Butyrate Improves Insulin Sensitivity and Increases Energy Expenditure in Mice

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**Objective**- To examine the role of butyric acid, a short chain fatty acid formed by fermentation in the large intestine, in the regulation of insulin sensitivity in mice fed a high fat diet.

**Research design and methods**- In dietary obese C57BL/6J mice, sodium butyrate was administrated through diet supplementation at 5% w/w in the high fat diet (HFD). Insulin sensitivity was examined with insulin tolerant test and HOMA IR. Energy metabolism was monitored in a metabolic chamber. Mitochondrial function was investigated in brown adipocytes and skeletal muscle in the mice.

**Results**- On the HFD diet, supplementation of butyrate prevented development of insulin resistance and obesity in C57BL/6 mice. Fasting blood glucose, insulin, insulin tolerance were all reserved in the treated mice. The body fat content was maintained at 10% without a reduction in food intake. Adaptive thermogenesis and fatty acid oxidation were enhanced. An increase in mitochondria function and biogenesis was observed in the skeletal muscle and brown fat. The type 1 fiber was enriched in the skeletal muscle. PGC-1α expression was elevated at mRNA and protein levels. AMPK and p38 activities were elevated. In the obese mice, supplementation of butyrate led to an increase in insulin sensitivity and reduction in adiposity.

**Conclusions**- Dietary supplementation of butyrate can prevent and treat diet-induced insulin resistance in mouse. The mechanism of butyrate action is related to promotion of energy expenditure and induction of mitochondria function.
Recent studies suggest that natural compounds represent a rich source for small thermogenic molecules, which hold potential in the prevention and treatment of obesity and insulin resistance. Several natural products, such as resveratrol (1; 2), bile acid (3), and genipin (4), have been reported to increase thermogenic activities in animal or cellular models. In the current study, we provide evidence for the thermogenic activity and therapeutic value of a short chain fatty acid, butyric acid (BA), in a mouse model of metabolic syndrome. Butyric acid has four carbons in the molecule (CH₃CH₂CH₂-COOH) and becomes sodium butyrate after receiving sodium. Sodium butyrate (SB) is a dietary component found in foods such as cheese and butter. It is also produced in large amounts from dietary fiber after fermentation in the large intestine, where butyric acid is generated together with other short chain fatty acids (SCFAs) from non-digestible carbohydrates, such as non-starch polysaccharides, resistant starch and miscellaneous low-digestible saccharides (5; 6). The bioactivities of SB are related to inhibition of class I and class II histone deacetylases (HDACs) (7). HDACs regulate gene transcription through modification of chromatin structure by deacetylation of proteins including histone proteins and transcription factors. According to our knowledge, there is no report about butyrate in the regulation of insulin sensitivity or energy metabolism.

Dietary intervention is a potential strategy in the prevention and treatment of metabolic syndrome. PGC-1α (peroxisome proliferator-activated receptor g coactivator 1 alpha), a transcription coactivator, is a promising molecular target in the dietary intervention (1; 2). PGC-1α controls energy metabolism by interaction with several transcription factors, e.g. ERRα, NRF-1, NRF-2, PPARα, PPARδ and thyroid hormone receptor (TR) that direct gene transcription for mitochondrial biogenesis and respiration (8). In the muscle, PGC-1α increases oxidative (Type I) fiber differentiation and enhances fatty acid metabolism (9). In brown fat, PGC-1α stimulates adaptive thermogenesis through up-regulation of UCP-1 expression (10). A reduction in PGC-1α function is associated with mitochondrial dysfunction, reduction in fatty acid oxidation and risk for insulin resistance or type 2 diabetes (11-14). Dietary intervention of PGC-1α activity holds promise in the prevention and treatment of metabolic syndrome. However, our knowledge is limited in the dietary components or derivatives that are able to regulate the PGC-1 activity. In the current report, we provide evidence that SB induced the PGC-1 activity in the skeletal muscle and brown fat in mice.

RESEARCH DESIGN AND METHODS

Animal model. Male C57BL/6J (4 weeks in age) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). After one week quarantine, the C57BL/6J mice were fed on HFD (D12331, Research Diets, New Brunswick, NJ), in which 58% calorie is in fat. All of the mice were housed in the animal facility with a 12:12-h light-dark cycle and constant temperature (22–24°C). The mice were free to access water and diet. All procedures were performed in accordance with National Institute of Health guidelines for the care and use of animals and approved by the Institute Animal Care and Use Committee at the Pennington Biomedical Research Center.

Sodium butyrate (SB) administration. SB (#303410, Sigma) was incorporated into the HFD at 5% w/w. SB was blended into the diet using a food processor at 400 rpm. The SB-containing diet was pelleted and stored in a -20 °C freezer.
until usage. On the supplemented diet, mice may receive SB at 5g/kg/day at the normal daily rate of calorie intake.

**Intraperitoneal insulin tolerance (ITT).** ITT was conducted by intra-peritoneal (i.p.) injection of insulin (I9278, Sigma) at 0.75 U/kg body weight in mice after a 4 hour fast as being described elsewhere (15).

**Nuclear magnetic resonance.** Body composition was measured for the fat content using quantitative nuclear magnetic resonance (NMR) as previously described (15).

**Quantitative real-time RT-PCR.** Total RNA was extracted from frozen tissues (kept at -80 °C) using Tri-Reagent (T9424, Sigma) as described elsewhere (16). Taqman RT-PCR primer and probe were used to determine mRNA for PGC-1α (Mm00447183_m1), UCP-1 (Mm00494069_m1), PPARδ (Mm01305434_m1) and CPT1b (Mm00487200_m1). The reagents were purchased from Applied Biosystems (Foster City, CA). Mouse ribosome 18S rRNA_s1 (without intron-exon junction) was used as an internal control. Reaction was conducted with 7900 HT Fast real time PCR System (Applied Biosystems, Foster City, CA).

**Metabolic chamber.** Energy expenditure, respiratory exchange ratio (RER), spontaneous physical movement, and food intake were measured simultaneously for each mouse with the Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus, OH) as described previously (15).

**Body temperature in cold response.** Body temperature was measured in the cold room with ambient temperature at 4°C. Animals were sedated and restrained for less than 30 sec in the measurement. A Thermalert model TH-8 temperature monitor (Physitemp,Clifton,NJ) was used with probe placed in the rectum at 2.5 cm in depth.

**Western Blotting.** Fresh fat and muscles were collected and frozen immediately in liquid nitrogen. The whole cell lysate was prepared in a lysis buffer with sonication as described elsewhere (16). Antibodies and their sources are myoglobin (sc-25607, Santa Cruz), pAkt (Thr308, #9275, Cell signaling), Tubulin (ab7291, Abcam), pIRS-1 (Tyr632, sc-17196, Santa Cruz), myosin (M8421, Sigma), pAMPK (Thr172, #2531, Cell signaling) and pP38 (sc-7975, Santa Cruz). The antibodies to PGC-1α and UCP-1 were from Dr. Thomas Gettys at our institute.

**Muscle fiber type.** The fiber types in skeletal muscle were examined using two methods: succinate dehydrogenase (SDH) staining for ATPase and immunostaining of type I myosin heavy chain. In the SDH staining, mid-belly cross-sections of muscle were cut at 8 µm in a cryostat (-20 °C). After drying for 5 min at room temperature, the sections were incubated at 37 °C for 60 min in the incubation solution containing 6.5 mmol/l sodium phosphate monobasic, 43.5mmol/l sodium phosphate biphasic, 0.6 mmol/l nitroblue tetrazolium (74032,Sigma), and 50 mmol/l sodium succinate (14160, Sigma). The sections were rinsed three times (30 sec/time) in 0.9% saline, 5 min in 15% ethanol and then mounted with aqueous mounting medium (Dakocytoma).

**Immunohistostaining.** Fresh skeletal muscle was collected, embedded in gum tragacanth mixed with OCT freezing matrix, and quickly frozen in liquid nitrogen. The tissue slides were obtained through serial cross-section cutting at 8 µm thickness. The slide was blotted with the type I myosin heavy chain antibody (M8421, Sigma) at 1:200 dilution. After being washed, the slide was incubated with a biotinylated secondary antibody (BA-2000), and the color reaction was performed using the ABC elite reagent (PK-6101). The DAB substrate kit (SK-4100) was used to obtain signal for myosin I and AEC substrate kit (AEC101, Sigma) for PGC-1α.
Hematoxylin and Eosin (H&E) staining. Fresh tissues (BAT and inguinal fat) were collected at 16 weeks of age after 12 weeks on the SB-containing diet. The tissue was fixed in the 10% formalin solution (HT50-1-2, Sigma). The tissue slides were obtained through serial cross-section cutting at 8 µm in thickness and processed with a standard procedure.

Histone deacetylase assay and nuclear extract preparation. Histone deacetylase assay were conducted using a HDAC assay kit (#17-320, Upstate). Briefly, the muscle nuclear extract (10 µg) was incubated with [3H]-acetyl CoA (#TRK688, Amersham) radio labeled histone H4 peptide (25,000 CPM, as a substrate) at 37 °C for 12 hours by shaking. Released [3H]-acetate was measured using a scintillation counter. The nuclear extract was prepared according to a protocol described elsewhere (17).

Lipids in serum and feces: The serum fatty acids including butyrate were examined using a protocol described elsewhere (18). The detail is presented in Supplement 1 in the online appendix available at http://diabetes.diabetesjournals.org. The fatty acids in feces were determined using a protocol in a study by Schwarz (19). Triglyceride and cholesterol were measured in the whole blood with the Cardiochek portable test system.

Statistical Analysis. In this study, the data were presented as mean ± SE from multiple samples. All of the in vitro experiments were conducted three times at least. Student’s t test or two-way ANOVA was used in the statistical analysis with significance $P \leq 0.05$.

RESULTS

Energy metabolism. We first tested butyrate in prevention of dietary obesity. In the diet-induced obesity model, the butyrate supplementation started at the beginning of HFD feeding. The plain HFD was used in the control group. Calorie intake was monitored four times in the first ten weeks. After normalization with the body weight, the calorie intake was reduced with the increase in age. In the butyrate group, it was significantly higher at all of the time points (Fig. 1, A). The energy expenditure, oxygen consumption and substrate utilization were monitored using the metabolic chamber. In the butyrate group, the energy expenditure and oxygen consumption were elevated at the night time (Fig. 1, B and C). The respiratory exchange ratio (RER) was reduced during the day and night time (Fig. 1D), suggesting an increase in the fatty acid oxidation in response to butyrate. These data suggests that SB may increase energy expenditure in the DIO model.

Body weight and fat content was monitored in the study. In the control mice, the body weight was increased from 23 g to 40 g after 16 weeks on HFD (Fig. 1E), and the fat content (adiposity) was increased from 10% to 35% of the body weight (Fig. 1F). Accordingly, the lean mass was reduced from 80% to 65% (Fig. 1G). In the butyrate group, these parameters were not significantly changed during the 16 weeks on HFD (Fig. 1, E, F, and G), suggesting that butyrate prevented diet-induced obesity. Mouse growth was not influenced by butyrate as the body length was identical between the two groups. Dietary fat digestion and absorption in the gastrointestinal track was examined by measuring the fatty acid content in feces. The fat content was identical in the feces of two groups (Fig. 1H), suggesting that butyrate does not influence the fat absorption by the gastrointestinal track. Spontaneous physical activity was monitored in daytime and nighttime in the mice. The data suggests that the physical activity was not reduced by butyrate (Fig. 1I). An increased activity was observed in the butyrate group at the night time. These data suggest that dietary supplementation of butyrate protected the
mice from diet-induced obesity. This effect is associated with an increase in energy expenditure and fatty acid oxidation. The food intake and physical activity suggests that no toxicity was observed for butyrate in the mice.

**Insulin sensitivity.** The increase in energy metabolism suggests that butyrate may protect the mice from HFD-induced insulin resistance. To test this possibility, systemic insulin sensitivity was analyzed using fasting glucose, insulin and insulin tolerance. In the control group, the fasting glucose was increased significantly after 10 weeks on HFD (Fig. 2A). In the butyrate group, this increase was not observed (Fig. 2A). The fasting insulin was 50% lower in the butyrate group at 16 weeks on HFD (Fig. 2B). In the ITT test, the butyrate group exhibited much better response to insulin at all the time points (30, 60, 120 and 180 mins) (Fig. 2C). HOMA-IR was 60% lower in the butyrate group (Fig. 2D). These data suggests that insulin resistance was prevented in the butyrate group. Insulin signaling was examined in the skeletal muscle lysate with tyrosine 632 (Y632) phosphorylation of IRS-1 protein and Threonine 308 phosphorylation of Akt (Fig. 2E). Both signals were increased in the butyrate-treated mice (Fig. 2, E and F), suggesting a molecular mechanism of insulin sensitization.

**Brown adipose tissue (BAT).** The association of increased food intake with elevated energy expenditure led us to study the brown adipose tissue, which is responsible for adaptive thermogenesis in response to diet or cold (20-22). Diet-induced thermogenesis reduces obesity in both humans and animals (23). In the butyrate group, the increase in energy expenditure was observed at night when mice actively took food (Fig 1, A and B). The result suggests an increase in thermogenesis. To determine the thermogenic function, we conducted a cold-response experiment. The mice were exposed to a cold environment with an ambient temperature of 4 °C fro 90 mins. The body core temperature was monitored 3 times by measuring the rectal temperature. In the control mice, the body temperature was decreased with time and was recorded at 34.5 °C after 90 mins in the cold (Fig. 3A). In the butyrate-treated mice, the body temperature dropped to 35 °C transiently at 30 minutes, and then returned to 36 °C for the rest of time. These data suggests that the thermogenic function is enhanced in the butyrate group.

Brown fat is critical for the adaptive thermogenesis in mice. Morphology and gene expression were examined in the brown fat. Compared to the control mice, the size of brown adipocytes was much smaller in the butyrate group (Fig. 3B), suggesting a higher thermogenic activity that leads to the reduction in fat accumulation. Mitochondrial function is regulated by gene expression (24). To understand the molecular basis of the increased thermogenesis, we examined expression of two thermogenesis-related genes, such as PGC-1α and UCP-1 in BAT. mRNA of both genes was increased in the butyrate-treated mice (Fig. 3C). The increase was observed in their proteins in the brown fat (Fig. 3D). The increased gene expression provides a molecular basis for the enhanced thermogenesis by the butyrate treatment.

**Skeletal muscle.** To understand the cellular basis of enhanced fatty acid utilization in the butyrate group, we assessed the muscle fiber types in the study. PGC-1α was reported to induce transformation of skeletal muscle fiber from glycolytic type (Type II) into oxidative type (Type I) in transgenic mice (9). The types I fibers are distinct from the type II fibers in several properties (25). The type I fibers (oxidative and slow-twitch fibers) are rich in mitochondria, red in color and active in fat oxidation for ATP biosynthesis. The type II fibers (glycolytic and fast-twitch fibers) are relatively poor in mitochondria activity,
lighter in color and dependent on glycolysis in the ATP production. The butyrate effect on PGC-1α in BAT suggests that skeletal muscle fibers may be changed by butyrate.

Compared to the control group, the butyrate group exhibited a deep red color (Fig. 4A). The fiber type analysis was conducted in the vastus lateralis, gastrocnemius (rich in glycolytic fibers) and soleus (rich in oxidative fiber). The type I fiber was determined with the type I myosin heavy chain in immunohistostaining. The ratio of type I fibers were increased in all of the skeletal muscle of butyrate-treated mice (Fig. 4B). The increase was confirmed with the metachromatic dye-ATPase assay, in which the type I fibers were in blue color (Fig. 4C). To support the change in muscle morphology, the proteins of type 1 myosin heavy chain and PGC-1α were quantified in the muscle lysate in an immunoblot. A significant increase was observed in both proteins in the butyrate-treated mice (Fig. 4D). Myoglobin (another marker of oxidative type 1 fiber) was also increased by butyrate (Fig. 4D). A mean value of each protein was presented in the bar figure in Figure 4D. These data suggest that the ratio of type I fiber was increased by butyrate in the skeletal muscle.

AMPK and p38 activities were examined by their phosphorylation status. Their activities may contribute to elevation of the PGC-1α protein through enhancing protein stability (26-28). It was not clear if this mechanism was activated by butyrate. To test this possibility, we examined activity of AMPK and p38 in the skeletal muscle. An increase in their phosphorylation was observed in both proteins in the butyrate-treated mice (Fig. 4D). Myoglobin (another marker of oxidative type 1 fiber) was also increased by butyrate (Fig. 4D). A mean value of each protein was presented in the bar figure in Figure 4D. These data suggest that the ratio of type I fiber was increased by butyrate in the skeletal muscle.

Mitochondrial function. Mitochondrial function was examined in the skeletal muscle tissue and L6 muscle cells under butyrate treatment. Fatty acid oxidation was monitored in the gastrocnemius muscle with 14C-labeled palmitic acid. A 200% increase in 14C-labeled CO2 was observed in the butyrate-treated mice (Fig. 6A). The fatty acid oxidation was associated with expression of PGC-1α target genes, such as CPT-1b (carnitine palmitoyltransferase-1b) and COX-I (cytochrome c oxidase I) (9). Expression of these two genes was increased in the skeletal muscle of butyrate-treated mice (Fig. 6, B and C). The nuclear receptor PPARδ promotes fatty acids oxidation in skeletal muscle (29). PPARδ expression was also increased in the butyrate-treated mice (Fig. 6B). In cultured L6 cells, a similar increase was observed in fatty acid oxidation and gene expression after butyrate treatment (Fig. 6, D and E). These data consistently support the butyrate activity in the promotion of mitochondrial function.

HDAC activity in muscle. The butyrate concentration was analyzed in the serum collected from the butyrate group and control groups. In the fasted condition (overnight fast), the butyrate concentration was 7.23±0.93 µg/ml in the butyrate group and 5.71±0.38 µg/ml in the control. In the fed condition, the butyrate concentration was 9.40±1.36 µg/ml in the butyrate group versus 5.48±0.60 µg/ml in the control (P<0.05, n=5) (Fig. 6F). The data suggests that the dietary supplementation increased butyrate levels in the blood. It is likely that the metabolic activity of butyrate is related to inhibition of HDAC. Sodium butyrate inhibits the class I
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and class II histone deacetylases (HDACs). To test this possibility, the HDAC activity was examined in the skeletal muscle of mice at 16 weeks on HFD (Fig. 6F). The assay was conducted with the nuclear extracts of muscle samples. The HDAC activity was reduced by 50% in the butyrate group (Fig. 6G). TSA, a typical histone deacetylase inhibitor, was used as a positive control in the parallel treatment. The HDAC activity was decreased in the skeletal muscle of TSA-treated mice (Suppl. 2). These data suggested that the dietary supplementation of butyrate leads to suppression of HDAC activity in the body. Total triglyceride and cholesterol were examined in the blood. These lipids were reduced in the butyrate group (Fig. 6, H and I).

**Treatment of obesity with butyrate.** In the prevention studies, butyrate was administrated together with HFD during the induction of obesity. To test butyrate in the treatment of obesity and insulin resistance, we administrated butyrate to obese mice that had been on HFD for 16 weeks. After a 5-week treatment with butyrate, the obese mice lost 10.2% of their original body weight that dropped from 37.6g to 34.4g (Fig. 7A). In the control group, the body weight was increased by 15.8% (from 35.9g to 41.6g) during the same time period. Consistent with the change in body weight, the fat content was reduced by 10% in the butyrate group (Fig. 7B). Further, the fasting glucose was reduced by 30% from 131 to 98.6 mg/dl (p<0.016), HOMA-IR was reduced by 50%, and ITT was improved significantly in the butyrate group (Fig. 7C and D). These data suggested that butyrate is effective in the treatment of obesity and insulin resistance in the dietary obese model.

**DISCUSSION**

Metabolic activities of butyric acid were examined in this study in diet-induced obese mice. The most important observation is that butyrate supplementation at 5% w/w in HFD prevented development of dietary obesity and insulin resistance. It also reduced obesity and insulin resistance in obese mice. In the butyrate-treated mice, the plasma butyrate concentration was increased, and the blood lipids (triglycerides, cholesterol and total fatty acids) were decreased (Fig. 6, H-I and Suppl. 1). The change in insulin sensitivity may be a consequence of reduction in adiposity in our model. The increase in energy expenditure and fatty acid oxidation may be responsible for the anti-obesity effect of butyrate. The butyrate supplementation did not reduce food intake, fat absorption or locomotor activity, suggesting that there was no toxicity from butyrate. Butyrate was tested at 5% and 2.5% w/w in the HFD in this study. At the lower (2.5% w/w) dosage, a similar metabolic activity was observed (Suppl. 3). At 5% in HFD, butyrate increases the calorie content from 58% to 64.4% in the fat. The increase in fat calorie may not contribute to our observation of the anti-obesity activity for butyrate. A recent study of weight-loss diets suggests that the total calorie intake, not diet composition, is responsible for the weight reduction in a human study (30). At the cellular level, butyrate increased mitochondrial respiration as indicated by the increase in oxygen consumption and carbon dioxide production. At the molecular level, an increased expression of PGC-1α, PPARδ and CPT1b may be involved in the stimulation of mitochondrial function by butyrate.

The current study indicates that in vivo, butyrate is a novel activator of PGC-1α. The PGC-1α activity may be regulated by butyrate at three levels. The PGC-1α expression was increased in both mRNA and protein. The protein elevation was observed in brown fat, skeletal muscle and liver in the butyrate-treated mice. It may be a result of increased mRNA expression or extended half-life of the PGC-1α protein. The change in protein stability is supported by the activities
of AMPK and p38 in tissues and cells after butyrate treatment. These kinases phosphorylate the PGC-1α protein and inhibit its degradation (27; 28; 31-34). As a transcriptional coactivator, the PGC-1α transcription activity may be induced by the phosphorylation, which leads to removal of a suppressor protein (p160 myb) that is associated with PGC-1α in the basal condition (35). P38 acts at the downstream of AMPK in the phosphorylation of PGC-1α (36). Therefore, AMPK may increase PGC-1α phosphorylation through direct and indirect (p38) mechanisms. It is not clear how AMPK is activated by butyrate. Butyrate may act through induction of AMP levels in cells from an increased consumption of ATP. It was reported that butyrate increases ATP consumption (37). Induction of PGC-1α activity may be a molecular mechanism by which butyrate stimulates the mitochondrial function.

Inhibition of HDAC may contribute to the increased mRNA expression of PGC-1α, PPARδ and CPT1b. HDAC inhibition promotes gene expression through transcriptional activation, which is determined by the gene promoter activity. The promoter activation requires histone acetylation that opens the chromatin DNA to the general transcription factors for the transcription initiation and mRNA elongation. HDAC inhibits the gene promoter activity through deacetylation of the histone proteins. In the presence of butyrate, the promoter inhibition is prevented by the butyrate suppression of HDAC. The HDAC suppression will enhance the histone acetylation. This chromatin modification may occur in the gene promoters for PGC-1α, PPARδ and CPT1b for the up-regulation of gene transcription.

Butyrate induces type 1 fiber differentiation in the skeletal muscle. In skeletal muscle cells, inhibition of HDAC enhances myotube differentiation in vitro (28-30), and protects muscle from dystrophy in vivo (29-31). In transgenic mice, knockout of class II HDACs was shown to promote differentiation of type 1 (oxidative) fibers in skeletal muscle (32). This is consistent with our data that the type 1 fiber was increased by butyrate, which inhibits HADC activities in the skeletal muscle of the butyrate-treated mice. TSA, a typical histone deacetylase inhibitor, was tested in the parallel treatment with butyrate. TSA exhibited similar activity to butyrate in mice (Suppl. 2). TSA prevented dietary obesity, insulin resistance, and increased the type 1 fiber in the skeletal muscle. The activity was associated with elevation of PGC-1α protein. The current study suggests that the metabolic activities of butyrate may be dependent on the inhibition of HDAC.

In summary, dietary supplementation of butyrate can prevent and treat diet-induced obesity and insulin resistance in the mouse models of obesity. These activities of butyrate are similar to those of resveratrol (1; 2). The mechanism of butyrate action is related to promotion of energy expenditure and induction of mitochondrial function. Stimulation of PGC-1α activity may be a molecular mechanism of the butyrate activity. Activation of AMPK and inhibition of HDACs may contribute to the PGC-1α regulation. Butyrate and its derivatives may have potential application in the prevention and treatment of metabolic syndrome in human.

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REFERENCE


Figure legend

Fig. 1. Energy metabolism in response to sodium butyrate. Butyrate increased energy expenditure in C57BL/B6 mice. Energy expenditure was examined using the metabolic chamber at the first week and the tenth week on HFD (16 weeks in age). In this study, sodium butyrate was used at 5% w/w in HFD. A. Food intake. Food intake was monitored daily for 5 days at each time point. Average daily food intake (g) was converted into K calorie and normalized with body weight (kg) and 24 hours. B. Energy expenditure. The unit is K caloric per kilogram lean mass every hour. C. Oxygen consumption. The unit is ml volume oxygen in kilogram lean mass per hour. D. Substrate utilization. This is expressed by respiratory exchange ratio (RER), which is a volume ratio of oxygen consumed versus CO2 exhaled. E. Body weight. F. Body fat content in percentage of body weight. This was determined by NMR. G. Body muscle content in percentage of body weight. H. Lipid in feces. Feces were collected in the cages during a 24 hours period on HFD at 12 weeks. The total lipids were extracted and quantified (P>0.05, n=5). I. Spontaneous physical activity. The frequency of horizontal movement was shown for day and night time at 10 wks on HFD. For A-D, I, n=8 in control or butyrate group. For E-G, n=10 in control or butyrate group. Values are the mean ± SE. *P<0.05, **P<0.001 by Student’s t test.

Fig. 2. Insulin sensitivity in butyrate-treated mice. A. Fasting glucose. Tail vein blood was used for glucose assay after 16 hr fasting during the period of HFD feeding. B. Fasting insulin. The insulin was determined at 16 weeks on HFD in fasting condition with Lincoplex kit (#MADPK). C. ITT in butyrate-treated mice. ITT was done at 12 weeks on HFD (at 16 weeks of age). In figure A-C, data are presented as means ± SE (N=9). *P<0.05, **P<0.001 by Student’s t test. D. The homeostasis model assessment (HOMA). After overnight fast, blood glucose and insulin were measured and used to determine insulin sensitivity through HOMA IR (IR=fasting insulin mU/ml X fasting glucose mg/dL ÷ 405). Values are the means ± SE (N=8 mice). ** P<0.001. E. Insulin signaling. The gastrocnemius muscle was isolated after insulin (0.75 U/kg) injection in mice for 30 minutes and used to prepare the whole cell lysate for immunoblot. The mice on HFD for 13 weeks were used in the signaling assay. F. The blot signal in the panel E was quantified and presented after normalization with protein loading. **P<0.001 (n=2).

Fig. 3. Brown adipose tissue response to sodium butyrate. A. Adaptive thermogenesis in cold environment. Rectum temperature was measured when the mice were exposed to 4 °C ambient temperature in a cold room at 10 weeks on HFD. Details of the procedure are described in materials and methods. B. Hematoxylin and eosin staining (H&E staining). The staining was conducted in the brown adipose tissues collected at 13 weeks on HFD. Photograph was taken at magnification 100X. C. Brown adipose tissues were collected at 13 weeks on HFD. Gene expression was examined by qRT-PCR. mRNA of PGC-1α and UCP-1 in brown fat of mice treated with butyrate. D. Immunoblot. Brown adipose tissues were collected at 13 weeks of butyrate treatment. The whole cell lysate (100 µg) was resolved in SDS-PAGE and blotted with PGC-1α and UCP-1 antibodies. Data are presented as means ±SE (n=9 mice). * P<0.05.

Fig.4. Oxidative fiber in skeletal muscle. A. Vastus lateralis muscle. The tissue was isolated from mice that were fed on HFD for 13 weeks. B. Oxidative fiber (Type I fibers) in serial cryostat sections of muscle. The muscle tissue slides were made from vastus lateralis, gastrocnemius (gastr.) and soleus muscle. They were stained with antibody against type I myosin heavy chain.
for oxidative fibers as indicated by the brown color. The photograph was taken with 20X magnification. C. Succinate dehydrogenase staining of oxidative fibers. The oxidative fibers were stained in serial cryostat sections of the vastus lateralis and gastrocnemius (gastr.) muscle as indicated by dark blue color in the photomicrograph. D and E. Quantification of proteins in immunoblot. The whole cell lysate was prepared from muscle tissues and analyzed in an immunoblot. Signals for PGC-1α, type I myosin heavy chain (Myosin), myoglobin, phospho-AMPK and phospho-p38 were blotted with specific antibodies. A representative blot is shown. Relative signal strength was quantified for each band and expressed in the bar figure. The results are the mean ±SE (n=8 mice). *P<0.01, **P<0.001 (vs. control).

Fig. 5. Effect of butyrate on L6 muscle cells and liver tissues. A. AMPK and PGC-1α in L6 cells. Differentiated L6 myotubes were starved in 0.25% BSA DMEM for overnight. The cells were treated with 500 µM of sodium butyrate for 4 hours and analyzed in an immunoblot. A mean value of triplicate experiments is shown in the bar figure. B. AMPK and PGC-1α in liver. The whole cell lysate was prepared from liver tissues collected from mice on HFD for 13 weeks and analyzed in an immunoblot. In the experiments, pAMPK, pP38 and PGC-1α were blotted with the specific antibodies. A representative blot is shown. A mean value of five mice is shown in the bar figure (N=5). * P < 0.05, ** P < 0.001.

Fig. 6. Mitochondrial function and blood lipids. Vastus lateralis muscle and blood samples were collected from mice at 13 weeks on HFD (18 weeks in age) and examined for fatty acid oxidation, gene expression and blood lipids. A. Fatty acid oxidation in muscle. The Y axis represents fold change in 14C-labeled CO2. B. Gene expression in muscle. Relative fold change in mRNA was used to indicate gene expression. C. Mitochondrial DNA COX I determined by Sybr green RT-PCR. D. Fully differentiated L6 cells were treated with 500 µM butyrate for 16 hours, fatty acid oxidation was measured. E. Gene expression in L6 cells. Relative fold change in mRNA was used to indicate gene expression. F. Butyrate in serum. G. Histone deacetylase activity in muscle. H. Triglyceride in blood. I. Total cholesterol in blood. Data are presented as means ±SE (n=6). * P<0.05; ** P<0.001.

Fig. 7. Treatment of obesity with butyrate. Obesity was induced in C57BL/6J mice fed on HFD for 16 weeks (21 weeks in age). The obese mice were then treated with butyrate through food supplement for 5 weeks. A. Body weight. Body weight was shown at the beginning and end of the 5 week butyrate treatment. B. Fat content. Fat content was determined in the body using NMR at the end of 5 week treatment with butyrate. C. ITT. At the end of 5 weeks, ITT was performed after 4 hours fast. D. The homeostasis model assessment (HOMA). Values are the means ± SE (N=8 in each group). * P<0.05.
Figure 1.
Figure 2.

A. Fasting glucose

B. Fasting insulin

C. ITT in butyrate mice

D. HOMA IR

E. Insulin signaling

F. Signal Quantification
Figure 3.

A. **Cold exposure**

B. **H&E staining in BAT**

C. **mRNA expression in BAT**

D. **Proteins in BAT**
Figure 4.

A. Vastus Lateralis

B. Type I Myosin Heavy Chain

C. SDH Staining of Type I Fiber

D. Type I Marker

E. Protein Quantification
Figure 5.

A  AMPK in L6 cells

B  AMPK pathway in liver

- pAMPK
- pP38
- PGC-1
- Tubulin

Control  Butyrate

- pAMPK
- pP38
- PGC-1
- Tubulin

Relative signal strength

- pAMPK
- pP38
- PGC-1a

Control  Butyrate

- pAMPK
- pP38
- PGC-1
- Tubulin

Relative signal strength

- pAMPK
- pP38
- PGC-1a
Figure 6.
Figure 7.