion in NPC1. *Am. J. Hum. Genet.*, **63**, 52-54.

11) Jan, M.M. and Camfield, P.R. (1998) Nova Scotia Niemann-Pick disease (type D) : clinical study of 20 cases. *J. Child. Neurol.*, **13**, 75-78.

12) Pentchev, P.G., Comly, M.E., Kruth, H.S., *et al*. (1987) Group C Niemann-Pick disease : faulty regulation of low-density lipoprotein uptake and cholesterol storage in cultured fibroblasts. *FASEB. J.*, **1**, 40-45.

13) Vanier, M.T., Wenger, D.A., Comly, M.E., Rousson, R., Brady, R.O. and Pentchev, P.G. (1988) Niemann-Pick disease group C : clinical variability and diagnosis based on defective cholesterol esterification. A collaborative study on 70 patients. *Clin. Genet.*, **33**, 331-348.

14) Vanier, M.T., Rodriguez-Lafrasse, C., Rousson, R., Gazzah, N., Juge, M.C., Pentchev, P.G., Revol, A. and Louisot, P. (1991) Type C Niemann-Pick disease : spectrum of phenotypic variation in disruption of intracellular LDL-derived cholesterol processing. *Biochim. Biophys. Acta*, **1096**, 328-337.

15) Thomas, G.H., Tuck-Muller, C.M., Miller, C.S. and Reynolds, L.W. (1989) Correction of sphingomyelinase deficiency in Niemann-Pick type C fibroblasts by removal of lipoprotein fraction from culture media. *J. Inherit. Metab. Dis.*, **12**, 139-151.

16) Reagan, J.W. Jr., Hubbert, M.L. and Shelness, G.S. (2000) Posttranslational regulation of acid sphingomyelinase in niemann-pick type C1 fibroblasts and free cholesterol-enriched chinese hamster ovary cells. *J. Biol. Chem.*, **275**, 38104-38110.

17) Leventhal, A.R., Chen, W., Tall, A.R. and Tabas, I. (2001) Acid sphingomyelinase-deficient macrophages have defective cholesterol trafficking and efflux. *J. Biol. Chem.*, **276**, 44976-44983.

18) Brown, R.E. (1998) Sphingolipid organization in biomembranes : what physical studies of model membranes reveal. *J. Cell. Sci.*, **111**, 1-9.

19) Devlin, C., Pipalia, N.H., Liao, X., Schuchman, E.H., Maxfield, F.R. and Tabas, I. (2010) Improvement in Lipid and Protein Trafficking in NPC1 Cells by Correction of a Secondary Enzyme Defect. *Traffic*, **11**, 601-615.

20) Patterson, M.C., Vecchio, D., Prady, H., Abel, L. and Wraith, J.E. (2007) Miglustat for treatment of Niemann-Pick C disease : a randomised controlled study. *Lancet Neurol.*, **6**, 765-772.

21) Wu, J., Cheng, Y., Jönsson, B.A., Nilsson, A. and Duan, R.D. (2005) Acid sphingomyelinase is induced by butyrate but does not initiate the anticancer effect of butyrate in HT29 and HepG2 cells. *J. Lipid Res.*, **46**, 1944-1952.

22) Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R. and Van Immerseel, F. (2010) From the gut to the peripheral tissues : the multiple effects of butyrate. *Nutr. Res. Rev.*, **23**, 366-384.

23) Canani, R.B., Costanzo, M.D., Leone, L., Pedata, M., Meli, R. and Calignano, A. (2011) Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J. Gastroenterol.*, **17**, 1519-1528.

24) Oyama, K., Takahashi, T., Shoji, Y., Oyamada, M., Noguchi, A., Tamura, H., Takada, G. and Kanbayashi, T. (2006) Niemann-Pick disease type C : cataplexy and hypocretin in cerebrospinal fluid. *Tohoku J. Exp. Med.*, **209**, 263-267.

25) Takada, G., Satoh, W., Komatsu, K., Konn, Y., Miura, Y. and Uesaka, Y. (1987) Transitory type of sphingomyelinase deficient Niemann-Pick disease : clinical and morphological studies and follow-up of two sisters. *Tohoku J. Exp. Med.*, **153**, 27-36.

26) Takahashi, T., Desnick, R.J., Takada, G. and Schuchman, E.H. (1992) Identification of a missense mutation (S436R) in the acid sphingomyelinase gene from

a Japanese patient with type B Niemann-Pick disease. *Hum. Mutat.*, **1**, 70-71.

27) Takahashi, T., Akiyama, K., Tomihara, M., Tokudome, T., Nishinomiya, F., Tazawa, Y., Horinouchi, K., Sakiyama, T. and Takada, G. (1997) Heterogeneity of liver disorder in type B Niemann-Pick disease. *Hum. Pathol.*, **28**, 385-388.

28) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.

29) Chatterjee, S. and Ghosh, N. (1991) Purification of neutral sphingomyelinase from human urine. *Methods Enzymol.*, **197**, 540-547.

30) Kim, S.J., Lee, B.H., Lee, Y.S. and Kang, K.S. (2007) Defective cholesterol traffic and neuronal differentiation in neural stem cells of Niemann-Pick type C disease improved by valproic acid, a histone deacetylase inhibitor. *Biochem. Biophys. Res. Commun.*, **360**, 593-599.

31) Pipalia, N.H., Cosner, C.C., Huang, A., Chatterjee, A., Bourbon, P., Farley, N., Helquist, P., Wiest, O. and Maxfield, F.R. (2011) Histone deacetylase inhibitor treatment dramatically reduces cholesterol accumulation in Niemann-Pick type C1 mutant human fibroblasts. *Proc. Natl. Acad. Sci. USA*, **108**, 5620-5625.