Short-Chain Fatty Acids protect against High-Fat Diet-Induced Obesity via a PPARγ-dependent switch from lipogenesis to fat oxidation

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ABSTRACT

Short-chain fatty acids (SCFAs) are the main products of dietary fiber fermentation and are believed to drive the fiber-related prevention of the metabolic syndrome. Here we show that dietary SCFAs induce a peroxisome proliferator-activated receptor (PPAR) γ-dependent switch from lipid synthesis to utilization. Dietary SCFA supplementation prevented and reversed high-fat diet-induced metabolic abnormalities in mice by decreasing PPARγ expression and activity. This increased the expression of mitochondrial uncoupling protein 2 and raised the AMP/ATP ratio, thereby stimulating oxidative metabolism in liver and adipose tissue via AMP-activated protein kinase. The SCFA-induced reduction in body weight and stimulation of insulin sensitivity were absent in mice with adipose-specific disruption of PPARγ. Similarly, SCFA-induced reduction of hepatic steatosis was absent in mice lacking hepatic PPARγ. These results demonstrate that adipose and hepatic PPARγ are critical mediators of the beneficial effects of SCFA on the metabolic syndrome, with clearly distinct and complementary roles. Our findings indicate that SCFAs may be used therapeutically as cheap and selective PPARγ modulators.
The shift in Western and developing countries from a traditional high-fiber, low-fat, low-calorie diet towards a low-fiber, high-fat, high-calorie diet is accompanied by a growing prevalence of obesity and insulin resistance (1, 2). Dietary fiber supplementation on the other hand has been shown to reduce body weight, insulin resistance and dyslipidemia (3-5). The main products of intestinal fermentation of dietary fibers are short-chain fatty acids (SCFAs), of which acetate, propionate and butyrate are the most abundant (6). While all three SCFAs are rapidly assimilated into host carbohydrates and lipids - providing ~10% of our daily energy requirements (7) - there are clear differences in the way in which each is metabolized: propionate is primarily a precursor for gluconeogenesis, while acetate and butyrate are rather incorporated into fatty acids and cholesterol (8). Besides serving as an energy source, SCFAs also regulate metabolism by inhibition of histone deacetylases and chain-length dependent activation of the endogenous G-protein-coupled receptors (GPR) 41 and 43 (9-11). The longer butyrate is more selective for GPR41, the shorter acetate is more selective for GPR43, while propionate binds to both receptors (12, 13). Recently, it was shown that the SCFAs propionate and butyrate increased intestinal gluconeogenesis (IGN) resulting in beneficial effects on glucose and energy homeostasis (14). The mechanism, however, was different for the different SCFAs as butyrate acted through a cAMP-dependent mechanism, while propionate, itself a substrate of IGN, activated IGN gene expression via a gut-brain neural circuit involving GPR41.

Despite the differences between the metabolism and GPR affinities of the three SCFAs, they ameliorate high-fat diet-induced obesity and insulin resistance to a similar extent when given as a dietary supplement (15-17). This suggests that there is a common, GPR-independent molecular mechanism for the beneficial effects of acetate, propionate and butyrate. A GPR-independent mechanism is in line with recent findings that GPR41-deficient mice are still
sensitive to effects of SCFA on body weight and insulin sensitivity (17). It is likely that a common mechanism may involve AMP-activated protein kinase (AMPK), since acetate and butyrate have been shown to increase energy expenditure by activating AMPK in liver and muscle tissue (15, 18) while propionate activates AMPK in colon cancer cells and reduces lipid synthesis in isolated rat hepatocytes (19, 20). The mechanism by which SCFAs activate AMPK is, however, unknown.

In this study we identify a unifying, AMPK-dependent mechanism by which the three SCFAs mediate the beneficial effects of dietary fiber on the metabolic syndrome. We reveal a cascade of events that starts with downregulation of peroxisome proliferator-activated receptor (PPAR) - γ activity and in which liver and adipose PPARγ play distinct, complementary roles.
RESEARCH DESIGN AND METHODS

Animals and Experimental Design

Male C57Bl/6J mice (Charles River, L’Arbresle Cedex, France), 2 months of age, were housed in a light- and temperature-controlled facility (lights on from 6:30 a.m. to 6:30 p.m., 21 °C) with free access to water and food. The experimental groups were fed a high-fat semi-synthetic diet (D12451, Research Diet Services, Wijk Bij Duurstede, The Netherlands) in which 45% of calories were from palm oil fat. For the SCFA diets, sodium acetate (S2889; Sigma), sodium propionate (P1880; Sigma) or sodium butyrate (303410; Sigma) was incorporated into the diet at 5% (w/w). A normal-fat control group received chow diet (RMH-B, Hope Farms, Woerden, The Netherlands). Mice in which exons 1 and 2 of the PPARγ gene were loxP-flanked (PPARγ f/f) were kindly provided by Ronald M. Evans (Salk Institute) and have been described previously (21). PPARγ lox/lox mice were crossed with C57Bl/6J transgenic mice expressing Cre recombinase under the control of either the albumin promoter which is expressed in liver (L-KO) or the aP2 promoter which targets adipose tissue (A-KO). Experimental procedures were approved by the Ethics Committee for Animal Experiments of the University of Groningen.

Lipogenesis and β-oxidation

In vivo lipogenesis was determined by incorporation of [1-13C]-acetate into palmitate by providing 2% (w/v) [1-13C]-acetate in drinking water for 24h as described previously (22). Fatty acid β-oxidation capacity was determined in fresh liver and adipose homogenates according to Hirschey et al. (23). Briefly, tissue was homogenized in sucrose/Tris/EDTA buffer, incubated for
30 min in the reaction mixture (pH 8.0) containing [1-\(^{14}\)C]palmitic acid, and trapped [\(^{14}\)C]CO\(_2\) was measured.

**Insulin tolerance and sensitivity**

Intraperitoneal glucose tolerance was tested following intraperitoneal injection of glucose at 2 g/kg body weight after an overnight fast. Intraperitoneal insulin tolerance (ITT) was tested following intraperitoneal injection of insulin (NovoRapid) at 0.75 units/kg body weight after a 4-h fast. Hyperinsulinemic-euglycemic clamp studies were performed as previously described (24).

**Plasma and tissue sampling**

The mice were fasted from 6 to 10 a.m. Blood glucose concentrations were measured using a EuroFlash meter (Lifescan Benelux, Beerse, Belgium). Mice were subsequently sacrificed by cardiac puncture under isoflurane anesthesia. Liver and epididymal fat pads were weighed, snap-frozen in liquid nitrogen and stored at -80 °C. Part of the fat tissue was fixed in 4% paraformaldehyde in PBS and embedded in paraffin. For adipocyte histology, 3 µm paraffin sections were stained with hematoxylin and eosin and analyzed at 20× magnification. Blood was centrifuged (4000 x g for 10 min at 4 °C) and plasma was stored at -20 °C. Plasma NEFA concentrations were determined using a commercially available kit (Roche Diagnostics, Mannheim, Germany). Plasma leptin and insulin levels were determined using ELISA (ALPCO Diagnostics, Salem, United States). Hepatic TG content was determined using a commercial available kit (Roche) after lipid extraction (25).

Hepatic malonyl-CoA and adenosine concentrations were determined by HPLC according to Demoz *et al.* (26) and Miller *et al.* (27), respectively. CPT-1 activity was
determined in liver homogenates at different dilutions (20, 10, 5, 2 and 1 ng/uL) according to van Vlies et al. (28) with 50 µM palmitoyl CoA and 2 mM L-carnitine as substrates. The reaction was followed in time by quenching the reaction at 0, 2, 5 and 10 minutes.

**Indirect calorimetry**

Oxygen consumption, energy expenditure, respiratory exchange ratio, food intake and activity patterns were measured simultaneously for each mouse using a Comprehensive Laboratory Animal Monitoring System (TSE Systems GmbH, Bad Homburg, Germany). The energy balance was determined by measuring the energy content of diet and that of dried, homogenized feces using a bomb calorimeter (CBB 330, standard benzoic acid 6320 cal g⁻¹, BCS-CRM no.90N).

**Oxygen consumption rates in liver mitochondria**

Mitochondria were isolated from fresh liver tissue according to Mildaziene et al. (29). The rates of oxygen consumption in isolated liver mitochondria were measured at 37 °C using a two-channel high-resolution Oroboros oxygraph-2k (Oroboros, Innsbruck, Austria) in mitochondrial respiration medium (30) with palmitoyl CoA as substrate. Maximal ADP-stimulated oxygen consumption (i.e. state 3) was achieved by adding 4.8 U ml⁻¹ hexokinase, 12.5 mM glucose and 1 mM ATP. Resting state (i.e. state 4) oxygen consumption rate was determined after blocking ADP phosphorylation with 1.25 µM carboxyatractyloside. Respiratory control ratio (RCR) was calculated by dividing oxygen consumption rate in state 3 by oxygen consumption rate in state 4.

**HepG2, 3T3-L1 and C2C12 experiments**
HepG2, 3T3-L1 and C2C12 cells, purchased from American Type Culture Collection (Manassas, VA), were maintained at 37 °C in 5% CO$_2$ in Dulbecco’s Modification of Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For HepG2 experiments, cells were plated in DMEM with 10% FBS and 1% penicillin/streptomycin in 6-wells plates and incubated with 0.1, 1 or 3 mM sodium acetate, sodium propionate, or sodium butyrate for 24 hours with or without 100 nM rosiglitazone (Sigma) or 10 µM GW9662 (Sigma) as indicated. For 3T3-L1 experiments, cells were differentiated in 6-wells plates according to Zebisch et al. (31) and incubated with 0.1, 1 or 3 mM sodium acetate, sodium propionate, or sodium butyrate for 24 hours with or without 100 nM rosiglitazone or 10 µM GW9662 as indicated. For C2C12 experiments, cells were differentiated with 2% horse serum in 6-wells plates according to Fujitjia et al. (32) and incubated with 3 mM sodium acetate, sodium propionate, or sodium butyrate for 24 hours.

**Gene expression levels and immunoblot analysis**

RNA was extracted from liver and adipose tissue using Tri reagent (Sigma-Aldrich, St. Louis, MO) and converted into cDNA by a reverse transcription procedure using M-MLV and random primers according to the manufacturer’s protocol (Sigma-Aldrich). For quantitative PCR (qPCR), cDNA was amplified using the appropriate primers and probes. The sequence of the other primers can be found in Table S1. mRNA levels were calculated relative to 36b4 expression and normalized for expression levels of mice fed a high-fat diet.

For immunoblot analysis, whole-cell lysate was prepared in lysis buffer and the protein concentrations were determined using the BCA Protein Assay kit (Pierce). Individual samples were mixed with loading buffer, heated for 5 min at 96 °C and subjected to SDS-PAGE.
Antibodies and their sources were as follows: AMP kinase (AMPK, no. 2532; Cell Signaling), phosphorylated AMP kinase (pAMPK Thr172, no. 2531; Cell Signaling), acetyl CoA carboxylase (ACC, no. 45174; Abcam), phosphorylated acetyl CoA carboxylase (pACC S79, no. 31931; Abcam), uncoupling protein 2 (UCP2, no. 6525; Santa Cruz), peroxisome proliferator-activated receptor γ (PPARγ, no. 2435; Cell Signaling) and fatty acid synthase (FASN, no. 3180; Cell Signaling). As loading control, β-actin (no. 2066; Sigma) was used for liver and adipose tissue and TOM20 (no. 11415; Santa Cruz) for isolated liver mitochondria. Finally, horseradish peroxidase-conjugated anti-rabbit from donkey (Amersham Pharmacia Bioscience) or horseradish peroxidase-conjugated anti-goat from donkey (Dako, Glostrup, Denmark) and SuperSignal West Pico Chemiluminescent Substrate System (Pierce) were used. The immunoblots were analyzed by densitometry using Image Lab software (Bio-Rad).

**Statistical analysis**

All data are presented as mean values ± SEM. Statistical analysis was assessed by one-way ANOVA using the Tukey test for post-hoc analysis. Statistical significance was reached at a $p$ value below 0.05.
RESULTS

Prevention and Treatment of High-Fat Diet-Induced Obesity and Insulin Resistance by SCFAs

To examine the effects of SCFAs on the development of obesity and insulin resistance, wild-type C57Bl/6J mice were fed a high-fat diet (HFD) with acetate, propionate or butyrate (5% w/w) for 12 weeks. This provides the animals with a physiological amount of SCFA, since the contribution of SCFA to the total energy intake has been estimated ~10% (7). The substantial raise in body weight that was observed in controls, fed a HFD only, was attenuated by all three SCFAs to a similar extent (Fig. 1A). This coincided with reductions in white adipose tissue (WAT) mass, adipose cell size and plasma leptin concentrations (Figs. S1A-C). The lower body weight in the SCFA groups was not due to alterations in food intake or physical activity, since neither of these was significantly affected by SCFA supplementation (Figs. S1D-E). We did, however, observe enhanced energy expenditure in the SCFA-treated mice (Figs. 1B and S1F) as well as a shift from carbohydrate to fatty-acid oxidation, as indicated by lower respiratory exchange ratios (RER) (Fig. 1C). This suggests that SCFAs reduce HFD-induced body weight gain by enhancing energy expenditure through increased lipid oxidation.

SCFA-fed and control-fed mice had similar fasting blood glucose levels and glucose tolerance (Figs. S1G-I), but much lower fasting insulin levels (Fig. 1D). Together with enhanced disposal of glucose upon insulin injection (Fig. S1J), this points to higher insulin sensitivity in these mice. Therefore, we performed hyperinsulinemic-euglycemic clamp studies under matched insulin exposure. The glucose-infusion rate required for maintaining euglycemia (a measure of whole-body insulin sensitivity) in SCFA-fed mice was approximately 1.5-fold higher than that in
control mice (Fig. 1E). While the hepatic glucose production rate during the clamp was similar in all groups (Ra in Fig. 1F), the degree to which insulin stimulated the rate of glucose uptake by peripheral tissues (primarily muscle and adipose tissue) was much higher in SCFA-fed mice (Rd in Fig. 1F). This indicates improved peripheral insulin sensitivity in these mice. Collectively, our observations demonstrate that supplementation of any of the three SCFAs enhances the insulin sensitivity of HFD-fed mice to a similar extent.

SCFA supplementation completely prevented the HFD-induced obesity and insulin resistance as indicated by similar body weight, WAT mass, plasma leptin and insulin levels, and peripheral insulin sensitivity compared to mice fed a normal-fat chow diet (Figs. S2A-G). The HFD did not significantly affect the basal glucose level, which was similar on chow and HFD (Fig. S2D).

Finally, we wondered whether SCFAs could also be used to treat existing obesity and insulin resistance. To this end, we first fed mice an HFD for 12 weeks to induce obesity and then supplemented the HFD with SCFAs for 6 weeks. Indeed, after this treatment the SCFA-supplemented mice showed significantly lower body weight and WAT mass than controls as well as a shift towards fatty-acid oxidation and enhanced insulin sensitivity, without affecting the food intake (Figs. S3A-I). After 18 weeks the HFD control had the same basal glucose level as the 12 weeks HFD and chow controls (cf. Figs. S3E and S2D).

**SCFAs Stimulate Mitochondrial Fatty-Acid Oxidation by Activation of the UCP2-AMPK-ACC Pathway**

Control mice on a HFD had high plasma concentrations of non-esterified fatty acids (NEFAs) and high liver concentrations of triglycerides (hepatic steatosis). These aspects of the metabolic
syndrome were reduced when SCFAs were either supplemented from the beginning (Figs. S4A-B) or after the mice were already obese (Figs. S3H-I). This prompted us to study hepatic fatty-acid synthesis and oxidation. SCFA-fed mice had lower transcript levels of genes involved in hepatic lipogenesis and a lower concentration of hepatic fatty acid synthase protein (FASN) (Figs. S4C-D). In agreement, these mice had a 2-fold reduction in \textit{in vivo} hepatic lipid synthesis (Fig. 2A). The capacity for hepatic lipid oxidation in SCFA-fed mice was 2-fold higher than that of controls (Fig. 2B). Clearly, hepatic lipid metabolism was shifted towards a more oxidative state.

We wondered whether the metabolic effects of SCFAs might be mediated through activation of AMPK, which is known to shift metabolism from lipid synthesis to oxidation (33). Indeed, we observed increased phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase (ACC) (Fig. 2C), without affecting the total hepatic AMPK and ACC levels (Fig. S4E). Phosphorylation inactivates ACC and should lead to lower concentrations of its product malonyl-CoA, an endogenous inhibitor of carnitine palmitoyltransferase I (CPT-1), the first enzyme in the beta-oxidation of fatty acids (34). Consistently, SCFA treatment reduced hepatic malonyl-CoA concentrations (Fig. S4F) and increased the enzyme capacity ($V_{max}$) of CPT-1 in diluted liver homogenates (Fig. 2D). This implies that CPT-1 is stimulated by SCFAs via a dual mechanism: by raising its $V_{max}$ and by reducing the concentration of its inhibitor.

SCFA-fed mice had decreased hepatic ATP concentrations and increased AMP/ATP ratios (Figs. S4G-H). The latter is a sensitive reflection of the energetic state of the cell and a direct activator of AMPK (35). Reduced ATP concentrations can be a result of increased mitochondrial proton leakage, leading to mitochondrial uncoupling and subsequently reduced ATP synthesis (36). Therefore, we examined oxygen consumption by isolated liver mitochondria
using palmitoyl CoA as a respiratory substrate, both in the presence of ADP (state 3) and in the presence of an inhibitor of ATP production (state 4). We observed lower respiratory control ratios (RCR, the rate of state 3 divided by state 4 respiration) in liver mitochondria from SCFA-fed mice. This could be attributed to an increased state 4 respiration rate (Fig. 2E), demonstrating that there is intrinsic uncoupling of mitochondrial oxidative phosphorylation in the livers of these mice. In line with this, SCFA feeding led to increased expression of uncoupling protein (UCP) 2 (Fig. 2F), suggesting that proton leak via UCP2 may be responsible for the observed uncoupling (36).

**Activation of the UCP2-AMPK-ACC Pathway by SCFAs is Dependent on PPARγ**

Next we studied how SCFAs activate the UCP2-AMPK-ACC pathway. Possible candidates were the peroxisome proliferator-activated receptors (PPAR) α and γ, which are known regulators of UCP2 expression, fatty-acid oxidation and whole-body lipid metabolism (37-40). SCFAs did not significantly affect expression of PPARα, while expression of target genes involved in fatty acid oxidation was decreased rather than increased (Figs. S5A-C). This suggests that PPARα is not responsible for the enhanced fatty-acid oxidation capacity of SCFA treated mice. In contrast, SCFAs did reduce expression of PPARγ – and its target genes Cd36, Lpl, Fabp4 and Pltp – in liver and adipose tissue, but not in muscle (Figs. S5D-G). A reduced expression of PPARγ expression or activity stimulates UCP2 expression and fatty acid oxidation and reduces lipogenesis and hepatic triglyceride levels (41, 42) suggesting that PPARγ may well be the mediating factor between SCFAs and the UCP2-AMPK-ACC pathway. PGC-1α mRNA expression in liver, adipose and muscle tissue and UCP-1 mRNA expression in brown adipose
tissue did not change upon SCFA feeding (Figs. S5H-I), suggesting that neither PGC-1α nor UCP-1 play a role in the SCFA-induced effect.

To find out whether PPARγ may be causally involved in the induction by SCFAs of the UCP2-AMPK-ACC signaling pathway, we first investigated this in vitro in liver cells (HepG2), differentiated adipose cells (3T3-L1) and muscle cells (C2C12). Treating cells for 24h with 3mM of any of the SCFAs reduced mRNA and protein levels of PPARγ and its target genes in HepG2 and 3T3-L1 cells, but not in C2C12 cells (Figs. 3A-C). SCFAs also enhanced the activity of the UCP2-AMPK-ACC signaling pathway in HepG2 and 3T3L1 cells, but not in C2C12 cells (Figs. 3D-F), in line with our in vivo results. The induction of the UCP2-AMPK-ACC pathway by SCFAs was abolished when the partial repression of PPARγ expression and activity was compensated by the PPARγ agonist rosiglitazone (Fig. S6A). On the other hand, complete inhibition of the activity of PPARγ by the PPARγ antagonist GW9662 did not affect the SCFA-induced reduction in PPARγ expression but did abolish the accompanying increase in the activity of the UCP2-AMPK-ACC pathway (Fig. S6B). Apparently, either activation or inhibition of the activity of PPARγ abolished the SCFA-induced increase of the activity of the UCP2-pAMPK-pACC pathway.

The SCFA concentrations of 3 mM used here represent typical intestinal concentrations rather than portal blood concentrations (43), although even portal SCFA concentrations may exceed 2 mM postprandially after digesting a fiber-rich diet (44-46). Therefore, we also incubated HepG2 and differentiated 3T3-L1 cells at different concentrations of SCFAs. In both HepG2 and 3T3-L1 cells there was only a significant effect on PPARγ expression after 24h exposure to 3 mM SCFAs, while 1 and 0.1 mM showed no reduction compared to control (Figs. S6C-D).
Altogether, these results indicate that the activation of the UCP2-pAMPK-pACC pathway by SCFAs in HepG2 and 3T3-L1 cells was due to the observed reduction of PPARγ expression and activity, while PPARα had no role in these SCFA-induced effects.

**Hepatic PPARγ mediates the SCFA-Induced Reduction in Hepatic Steatosis, while Adipose PPARγ mediates SCFA effects on HFD-Induced Obesity and Insulin Resistance**

To distinguish between the role of PPARγ in the liver and adipose tissue, mice with a liver-specific (L-KO) or adipose-specific knock-out (A-KO) of PPARγ were fed an HFD, with or without SCFA supplementation. The SCFA-induced effects in these KO mice were compared to those of the WT above to analyze whether they were mediated by liver or adipose PPARγ.

Like the WT mice, also the L-KO mice showed a reduction of body weight, WAT mass and insulin levels in response to SCFA supplementation, demonstrating that these effects were not mediated by hepatic PPARγ (Figs. 4A-B and S7A-C). This is in line with our previous observation that SCFAs increased peripheral, but not hepatic insulin sensitivity (Fig. 1F). Whereas in WT mice the RER values and plasma NEFA concentrations were reduced by SCFAs, they were increased by SCFAs in L-KO mice (Figs. S7D-E). In addition, the SCFA-induced increase in hepatic lipid oxidation capacity and the concomitant reduction in hepatic triglycerides that was observed in WT mice, were abolished in PPARγ L-KO mice (Figs. 4C-D). Finally, we were no longer able to detect any differences in hepatic protein levels of UCP2, pAMPK and pACC between the SCFA-fed and non-SCFA-fed PPARγ L-KO mice (Fig. 4E), nor did we see any effects on the target genes of PPARγ (Fig. S7F). These results suggest that the beneficial effect of SCFAs on liver lipid metabolism was directly mediated by hepatic PPARγ.
Our results with the PPARγ A-KO mice were almost opposite to those with the L-KO mice. Disruption of adipose PPARγ abolished the effects of SCFAs on body weight gain, WAT mass and insulin levels (Figs. 5A-B and S8A-C). In contrast to our observations in the PPARγ L-KO mice, in the PPARγ A-KO mice SCFAs had no effect on RER and plasma NEFA concentrations (Figs. S8D-E), while the strong SCFA-induced reduction in liver triglycerides was preserved in these mice (Fig. 5C). Finally, there was no increase in adipose lipid oxidation capacity and in adipose protein levels of UCP2, pAMPK and pACC, when SCFA-fed and non-SCFA-fed PPARγ A-KO mice were compared (Figs. 5D-E), nor did we see any effects of SCFAs on the target genes of PPARγ (Fig. S8F). The differences between the L-KO and the A-KO are further reflected by the fact that the adipose tissue of the L-KO mice preserved the SCFA-induced effects on lipid oxidation capacity, PPARγ protein levels, transcript levels of the PPARγ target genes and the concomitant increase of UCP2, pAMPK and pACC protein levels (Figs. 4D-E and S7G), while the A-KO mice preserved these characteristics in liver tissue (Figs. 5D-E and S8G).

Taken together, our results indicated that SCFA-induced protection against HFD-induced obesity and insulin resistance is impaired by adipose PPARγ deficiency, while hepatic PPARγ deficiency impairs the SCFA-induced reduction in hepatic steatosis.
Discussion

In this study we demonstrate that the beneficial metabolic effects of SCFAs – protection against HFD-induced obesity and improved insulin sensitivity – are mediated by downregulation of PPARγ. Our results are in line with other studies in which similar physiological effects of SCFAs have been found (15, 17). However, by identifying PPARγ as a central regulator of the systemic response, we are the first to integrate the role of all three major SCFAs (acetate, propionate and butyrate) into a single mechanism.

The combined results of our in vivo and in vitro experiments suggest an SCFA-induced cascade in which downregulation of PPARγ activates an UCP2-AMPK-ACC network. This cascade shifts metabolism in adipose and liver tissue from lipogenesis to fatty-acid oxidation. SCFAs activate both the supply of fatty-acid substrates via CPT1 and the demand for their oxidation products via uncoupling of the respiratory chain. This is a clear example of what has been described as multisite modulation, i.e. the phenomenon that metabolic fluxes can only be altered by simultaneous modulation of multiple enzymes in a pathway (47).

The observed mitochondrial uncoupling may – at least in part – be explained by the increased expression of UCP2. UCP2 belongs to a family of mitochondrial uncoupling proteins comprising UCP1, 2 and 3, all three with proton leak activity (48, 49). Whereas UCP1 has clear-cut proton-conducting activity, UCP2 catalyzes the proton-driven exchange of phosphate for small dicarboxylic acids across the mitochondrial inner membrane (50). This allows UCP2 to dissipate the proton gradient across the mitochondrial inner membrane in a substrate-dependent manner.

SCFAs activate the expression of leptin via the SCFA receptor GPR43 (51). Yet, we found reduced serum levels of the satiety factor leptin in SCFA-treated animals. This can be
explained from the overall decrease in WAT mass. The same observation was made previously in mice that overexpress GPR43 (52). Despite their lower leptin levels, the food intake of SCFA treated animals was not increased. This may be due to other satiety factors, which we did not investigate further.

In addition to our finding that PPARγ is a central regulator of the beneficial effects of SCFAs, we also demonstrated that liver and adipose tissue contribute independently to this mechanism: the beneficial effects on whole body fat accumulation, hepatic steatosis, hypertriglyceridemia and insulin sensitivity appear to be mediated differently in these two tissues. While an SCFA-induced reduction of PPARγ in the liver reduced hepatic triglyceride concentrations, the same reduction in adipose tissue reduced body weight and improved insulin sensitivity. The fact that cultured adipose or liver cells respond to SCFAs in the same way, by reducing PPARγ and activating the UCP2-AMPK-ACC network, suggests that the organs respond directly to the SCFAs that have been taken up into the body. Recently, we showed that the fluxes of SCFA uptake into the body – but not the SCFA concentrations in the cecum – are correlated with the physiological effects of dietary fiber (53). This corroborates the idea that SCFAs need to be taken up and do not exert their full effect on organ physiology through intestinal receptors. In addition, it was recently shown that the intestinal gluconeogenesis pathway is also an important mediator of SCFA-induced reduction of body weight and insulin resistance (14) as PPARγ in our study. De Vadder et al. showed - by capsaicin-induced periportal nervous deafferentation - that propionate exerts its effect via a gut-brain communication axis. Intestinal knockout of the gluconeogenic gene G6Pc abolished the benefits of fiber and SCFAs on body weight and glucose homeostasis, as our adipose knockout of PPARγ. How this intestinal pathway relates to the PPARγ pathway in liver and adipose is currently unclear. Similarly, it
remains to be established whether and how the PPARγ-dependent pathway interacts with GPR41 and/or GPR43 signaling.

That we established PPARγ as a central mediator of the beneficial effects of SCFAs has possible consequences for the treatment of metabolic disorders. A key finding in the PPARγ field is that adipose mass increases almost proportionally to PPARγ activity, while either inhibition or activation of PPARγ sensitizes the body for insulin (54). With regard to treatment, the most suitable molecules are most likely those that uncouple these different functions of PPARγ such that the receptor sensitizes the body for insulin, without any undesired adipogenesis. Such selective PPARγ modulators (SPPARMs) have been proposed by others (55) and our results suggest that SCFAs act as highly effective endogenous SPPARMs. The few human studies that have been conducted, showed that intravenous administration of acetate or propionate reduces plasma free fatty acids in humans (56, 57), while 12 weeks of dietary vinegar (acetate) supplementation in obese subjects led to lower body weight, body fat mass and serum triglycerides levels than in the placebo-controlled group (16). This makes the inexpensive SCFAs very attractive compounds to prevent and reverse HFD-induced obesity and insulin resistance.

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**Author Contributions** G.D.B. conceived study, designed and performed experiments, analyzed data, and wrote the manuscript. A.B., A.G., K.V.E., R.H., M.H.O. and T.H.V.D. performed experiments, analyzed data, and provided input into the manuscript. J.W.J., A.K.G., D.-J.R., and B.M.B. conceived study, interpreted data, and edited the manuscript. B.M.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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FIGURE LEGENDS

Figure 1 - SCFAs protect against high-fat diet-induced obesity and insulin resistance. Eight-week-old male C57Bl/6J mice were fed a high-fat diet supplemented with acetate, propionate or butyrate (5% w/w). (A) Body weight was monitored for a period of 12 weeks on the indicated diets. *p<0.05 acetate vs control, #p<0.05 propionate vs control, $p<0.05$ butyrate vs control. (B-C) Energy expenditure and RER were evaluated using indirect calorimetry data in animals after 10 weeks on the indicated diets. (D) Blood insulin levels were measured in animals after 12 weeks on the indicated diets and after a 4h fast. (E-F) Hyperinsulinemic-euglycemic clamp studies were performed in animals fed the indicated diets for 10 weeks. Glucose infusion rate (GIR), glucose production rate (Ra) and glucose uptake rate (Rd) were calculated after the test. Values are means ± SEM for n=7-8; *p<0.05, **p<0.01, ***p<0.001 vs control.

Figure 2 - SCFAs enhance oxidative metabolism. (A) Hepatic lipogenesis determined in vivo by incorporation of [1-13C]-acetate dissolved in drinking water after 12 weeks of HFD with or without SCFA. (B) Hepatic β-oxidation determined ex vivo in liver homogenates by trapping 14C-labeled CO2 produced during incubation with [1-14C]-palmitic acid. (C) Hepatic pAMPK and pACC protein levels were analyzed by western blot of tissue lysates from mice after 12 weeks on diet. (D) CPT-1 activity was analyzed by measurement of palmitoyl-carnitine produced in total liver homogenates during incubation with palmitoyl CoA and L-carnitine as substrates. (E) Liver mitochondria were isolated and maximal ADP-stimulated oxygen consumption (i.e. state 3) and oxygen consumption in the presence of oligomycin inhibition of ATP synthesis (i.e. state 4) were determined. (F) Mitochondrial UCP2 protein levels were analyzed by western blot of tissue
lysates from mice after 12 weeks on the indicated diets. Values are means ± SEM for n=7-8; *p<0.05, **p<0.01, ***p<0.001 vs control.

**Figure 3** - Activation of the UCP2-AMPK-ACC Pathway by SCFAs Depends on PPARγ. HepG2 cells, differentiated 3T3-L1 cells and differentiated C2C12 cells were incubated with 3 mM SCFAs for 24 hours in the presence of 100 nM rosiglitazone or 10 µM GW9662 as indicated. (A-C) mRNA expression of PPARγ and target genes was assessed via qPCR in HepG2, differentiated 3T3-L1 and C2C12 cells after 24h incubation with SCFAs. (D-F) PPARγ, UCP2, pAMPK and pACC protein levels were assessed by western blot in HepG2, differentiated 3T3-L1 and C2C12 cells after 24h incubation with SCFAs. Values are means ± SEM for n=6; *p<0.05 vs control.

**Figure 4** - Hepatic PPARγ deficiency impairs the SCFA-induced reduction in hepatic steatosis. Eight-week-old male liver-specific PPARγ knock-out mice were fed a HFD supplemented with acetate, propionate or butyrate (5% w/w). (A) Body weight was measured for a period of 10 weeks. *p<0.05 acetate vs control, #p<0.05 propionate vs control, $p<0.05 butyrate vs control. (B) Insulin tolerance tests were performed on mice after 9 weeks on the indicated diets and after a 4h fast. (C) Liver triglycerides in mice after 10 weeks on the indicated diets. (D) Fatty-acid β-oxidation in liver and white adipose tissue was measured in mice after 10 weeks on the indicated diets. (E) PPARγ, UCP2, pAMPK and pACC protein levels were assessed by western blot in liver and white adipose tissue lysates. Values are means ± SEM for n=6-8; *p<0.05 vs control.
**Figure 5** - Adipose PPARγ deficiency impairs the SCFA-induced protection against HFD-induced obesity and insulin resistance. Eight-week-old male adipose-specific PPARγ knock-out mice were fed a HFD supplemented with acetate, propionate or butyrate (5% w/w). (A) Body weight was measured for a period of 10 weeks on the indicated diets. \*p<0.05 acetate vs control, \#p<0.05 propionate vs control, \$p<0.05 butyrate vs control. (B) Insulin tolerance tests were performed on mice after 9 weeks on the indicated diets and after a 4h fast. (C) Liver triglycerides in mice after 10 weeks on the indicated diets. (D) Fatty-acid β-oxidation in liver and white adipose tissue was measured in mice after 10 weeks on the indicated diets. (E) PPARγ, UCP2, pAMPK and pACC protein levels were assessed by western blot in liver and white adipose tissue lysates. Values are means ± SEM for n=6-8; \*p<0.05 vs control.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
# SUPPLEMENTARY DATA

Table S1 - List of oligonucleotide primer pairs used in qPCR analysis.

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SUPPLEMENTAL FIGURE LEGENDS

**Figure S1** - SCFA treatment increases energy expenditure and insulin sensitivity. Eight-week-old male C57Bl/6J mice were fed a high-fat diet mixed either with acetate, propionate or butyrate (5% w/w). (A-B) White adipose tissue mass and morphology after 12 weeks on diet. (C) Plasma leptin levels after 12 weeks on diet. (D) Energy balance was determined by measuring the energy content of the diet and dried homogenized feces. Uptake is defined as the difference between intake and output. (E) Locomotor activity was evaluated by beam breaks using indirect calorimetry cages after 10 weeks on high-fat diet. (F) VO$_2$ was evaluated using indirect calorimetry data after 10 weeks on high-fat diet. (G) Blood glucose levels measured in animals with their respective diets for 12 weeks after a 4h fast. (H) After 11 weeks on their respective diets an intraperitoneal glucose tolerance test was performed in mice that were fasted overnight. *p<0.05 acetate vs control, #p<0.05 propionate vs control, $p<0.05$ butyrate versus control. (I) Area under the curve of the intraperitoneal glucose tolerance test. (J) Insulin tolerance tests were performed on mice for 11 weeks on their respective diets after a 4h fast. Values are means ± SEM for n=7-8; *p<0.05, ***p<0.001 vs control.

**Figure S2** - SCFA treatment reduces HFD-induced obesity and insulin resistance to normal chow diet levels. Eight-week-old male C57Bl/6J mice were fed a normal chow diet or a high-fat diet mixed either with acetate, propionate or butyrate (5% w/w) for 10 weeks. (A-B) Body weight and white adipose mass after 10 weeks of diet. (C-E) Plasma leptin, glucose and insulin levels after 10 weeks of diet. (F-G) Hyperinsulinemic-euglycemic clamp studies were performed in animals fed the indicated diets for 10 weeks. Glucose infusion rate (GIR), glucose production
rate (Ra) and glucose uptake rate (Rd) were calculated after the test. Values are means ± SEM for n=7-8; ***p<0.001 vs HFD.

**Figure S3** - SCFAs reverse HFD-induced obesity and insulin resistance. Eight-week-old male C57Bl/6J mice were fed a high-fat diet for 12 weeks and switch to a high-fat diet supplemented with acetate, propionate or butyrate (5% w/w) for 6 weeks. The start of the SCFA treatment is indicated as time point zero. (A) Body weight was measured before and after the 6-week intervention with the indicated diets. (B) Food intake was measured after 5 weeks’ intervention with the indicated diets. (C) White adipose tissue mass of mice after the 6-week intervention with the indicated diets. (D) RER was evaluated using indirect calorimetry data in mice after 5 weeks’ intervention with the indicated diets. (E-F) Blood glucose and insulin levels measured in animals after the 6-week intervention with the indicated diets and after a 4h fast. (G) Insulin tolerance tests were done in mice after 5 weeks’ intervention with the indicated diets and after a 4h fast. *p<0.05 acetate vs control, #p<0.05 propionate vs control, $p<0.05 butyrate vs control. (H-I) Plasma NEFA concentrations and liver triglycerides in mice after the 6-week intervention with the indicated diets. Values are means ± SEM for n=7-8; *p<0.05, **p<0.01, ***p<0.001 vs control.

**Figure S4** - SCFA treatment switch hepatic metabolism from lipogenesis to fat oxidation. Eight-week-old male C57Bl/6J mice were fed a high-fat diet mixed either with acetate, propionate or butyrate (5% w/w). (A-B) Plasma NEFA concentrations and liver triglycerides in mice after 12 weeks on diet. (C) Hepatic mRNA expression of lipogenic genes was assessed via qPCR. (D) Hepatic FAS protein expression was assessed by western blot. (E) Hepatic total AMPK and ACC
protein expression was assessed by western blot. (F-G) Hepatic malonyl CoA and adenine nucleotides were determined by HPLC. (G) Hepatic AMP/ATP ratio triglycerides in mice after 12 weeks on diet. Values are means ± SEM for n=7-8; *p<0.05, **p<0.01, ***p<0.001 vs control.

**Figure S5** - SCFA treatment affects PPARγ in liver and adipose tissue. Eight-week-old male C57Bl/6J mice were fed a high-fat diet mixed either with acetate, propionate or butyrate (5% w/w). (A-C) mRNA expression of PPARα and its target genes was assessed via qPCR in liver, adipose and muscle tissue of mice after 12 weeks on the diet. (D-F) mRNA expression of PPARγ and target genes was assessed via qPCR in liver, adipose and muscle tissue of mice after 12 weeks on the diet. (G) Protein expression of PPARγ was analyzed by western blot in liver, adipose and muscle tissue of mice after 12 weeks on the diet. (H) mRNA expression of PGC-1α was assessed via qPCR in liver, adipose and muscle tissue. (I) mRNA expression of UCP-1 was assessed via qPCR in brown adipose tissue. Values are means ± SEM for n=6-8; *p<0.05 vs control.

**Figure S6** - Activation of the UCP2-AMPK-ACC pathway by SCFAs depends on PPARγ activity. HepG2 cells and differentiated 3T3-L1 cells were incubated with 3 mM SCFAs for 24 hours in the presence of 100 nM rosiglitazone or 10 µM GW9662 as indicated. (A) PPARγ, UCP2, pAMPK and pACC protein levels were assessed by western blot in HepG2 and differentiated 3T3-L1 cells after incubation with SCFAs and the PPARγ agonist rosiglitazone (Rosi). (B) PPARγ, UCP2, pAMPK and pACC protein levels were assessed by western blot in HepG2 and differentiated 3T3-L1 cells after incubation with SCFAs and the PPARγ antagonist
GW9662. (C-D) mRNA expression of PPARγ was assessed via qPCR in HepG2 and differentiated 3T3-L1 cells after 24h incubation with 0.1, 1 and 3 mM SCFAs. Values are means ± SEM for n=6; *p<0.05 vs control.

**Figure S7** - SCFA treatment affects WAT metabolism in L-KO PPARγ mice. Eight-week-old male liver-specific PPARγ knock-out mice were fed a high-fat diet mixed either with acetate, propionate or butyrate (5% w/w). (A) White adipose tissue mass after 10 weeks on diet. (B-C) Blood glucose and insulin levels measured in animals after 10 weeks on their respective diets and after a 4h fast. (D) RER was evaluated using indirect calorimetry data in mice after 9 weeks on diet. (E) Plasma NEFA concentrations in mice after 10 weeks on their respective diets. (F-G) mRNA expression of PPARγ and target genes was assessed via qPCR in liver and adipose tissue of mice after 10 weeks on diet. Values are means ± SEM for n=7-8; *p<0.05, **p<0.01, ***p<0.001 vs control.

**Figure S8** - SCFA treatment affects liver metabolism in A-KO PPARγ mice. Eight-week-old male adipose-specific PPARγ knock-out mice were fed a high-fat diet mixed either with acetate, propionate or butyrate (5% w/w). (A) White adipose tissue mass after 10 weeks on diet. (B-C) Blood glucose and insulin levels measured in animals after 10 weeks on their respective diets and after a 4h fast. (D) RER was evaluated using indirect calorimetry data in mice after 9 weeks on diet. (E) Plasma NEFA concentrations in mice after 10 weeks on their respective diets. (F-G) mRNA expression of PPARγ and target genes was assessed via qPCR in adipose and liver tissue of mice after 10 weeks on diet. Values are means ± SEM for n=7-8; *p<0.05 vs control.
Figure S1
Figure S2
Figure S3
Figure S4
Figure S5
Figure S6
Figure S7
Figure S8