Tributyrin attenuates obesity-associated inflammation and insulin resistance in high-fat-fed mice

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Vinolo MA, Rodrigues HG, Festuccia WT, Crisma AR, Alves VS, Martins AR, Amaral CL, Fiamoncini J, Hirabara SM, Sato FT, Fock RA, Malheiro G, dos Santos MF, Curi R. Tributyrin attenuates obesity-associated inflammation and insulin resistance in high-fat-fed mice. Am J Physiol Endocrinol Metab 303: E272–E282, 2012. First published May 22, 2012; doi:10.1152/ajpendo.00053.2012.—The aim of this study was to investigate whether treatment with tributyrin (Tb; a butyrate prodrug) results in protection against diet-induced obesity and associated insulin resistance. C57BL/6 male mice fed a standard chow or high-fat diet were treated with Tb (2 g/kg body wt, 10 wk) and evaluated for glucose homeostasis, plasma lipid profile, and inflammatory status. Tb protected mice against obesity and obesity-associated insulin resistance and dyslipidemia without food consumption being affected. Tb attenuated the production of TNFα and IL-1β by peritoneal macrophages and their expression in adipose tissue. Furthermore, in the adipose tissue, Tb reduced the expression of MCP-1 and infiltration by leukocytes and restored the production of adiponectin. These effects were associated with a partial reversal of hepatic steatosis, reduction in liver and skeletal muscle content of phosphorylated JNK, and an improvement in muscle insulin-stimulated glucose uptake and Akt signaling. Although part of the beneficial effects of Tb are likely to be secondary to the reduction in body weight, we also found direct protective actions of butyrate reducing TNFα production after LPS injection and in vitro by LPS- or palmitic acid-stimulated macrophages and attenuating lipolysis in vitro and in vivo. The results, reported herein, suggest that Tb may be useful for the treatment and prevention of obesity-related metabolic disorders.

butyrate; macrophages; diabetes; cytokines; white adipose tissue

THE CHRONIC LOW-GRADE INFLAMMATION associated with obesity plays a central role as a link between excessive fat accumulation and the development of pathologies (20). In obesity, adipose tissue is markedly infiltrated by proinflammatory macrophages and other leukocytes that secrete proinflammatory cytokines and chemokines (20, 41, 50). The overproduction of these inflammatory mediators together with changes in adipokine production and nonesterified fatty acid (NEFA) levels leads to the development of insulin resistance. In addition to adipose tissue, several organs, including the liver (1) and hypothalamus (28), develop a proinflammatory profile in response to the excessive nutrient supply. This systemic inflammatory state is associated with the activation of intracellular signaling pathways such as JUN NH2-terminal kinase (JNK) and IκB kinase-β (IKKβ) that in turn phosphorylate serine residues in the insulin receptor substrate, inhibiting tyrosine phosphorylation and the interaction with phosphatidylinositol-3 kinase (19, 21).

In accord with an important role of inflammation in the development of obesity-associated diseases, whole body deletion of intracellular kinases activated upon inflammation, namely JNK (18) and IKKβ (1), and pharmacological treatment with anti-inflammatory agents such as ω-3 fatty acids and salicylate, a salicylate derivate, were demonstrated to protect mice from the deleterious effects of high-fat feeding and obesity on insulin sensitivity (16, 32).

Butyrate is a short-chain fatty acid (SCFA) produced during fermentation of fibers and other substrates by an anaerobic bacteria resident in the gastrointestinal tract (36). In addition to its role as an energetic substrate, butyrate regulates leukocyte functions, including production of proinflammatory mediators and leukocyte recruitment (33, 38, 45, 46, 48). At least two different mechanisms seem to be involved in these effects, inhibition of histone deacetylase (HDAC) activity and activation of a G protein-coupled receptor (GPR), namely GPR43 (47). Regarding the production of inflammatory mediators, most of the studies demonstrated that butyrate attenuates their production by leukocytes. In line with this anti-inflammatory action, promising results have been obtained using butyrate in inflammatory conditions such as inflammatory bowel disease (47).

Elevation of SCFA availability by increasing dietary fiber intake or diet supplementation with butyrate may prevent the development of metabolic disarrangements and the insulin resistance associated with obesity (12, 13, 31, 43). Dietary supplementation with butyrate (at 5% wt/wt) prevented the development of insulin resistance and obesity. These effects have been related to the promotion of energy expenditure through enhanced mitochondrial expression of PGC-1α and uncoupling protein 1 (UCP1) (13). A recent study has shown that butyrate supplementation of milk formula given to preweaning calves improved insulin sensitivity (23).

Tributyrin (Tb) is a triglyceride containing three butyrate moieties that has been evaluated with the aim of overcoming pharmacokinetic problems associated with butyrate administration, including its rapid metabolism. After oral administration, Tb is rapidly absorbed and hydrolyzed to butyrate by...
plasmatic esterases; the peak of Tb and butyrate concentrations after oral administration of Tb is at 25 and 30 min, respectively (42). This triglyceride presents more favorable pharmacokinetics compared with butyrate and low toxicity (8). In addition, Tb also presents direct effects on cells and is more potent than butyrate (14).

In the present study, we investigated whether treatment with Tb results in protection against diet-induced obesity and associated insulin resistance. For this, mice fed a high-fat diet treated with Tb were evaluated for glucose homeostasis, plasma lipid profile, and inflammatory status. To further characterize the effects of Tb/butyrate, experiments with RAW 264.7 macrophages and in vivo in models of endotoxiaemia and lipolysis-induced leukocyte recruitment to adipose tissue were also performed.

MATERIALS AND METHODS

Animals. All experimental protocols were approved by the Animal Care Committee of the Institute of Biomedical Sciences, University of Sao Paulo. Male 8-wk-old C57BL/6 mice were kept individually in cages at 23°C on a 12:12-h light-dark cycle with food and water ad libitum. After adaptation (1–2 wk), mice were randomly assigned in one of four groups: control (C) and tributyrin (Tb) received a standard rodent chow diet (total energy: 75.8% carbohydrates, 9.5% fat, and 14.7% protein), and high-fat diet (HFD) and HFD + Tb groups were fed a diet rich in lard (total energy: 26.0% carbohydrates, 59.1% fat, and 14.9% protein). Animals were treated with Tb at 2.0 g/kg body wt (Tb and HFD + Tb groups) or water (C and HFD groups) by gavage three times/wk at intervals of 48 h during the entire protocol (10 wk). The Tb dose was chosen on the basis of preliminary observations, indicating its effectiveness to prevent insulin resistance induced by palmitate in isolated skeletal muscle of rats (data not shown) and on previous study (42).

Body weight and food intake were recorded three times/wk. At the end of the protocol, 6-h-fasted mice were euthanized by cervical dislocation after an intramuscular injection of ketamine (100 mg/kg body wt) and xylazine (50 mg/kg body wt) for tissue harvesting. Energy efficiency was calculated by the following formula: energy efficiency = body weight gain/energy intake. The energy intake per mouse was determined using the following conversions: standard chow = 3.80 kcal/g; HFD = 5.34 kcal/g.

Oxygen consumption/carbon dioxide production and respiratory quotient determination. Oxygen consumption/carbon dioxide production was measured in fed animals through a computer-controlled, open-circuit calorimeter system LE405 Gas Analyzer (Panlab-Harvard Apparatus, Holliston, MA). Mice were singly housed in clear respiratory chambers, and room air was passed through chambers at a flow rate of 0.8 l/min. The air flow within each chamber was monitored by an Air Supply and Switching sensor (Panlab-Harvard Apparatus). Gassensors were calibrated prior to the onset of experiments. The analyses were performed during a period of 24 h. Sample air was passed sequentially through O2 and CO2 sensors for determination of O2 and CO2 content, from which measurements of oxygen consumption (VO2) and carbon dioxide production (VCO2) were estimated. VO2 and VCO2 were calculated by Metabolism Version 2.2 software. The day and night values of VO2 and VCO2 were calculated as the sum of the values of 6 h of measurement in each period. Respiratory quotient (RQ) was calculated using VCO2/VO2.

Blood analyses. Blood glucose, triglycerides, total cholesterol, HDL, and the activities of alanine (ALT) and aspartate (AST) transaminases were determined using a commercial kit (Laiest Diagnostica). NEFA were measured using the HR NEFA series kit (Wako diagnostic, Richmond, VA) according to the manufacturer’s instructions. The LDL cholesterol concentration was determined using the formula of Friedewald et al. (11). Resistin, leptin, macrophage chemoattractant chemokine-1 (MCP-1), and TNFα were measured by Luminex-based bead array method using a LINCOplex simultaneous multianalyte detection system (Linco Research) according to the manufacturer’s instruction. Insulin was determined using rat/mouse insulin ELISA (Millipore).

Glucose tolerance test. Glucose tolerance test (GTT) was performed on the 8th wk of treatment. After 6 h of fasting and blood collection (time 0), mice were injected intraperitoneally (ip) with a 50% glucose solution (2.0 g/kg body wt). Blood samples were collected at 15, 30, 45, 60, and 90 min after glucose injection for determination of glycemia.

Insulin tolerance test. Blood samples were collected before or 10, 20, 30, 40, and 50 min after ip insulin injection (0.75 U/kg) for serum glucose determination. The constant for glucose disappearance rate during the test (Kins) was calculated using the 0.693/t1/2 formula. The glucose t1/2 was calculated from the slope of the least-square analysis of the plasma glucose concentrations during the linear decay phase.

Peritoneal macrophage isolation and culture. Cells were obtained by washing the peritoneal cavity with 5 ml of RPMI-1640 culture medium (Cultilab, Sao Paulo, Brazil). Macrophage-rich cultures (>90% of the cells were F4/80+) were obtained by incubating peritoneal cells in 24-well polystyrene culture plates for 2 h at 37°C in a 5% CO2 humidified air environment (37). Nonadherent cells were then incubated with washing with RPMI. Adherent cells were then incubated with 1 µg/ml lipopolysaccharide (LPS; E. coli, serotype 0111:B4; Sigma Chemical) for 24 h. The supernatant was collected to determine the contents of the IL-1β, IL-6, IL-10, TNFα, and keratinocyte-derived chemokine (KC) by ELISA and nitric oxide (NO) by Griess assay (7).

In vitro evaluation of adipocyte production. The epididymal adipose depot was removed, cut in pieces of ~100 mg, and washed two times with sterile PBS. The explants were further cut into pieces of 10–15 mg, washed more two times with PBS, and then incubated in DMEM (Sigma Chemical) containing 10% fetal bovine serum at 37°C for 24 h. Culture medium was collected, and IL-1β, IL-6, and TNFα adiponectin and leptin contents were determined by ELISA.

Measurement of lipolysis in isolated adipocytes. Epididymal fat pads were minced with fine scissors and added to sterile polypropylene containers containing 10 ml of sterile DMEM with HEPES (25 mM), collagenase type II (125 mg/ml), and 4% BSA. The mixture was incubated for 30 min at 37°C in an orbital shaker at 150 oscillations/min. The isolated adipocytes were then filtered through a fine plastic mesh (150 µm), transferred to a conical (50 ml) plastic tube, and washed three times with the same buffer without collagenase. After the last wash, the medium was totally aspirated. Lipolysis was determined by incubating isolated adipocytes (aliquot of 40 µl) at 37°C in a final volume of 0.2 ml of DMEM with 2% fatty acid-free BSA for 120 min under constant shaking with or without isoproterenol (1 µmol/l). NEFA concentration in the infranatant was determined using the HR NEFA kit. Aliquots of adipocyte suspension were fixed with 10% formaldehyde for subsequent analyses of cell diameter. Ten photos of each sample were taken using an optical microscope, and the diameter of the cells (500 in total) was determined using the Image-Pro Plus program. NEFA levels were expressed in relation to the adipocyte number.

Liver histology and triacylglycerol measurements. Liver fragments (100 mg) were subjected to extraction for 16 h at 4°C with 4 ml of CHCl3/methanol (2:1, vol/vol). Two milliliters of 0.6% NaCl were added to the extract, and the mixture was centrifuged at 2,000 g for 20 min (22). The organic layer was collected and dried, and the residue was dissolved in isopropanol and assayed for triglyceride content. For histological analysis, a fragment of the liver of each animal was removed, fixed with 3.7% buffered formaldehyde solution for 8 h at room temperature, dehydrated, processed, and embedded in Paraplast (Sigma Chemical). Sections (7 µm) were stained with hematoxylin and eosin to evaluate tissue morphology as well as the degree of liver steatosis.

TRIBUTYRIN PREVENTS INSULIN RESISTANCE

E273

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00053.2012 • www.ajpendo.org
**Quantitative RT-PCR.** Total RNA from the epididymal adipose depot was extracted with Trizol reagent (Invitrogen Life Technologies) (5) and reverse transcribed to cDNA using the High-Capacity cDNA kit (Applied Biosystems). Gene expression was evaluated by real-time PCR (17) using a Rotor Gene (Qiagen) and SYBR Green as fluorescent dye. Primer sequences are shown in Table 1. Quantification of gene expression was carried out using a previously described method (27), with ubiquitin C gene as an internal control.

**Glucose metabolism in isolated soleus muscle.** Soleus muscles were rapidly and carefully isolated, weighed, preincubated at 35°C in Krebs-Ringer bicarbonate buffer, pH 7.4, and preagassed for 30 min with 95% O2-5% CO2 containing 5.6 mmol/l glucose with agitation at 120 oscillations/min. After 30 min, muscles were transferred to vials containing Krebs buffer supplemented with 0.3 μCi/ml 2-deoxy-D-[2,6-3H]glucose and 0.2 μCi/ml 2-deoxy-β-[2,6-3H]glucose in the presence or absence of insulin (7 mmol/l). Gasification was maintained for >15 min and then removed. After 1 h of incubation, muscles were washed in cold buffer, briefly dried on filter paper, and frozen in liquid N2. Muscles were processed for 2-deoxy-β-[2,6-3H]glucose uptake, β-[2,6-3H]glucose incorporation, and [14C]glycogen synthesis (6). Extracellular space was determined by incubating some muscles in the presence of 0.1 μCi/ml l-[1-14C]glucose and 5.6 mmol/l l-glucose. Glucose uptake was calculated by subtracting extracellular l-[1-14C]glucose from total 2-deoxy-β-[2,6-3H]glucose.

**Western blotting analysis.** Isolated soleus muscles and liver were homogenized in lysis buffer containing protease and phosphatase inhibitors and centrifuged (12,000 g, 10 min, 4°C). The supernatants were mixed with 4× SDS-PAGE sample buffer, and the protein content of homogenates was determined by the Bradford method (2). Equal amounts of protein were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Detection was carried out using antibodies against phospho-Akt (Ser437), phospho-JNK (T183/Y185), Akt, and JNK from Cell Signaling Technology (Boston, MA).

**LPS-induced endotoxemia.** Male adult Balb/c (Mus domesticus) mice were treated by gavage with water (control) or Tb (2.0 g/kg) 1 h before being challenged with an intravenous injection of LPS (1.25 μg, E. Coli B55:O5; Sigma Chemical) (9, 10). Mice were anesthetized, and blood was collected 1 h after LPS injection.

**Lipolysis activation.** Two protocols were used to activate lipolysis, namely pharmacologically β-adrenergic activation and food restriction. In the former, mice fed a standard diet were treated with either amide solution (3.34 g/kg body wt) or Tb (2.0 g/kg body wt) three times. In the latter, mice fed a standard diet were treated with either amide solution or Tb (2.0 g/kg body wt) 1 h before being challenged with an intravenous injection of LPS (1.25 μg, E. Coli B55:OS; Sigma Chemical) and transferred to polyvinylidene difluoride membranes. Detection was carried out using antibodies against phospho-Akt (Ser437), phospho-JNK (T183/Y185), Akt, and JNK from Cell Signaling Technology (Boston, MA).

<table>
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<tr>
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UBC, ubiquitin C; MCP-1, macrophage chemoattractant chemokine-1.

E274 TRIBUTYRIN PREVENTS INSULIN RESISTANCE

**RESULTS**

*Tb* reduced body weight gain in high-fat-fed mice. Mice fed a HFD were treated with *Tb* or water during the period of 10 wk. As depicted in Fig. 1, *Tb* treatment significantly attenuated the increase in body weight gain (−40%; Fig. 1, A and B) and visceral and subcutaneous adipose tissue masses (−20%; Fig. 1, C and D). HFD intake also resulted in a significant increase in liver weight (25%, from 1.20 ± 0.03 g in the control group to 1.50 ± 0.04 g in the HFD group), which was partially reversed by *Tb* (1.38 ± 0.04 g). No effect of *Tb* on the weight of skeletal muscle, heart, or brown adipose tissue (BAT) was observed (data not shown). The reduction in adiposity induced by *Tb* was associated with a significant attenuation in adipocyte hypertrophy (data not shown). The daily food intake of the experimental groups was 4.57 ± 0.17 (C), 4.51 ± 0.16 (Tb), 2.87 ± 0.17 (HFD), and 2.89 ± 0.22 (HFD + Tb) g/day−1·animal−1, and no change in the caloric intake was observed (Fig. 1E). Despite similar caloric intake, a significant reduction in energy efficiency (−31.6%; Fig. 1F) was observed in high-fat-fed mice after treatment with *Tb*. The mice in this group (HFD + Tb) also presented an increment on oxygen consumption/carbon dioxide production compared with HFD (Fig. 1, G and H), suggesting an increased energy expenditure. These changes were accompanied by a reduction in the RQ (Fig. 1).

*Tb* treatment partially prevented the changes in plasma leptin and resistin levels and reversed the increase in NEFA concentrations induced by high-fat diet (Table 2). Indeed, *Tb* partially reverted the hypertriglyceridemia and hypercholesterolemia induced by high-fat feeding (Table 2). Taken together, these results indicate that *Tb* improves the dyslipidemia associated with high-fat feeding.

*Tb* attenuated high-fat diet-induced insulin resistance. *Tb* treatment reduced fasting hyperglycemia, hyperinsulinemia, and insulin resistance, as evaluated by the homeostasis model assessment (HOMA) of insulin resistance (Table 2), in high-fat-fed mice. *Tb* also ameliorated glucose intolerance (Fig. 2, A and B) and partially reverted the insulin intolerance (Fig. 2, C and D) induced by high-fat feeding. Corroborating with these beneficial effects on glucose homeostasis, *Tb* prevented the reduction in insulin-stimulated skeletal muscle glucose uptake and incorporation into glycogen associated with diet-induced insulin resistance. Statistical analysis. Comparisons of the results were performed using Student’s *t*-test or one-way ANOVA and the Tukey multiple comparison posttest. The statistical test used in each of the analyses is described in the figure and table legends. Significance was set at *P* < 0.05. Results were obtained from at least three separate experiments and expressed as means ± SE.

**Table 1. The annealing temperature and sequences of the primers used**

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induced obesity (Fig. 3, A–C) or caused by palmitate (500 μM) treatment in vitro in skeletal muscle of lean mice (data not shown). In line with an amelioration of muscle/insulin resistance, Tb abolished the impairment in Akt activation by insulin found in high-fat-fed mice, as evaluated by the content of phosphorylated Akt (Ser437; Fig. 3, D and E). Tb attenuated macrophage and white adipose tissue production of inflammatory mediators.

Tb treatment significantly prevented the increase in the 24-h production of TNFα and IL-6 by peritoneal macrophages isolated from high-fat-fed mice under basal, nonstimulated conditions (Fig. 4, A and C). Upon LPS stimulation, Tb attenuated the higher production of TNFα, IL-1β, and IL-6, but not KC, by HFD macrophages (Fig. 4, A–D). Tb had no significant effect on macrophage production of IL-10 and NO (data not shown). In line with the reduced production of TNFα by peritoneal macrophages, Tb treatment significantly attenuated the increase in circulating TNFα (Fig. 4E) and IL-6 (data not shown) levels induced by LPS administration. To test whether the effects of Tb/butyrate on TNFα production by macrophages were due to a direct action, we evaluated the cytokine production in RAW 264.7 (a murine macrophage cell line) after incubation with various concentrations of butyrate. Palmitic acid- and LPS-stimulated production of TNFα by RAW 264.7 cells was attenuated partially by butyrate at 1 mM, suggesting a direct action of this SCFA. This effect of butyrate was mimicked by trichostatin A (TSA), a potent inhibitor of HDAC activity, indicating that this pathway may be involved (Fig. 4, F and G).

Tb treatment prevented the reduction, induced by the high-fat diet, in adiponectin production by fat explants (Fig. 5B), but it had no effect on leptin and IL-6 release (Fig. 5, A and C). The cytokines whose concentrations were below the limit of detection (TNFα, IL-1β, IL-10, and MCP-1) had their mRNA levels measured by quantitative PCR. In line with its anti-inflammatory actions, Tb treatment markedly attenuated the increase in white adipose tissue (WAT) TNFα, IL-1β, and MCP-1 mRNA levels in high-fat-fed mice (Fig. 5, D–F). The IL-10 expression was similar between experimental groups (data not shown). Tb treatment was associated with a marked effect on macrophage recruitment to WAT induced by the high-fat diet; reduction of...
M1 macrophage marker mRNA levels (F4/80 and CD11c) was observed in mice treated with Tb (Fig. 5, G and H). However, no effects of Tb were seen on mRNA levels of the M2 macrophage markers (MGL-2 and arginase 1; data not shown).

**Tb treatment suppressed lipolysis and macrophage recruitment to the WAT.** Rates of lipolysis are directly related to macrophage recruitment to adipose tissue (24). Therefore, we tested whether Tb affected leukocyte recruitment by modulating lipolysis. Tb treatment markedly attenuated the increase in basal and isoproterenol-stimulated lipolysis induced by high-fat feeding (Fig. 6A). In vitro, the treatment of adipocytes with butyrate or the GPR43 agonist (phenylacetamide-1) reduced isoproterenol-stimulated lipolysis (Fig. 6B). Corroborating with the data obtained in vitro, Tb treatment reduced circulating NEFA levels in response to isoproterenol administration in vivo (Fig. 6C).

Next, we investigated whether inhibition of adipose tissue lipolysis by Tb would affect macrophage recruitment in vivo. Tb prevented the increase in expression of the macrophage marker F4/80 in food-restricted and β-adrenergically stimulated mice, suggesting that it may affect macrophage recruitment by reducing WAT lipolysis (Fig. 6, D and E).

**Tb attenuated hepatic steatosis induced by high-fat diet.** Varying degrees of liver steatosis were observed in all high-fat-fed mice. Cells containing large fat inclusions were observed throughout the hepatic lobules; centrilobular hepatocytes, however, were more strongly affected, showing many fat vacuoles of different sizes. Infiltration of inflammatory cells was observed frequently. Tb significantly attenuated liver steatosis, although inflammatory cells and cells containing large fat inclusions, mostly in the centrilobular region, were still observed to a much lesser degree (data not shown). In accordance with these findings, a significant reduction in hepatic triglyceride accumulation was observed in mice treated with Tb (Fig. 7A). Tb treatment also reduced IL-1β and F4/80 expression in the livers of high-fat-fed mice (Fig. 7, B and C). No difference in TNFα, IL-6, or CD11c expression was observed between the experimental groups (data not shown).

**Tb suppressed JNK activation by high-fat feeding.** In accord with our initial hypothesis that the improvement in insulin sensitivity in high-fat-fed mice induced by Tb is due to an attenuation in the activity of intracellular kinases activated upon proinflammatory stimuli, we found that Tb treatment significantly reduced the activation of JNK in the liver and skeletal muscle induced by high fat diet, as indicated by the content of phosphorylated JNK (T183/Y185) (Fig. 8).

**DISCUSSION**

Butyrate is a SCFA with relevant effects on cancer and inflammation and on the development of obesity. The major problem of using this compound in vivo has been the difficulty in achieving and maintaining its concentrations in blood since this fatty acid is rapidly metabolized by colonocytes. To overcome this problem, we have used Tb, a produg of butyrate that presents low toxicity and has been shown to increase plasmatic butyrate levels up to 2.4 mM and to sustain butyrate concentrations above 0.1 mM for more than 24 h after oral administration (8, 29).

In the present study, Tb protected mice against obesity and obesity-associated insulin resistance and dyslipidemia. Data indicate that these effects are, at least in part, due to the anti-inflammatory actions of this compound. Accordingly, Tb reduced the expression of proinflammatory cytokines in adipose tissue and liver and attenuated the elevation in circulating levels of TNFα in response to LPS and the production of TNFα, IL-1β, and IL-6 by peritoneal macrophages isolated from high-fat-fed mice. In vitro, butyrate attenuated the production of TNFα stimulated by LPS and palmitic acid. Furthermore, Tb also reduced the expression of proinflammatory cytokines in adipose tissue and liver and attenuated the elevation in circulating levels of TNFα in response to LPS and the production of TNFα, IL-1β, and IL-6 by peritoneal macrophages isolated from high-fat-fed mice. These effects were associated with a reduction in hepatic steatosis, liver, and skeletal muscle content of phosphorylated JNK and an improvement in muscle insulin-stimulated glucose uptake and Akt signaling. In addition to the attenuation of inflammation, Tb may also protect mice from insulin resistance by reducing body weight gain and regulating the production of gut hormones, as demonstrated recently (26).

Tb treatment reduced body weight gain and fat accumulation induced by high-fat feeding, an effect that may be related to the increase in energy expenditure and oxygen consumption ob-

![Table 2. Serum determinations in high-fat-fed mice treated (HFD + Tb) or not (HFD) with Tb and the control groups (C and Tb)](Image)
served in these animals. A similar effect was also described in experimental models, using diets supplemented with butyrate or high contents of fibers (12, 13). In mice receiving the butyrate-supplemented diet, these effects seemed to be the result of a combination of increased spontaneous motor activity and BAT thermogenesis (13). In our study, no changes in BAT mRNA levels of the thermogenic markers UCP1, PGC-1α, and deiodinase-2 (data not shown) were observed, suggesting that Tb administration enhances energy expenditure by mechanisms different from the butyrate-supplemented diet.

The reduction in adiposity induced by Tb was associated with slight changes in circulating triglyceride and cholesterol levels and attenuation in the elevation in serum leptin and resistin levels associated with diet-induced obesity. Tb treatment also protected mice from glucose intolerance and insulin resistance induced by high-fat diet as evaluated by GTT, insulin tolerance test, HOMA, and muscle glucose uptake and insulin signaling. Extending these findings, Tb prevented skeletal muscle insulin resistance induced in vivo by high-fat diet and in vitro by prolonged incubation with palmitate. This latter
result indicates that Tb treatment protects skeletal muscle from insulin resistance independently of its effect on body weight. However, the precise mechanisms underlying this effect are still under investigation. Activation of AMP-activated protein kinase and PGC-1α directly by butyrate (13) or indirectly by attenuation of the production of proinflammatory cytokines (40) may be involved. Butyrate and other SCFAs may also contribute to the improvement of glucose metabolism by stimulating the secretion of glucagon-like peptide-1 through the receptors GPR41 and -43 (26, 44).

Excessive fat accumulation is associated with the recruitment and accumulation of leukocytes with different phenotypic and functional characteristics, such as the M1 proinflammatory macrophages in the adipose depot, which contribute to the low-grade inflammation and insulin resistance found in obesity (34). Tb attenuated M1 accumulation and production of proinflammatory cytokines in macrophages from Tb-fed mice compared to those of HFD-fed mice, as shown in Fig. 4. These findings suggest that Tb treatment may help to reduce the accumulation of proinflammatory macrophages in adipose tissue and contribute to the improvement of insulin sensitivity.

Fig. 4. Production of inflammatory mediators in vitro by macrophages and in vivo in lipopolysaccharide (LPS)-treated mice. Peritoneal macrophages were incubated for 24 h. The culture supernatants were then assayed for the production of cytokines. A–D: TNFα (A), IL-1β (B), IL-6 (C), and keratinocyte-derived chemokine (KC; D) quantification in the supernatants of nonstimulated (black bars) and LPS-stimulated macrophages (open bars). Results are means ± SE (n = 8–10/group). *P < 0.05 vs. C; †P < 0.05 vs. Tb; ‡P < 0.05 vs. HFD + Tb (ANOVA with Tukey’s posttest). E: serum concentration of TNFα was determined 1 h after intravenous LPS administration. Results are means ± SE (n = 6–7/group). *P < 0.05 vs. LPS condition (Student’s t-test). RAW 266.7 cells were preincubated for 1 h with butyrate or trichostatin A (TSA) and then stimulated with palmitic acid (PA) or LPS for 23 h. F and G: TNFα was then determined in the supernatant of LPS- (F) and PA-stimulated cells (G). Results are means ± SE (n = 5). *P < 0.05 vs PA- or LPS-stimulated cells without treatment (ANOVA with Tukey’s posttest).

Fig. 5. Production of adipokines and cytokines and expression of leukocyte markers in epididymal white adipose tissue (WAT). Leptin (A), adiponectin (B), and IL-6 (C) were measured in the 24-h culture supernatant of WAT explants (n = 9–10/group). mRNA expression of TNFα (D), macrophage chemattractant chemokine (MCP-1; E), IL-1β (F), F4/80 (G), and CD11c (H) was analyzed in WAT of high-fat-fed mice treated (HFD + Tb) or not (HFD) with Tb and their lean controls (C and Tb). Results are means ± SE (n = 6–8 animals/group). *P < 0.05 vs. C; †P < 0.05 vs. Tb; ‡P < 0.05 vs. HFD + Tb (ANOVA with Tukey’s posttest).
inflammatory mediators in the adipose tissue and liver of high-fat-fed mice. Although the molecular mechanisms involved in the initial activation of inflammatory cells in a high-fat diet are not totally understood, evidence exists in favor of a role of Toll-like receptor agonists such as saturated fatty acids (e.g., palmitic acid) (39) and LPS (3) in this process. Butyrate suppressed the response of LPS- and palmitate-stimulated macrophages and attenuated cytokine production after LPS administration. These results were also obtained with TSA, a potent inhibitor of HDAC, indicating that this pathway may be involved in the anti-inflammatory effect of butyrate. Indeed, previous studies have shown that butyrate and other inhibitors of HDAC reduce the activation of Toll-like receptor signaling in cells (e.g., neutrophils and macrophages) through

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Fig. 6. WAT lipolysis and macrophage recruitment. Nonesterified fatty acid (NEFA) released by adipocytes isolated from the experimental groups (A) or adipocytes incubated with butyrate or G protein-coupled receptor 43 agonist [phenylacetamide (phenyl)]. B) were measured after stimulation with isoproterenol in vitro. Results are means ± SE (n = 5–6). *P < 0.05 vs. control; #P < 0.05 vs. Tb; †P < 0.05 vs. HFD + Tb (ANOVA with Tukey’s post test). C) plasma NEFA levels after administration of isoproterenol (n = 6 animals/group). Results are means ± SE. *P < 0.05 vs. C (Student’s t-test). D and E: F4/80 expression in WAT of obese mice submitted to 30% food restriction (restr) for 3 days (D) or lean mice after isoproterenol (isop) administration (E) (n = 5–6 animals). Results are means ± SE. *P < 0.05 vs. C (ANOVA with Tukey’s post test).

Fig. 7. Measurement of triglyceride levels and inflammatory marker expression in liver. A: hepatic triglyceride contents were determined at the end of the experimental protocol. B and C: the mRNA expression of IL-1β (B) and F4/80 (C) was analyzed in livers of high-fat-fed mice treated (HFD + Tb) or not (HFD) with Tb and their lean controls (C and Tb). Results are means ± SE (n = 5–7 animals/group). *P < 0.05 vs. C; #P < 0.05 vs. Tb; †P < 0.05 vs. HFD + Tb according to ANOVA with Tukey’s posttest statistical analysis.
the modulation of intracellular pathways, including mitogen-activated protein kinase and NF-κB (4, 48).

SCFAs inhibit lipolysis through fatty acid-activated GPR43 (15). Although butyrate is a less potent agonist of GPR43 than acetate and propionate, the results of the present study and the data obtained by others (52) suggest that this fatty acid can also inhibit the release of fatty acids from WAT (lipolysis). The relevance of this effect is that the fatty acids released by WAT not only act directly or indirectly through stimulation of cytokine production (39) on other tissues inducing insulin resistance but also play a role in the recruitment of leukocytes to this tissue (24). The increased rate of NEFA efflux from adipose tissue, induced by fasting or by pharmacological intervention, leads to macrophage accumulation in this tissue (24), an effect that was abolished by Tb. These results are suggestive of a role of the antilipolytic effect of butyrate in the reduction of inflammation in WAT. Tb treatment also reduced MCP-1, an important chemokine for macrophage infiltration of WAT (22). Tb treatment also attenuated the hepatic steatosis induced by a high-fat diet. This protective effect of Tb has also been demonstrated in endotoxemic rats and seems to involve inhibition of NF-κB (29).

Inflammatory mediators, including TNFα and IL-1β derived from adipose tissue, liver, and leukocytes and fatty acids released from the WAT, promote insulin resistance. Evidence that activation of the stress/inflammatory JNK and IKK/NF-κB pathways is the link between inflammation and insulin resistance has been presented (30, 51). The attenuation of JNK activation in the liver and skeletal muscle of Tb-treated mice may be related to the reduction in inflammatory mediators and NEFA levels. Another possibility is that Tb effects are secondary to its actions on colon since butyrate has been shown to modulate gene expression in colonocytes and to enhance the intestinal barrier (25, 35). Although further studies are necessary to characterize in detail the effect of Tb and butyrate in the colon and their consequences in the whole organism, the fact that Tb did not change plasmatic levels of LPS (data not shown) found in high-fat feeding mice indicates that the beneficial effects of Tb are not due to change in the intestinal permeability.

In the present study, we showed that Tb attenuates several metabolic changes observed in mice fed a high-fat diet, including high plasma lipid (cholesterol, triglycerides, and NEFA) levels and increased production of inflammatory mediators (e.g., TNFα, IL-1β, and MCP-1). In addition to the effects of Tb secondary to the reduction in body weight, we demonstrated that Tb/butyrate has direct effects, such as the inhibition of proinflammatory mediator production by macrophages, protection of skeletal muscle against insulin resistance induced by palmitate, and inhibition of lipolysis and leukocyte accumulation in WAT. Unlike other studies performed using diets supplemented with butyrate or with high contents of fibers, we present data showing a protective effect of butyrate in obesity and insulin resistance after oral administration of Tb (3 times/wk). Despite the fact that the dose of Tb used in the present study is high, this triglyceride could be incorporated in food products or used in lower concentrations together with other compounds to achieve these beneficial effects in humans. The results reported herein and by others suggest that butyrate and its derivatives may be used therapeutically for the treatment and prevention of obesity-related metabolic disorders.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

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