Elucidating the mechanisms of action of short-chain fatty acids

den Besten, Gijs

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Dietary short-chain fatty acids protect against high-fat diet-induced obesity via a PPARγ-dependent switch from lipogenesis to fatty-acid oxidation
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. SCFA-induced decreases in body weight and increases in 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 SCFAs on the metabolic syndrome, with clearly distinct and complementary roles.
Introduction

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 (167, 168). Epidemiological studies have reported an inverse correlation between dietary fiber intake on the one hand, and body weight, insulin resistance and dyslipidemia on the other (169), suggesting that fiber supplements may have beneficial effects on metabolism. Indeed, numerous studies have shown that dietary fiber intervention reduces obesity and insulin resistance in both healthy individuals and metabolic syndrome patients (171, 178, 179). However, meta-analysis studies have also shown that the beneficial effects of different dietary fibers vary widely (240-242).

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 (5, 11). While all three SCFAs are rapidly assimilated into host carbohydrates and lipids - providing ~10% of our daily energy requirements (31) - 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 (30, Chapter 3). Besides serving as an energy source, SCFAs are also thought to be important regulators that mediate the beneficial effects of dietary fiber (6, Chapter 1). Known aspects of the underlying mechanism are that SCFAs inhibit histone deacetylase (243) and activate the endogenous G protein-coupled receptors (GPR) 41 and 43, both abundantly expressed in the distal small intestine, colon and adipose tissue (83, 115, 119). The individual SCFAs differ in their GPR-mediated effects because of their distinct relationship between chain length and receptor affinity: the longer butyrate is more selective for GPR41, the shorter acetate is more selective for GPR43, while propionate binds to both receptors (109, 110). By activating GPR43, both acetate and propionate increase the expression of glucagon-like peptide-1 (145, 244) and leptin (113, 118), thereby regulating adipogenesis (113), lipolysis (114) and inflammatory responses (245, 246). Propionate has been described to activate the sympathetic nervous system through GPR41 (247) and to cause apoptotic cell death and cell-cycle arrest in colon cancer cells through GPR43 (21, 217). Butyrate has been shown to regulate immune responses, energy metabolism and autophagy in a GPR-independent manner (18, 248).

Despite these differences in the GPR-mediated effects of SCFAs, when given as a dietary supplement all three SCFAs are able to ameliorate high-fat diet-induced obesity and insulin resistance (17, 122, 124). The fact that all three SCFAs exert similar beneficial effects on symptoms of the metabolic syndrome suggests that the underlying molecular mechanism is GPR-independent. This is in line with recent findings that GPR41 deficient mice were still sensitive to beneficial effects of SCFA on body weight and insulin sensitivity (122). In support of an alternative mechanism, acetate and butyrate have been shown to increase energy expenditure by activating AMP-activated protein kinase (AMPK) (17, 137). Similarly, propionate has been shown to activate AMPK in colon cancer cells (217) and to reduce lipid synthesis in isolated rat hepatocytes (79, 185). The mechanism by which SCFAs activate AMPK is, however, still unknown.

In this chapter we investigated the molecular mechanism by which SCFAs mediate the...
beneficial effects of dietary fiber on the metabolic syndrome. We reveal a cascade of events that is dependent on peroxisome proliferator-activated receptor (PPAR)-γ and in which liver and adipose tissue play distinct, complementary roles.

Materials 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. They 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). 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 (249). 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). Whole-body energy metabolism was assessed immediately after termination of the mice. Experimental procedures were approved by the Ethics Committee for Animal Experiments of the University of Groningen.

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. 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) and HOMA-IR was calculated as follows: IR = fasting insulin mU/L x fasting glucose mM ÷ 22.5. Hepatic TG content was determined using a commercial available kit (Roche) after lipid extraction (223).

Hepatic malonyl-CoA and adenosine concentrations were determined by HPLC according to Demoz et al. (250) and Miller et al. (251), respectively. CPT-1 activity was determined in liver homogenates at different dilutions (20, 10, 5, 2 and 1 ng/μL) according to van Vlies et al. (252) 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).

**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 (253).

**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 (194). Fatty acid β-oxidation capacity was determined in fresh liver and adipose homogenates according to Hirschey et al. (254). Briefly, tissue was homogenized in sucrose/Tris/EDTA buffer, incubated for 30 min in the reaction mixture (pH 8.0) containing [1-¹⁴C]palmitic acid, and trapped [¹⁴C]CO₂ was measured.

**Oxygen consumption rates in liver mitochondria**

Mitochondria were isolated from fresh liver tissue according to Mildaziene et al. (255). 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) 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% CO₂ 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 (657160; Greiner Bio-One) and incubated with 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. (256) and incubated with 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 Fujitia et al. (257) 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: phosphorylated AMP kinase (pAMPK Thr172, no. 2531; Cell Signaling), phosphorylated acetyl CoA carboxylase (pACC S79, no. 31931; Abcam), uncoupling protein 2 (UCP2, no. 6525; Santa Cruz), peroxisome proliferator-activated receptor γ (PPARY, 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

SCFAs protect against high-fat diet-induced obesity and insulin resistance

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. Controls received HFD without SCFA during the same period and showed a substantial raise in body weight (Figure 1A). Interestingly, all three individual dietary SCFAs attenuated this increase in body weight to a similar extent (Figure 1A). After 12 weeks this coincided with reductions in white adipose tissue (WAT) mass (Figures 1B and S1A) and plasma leptin concentrations compared to controls (Figure 1C).

To investigate the effect of dietary SCFAs on energy metabolism, mice were subjected to indirect calorimetry. The fact that neither caloric intake nor physical activity was significantly affected by SCFA supplementation (Figures 1D and S1B) indicates that the lower body weight seen in mice fed an SCFA-supplemented HFD was not due to alterations in food intake or physical activity. We did, however, observe enhanced energy expenditure in these mice (Figure 1E). Mice fed SCFAs displayed a shift towards increased fatty-acid
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 measured 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) White adipose tissue mass and plasma leptin levels in mice after 12 weeks on the indicated diets. (D) Energy balance was determined by measuring the energy content of the diet and dried homogenized feces by bomb calorimetry. Energy uptake is defined as the difference between intake and output. (E-G) Energy expenditure, VO2, and RER were evaluated using indirect calorimetry data in animals after 10 weeks on the indicated diets. (H) Blood glucose levels were measured in animals after 12 weeks on the indicated diets and after a 4h fast. (I) After 11 weeks on the indicated diets, an intraperitoneal glucose tolerance test was performed in mice that had been fasted overnight for 9h. *p<0.05 acetate vs control, #p<0.05 propionate vs control, $p<0.05 butyrate vs control. (J-K) Blood insulin levels were measured in animals after 12 weeks on the indicated diets and after a 4h fast. HOMA-IR was calculated by multiplying fasting insulin in mU/L with fasting glucose in mM and dividing by 22.5. (L) Insulin tolerance tests were performed in animals after 11 weeks on 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. (M-N) 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. All values in the figure are presented as mean ± SEM for n=7-8; *p<0.05, **p<0.01, ***p<0.001 vs control. This figure is complemented by Figures S1.
oxidation, as indicated by higher O2 consumption rates (Figure 1F) and lower respiratory exchange ratios (RER, defined as the rate of CO2 production divided by the rate of oxygen consumption) (Figure 1G). Altogether, these results indicate 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 (Figures 1H and 1I). Fasting insulin levels were much lower in SCFA-fed mice (Figure 1J). Together this translated into significantly lower HOMA-IR values (Figure 1K), which were calculated according the formula for homeostatic model assessment of insulin resistance (HOMA-IR, see Materials and Methods). Together with enhanced disposal of glucose upon insulin injection (Figure 1L), these experiments point to higher insulin sensitivity in these mice. To quantify insulin sensitivity and to discriminate between the effects of SCFAs on the liver and their effects on peripheral insulin action, 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 (Figure 1M). While the hepatic glucose production rate during the clamp was similar in all groups (Ra in Figure 1N), 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 Figure 1N). This indicates improved peripheral insulin sensitivity in these mice. No differences were observed between the three individual SCFAs. Collectively, these observations unequivocally demonstrate that SCFA supplementation enhances the insulin sensitivity of HFD-fed mice.

**SCFAs reverse high-fat diet-induced obesity and insulin resistance**

Given the promising results of SCFAs in the prevention of HFD-induced obesity and insulin resistance, we wondered whether SCFAs could also be used to treat these metabolic disorders. 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 period the SCFA-supplemented mice showed significantly lower body weight and WAT mass than controls (Figures 2A-B). The reduction in body weight in SCFA-fed mice was accompanied by a higher insulin sensitivity, as shown by increased glucose uptake by peripheral tissues upon insulin delivery and lower HOMA-IR values (Figures 2C-F). SCFA-fed mice also had lower RER values, indicating a shift towards increased fatty-acid oxidation (Figures 2G). These data show that all three SCFAs can be used not only to prevent but also to treat HFD-induced obesity and insulin resistance.

**SCFAs stimulate mitochondrial fatty-acid oxidation by activating the UCP2-AMPK-ACC pathway**

Control mice showed high concentrations of plasma non-esterified fatty acids (NEFAs) and hepatic triglycerides (hepatic steatosis). These aspects of the metabolic syndrome were reduced by SCFA supplementation (Figures 3A-B), while the liver weight to body weight ratios were similar in control and SCFA-fed mice (Figure S2A). The reduction in NEFA and hepatic triglycerides was also observed when SCFAs were fed to already obese mice (Figures 2H-I). Together with the reduction in RER, this suggested a shift from lipid synthesis to lipid
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) White adipose tissue mass of mice after the 6-week intervention with the indicated diets. (C) Insulin tolerance tests were done in mice after 5 weeks of 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. (D-E) Blood glucose and insulin levels measured in animals after the 6-week intervention with the indicated diets and after a 4h fast. (F) HOMA-IR was calculated from fasting glucose and insulin levels in mice after 6 weeks intervention with the indicated diets. (G) RER was evaluated using indirect calorimetry data in mice after 5 weeks of intervention with the indicated diets. (H-I) Plasma NEFA concentrations and liver triglycerides in mice after the 6-week intervention with the indicated diets. All values in the figure are presented as mean ± SEM for n=6-8; *p<0.05, **p<0.01, ***p<0.001 vs control.

oxidation in SCFA-fed mice, prompting 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) (Figures S2B-C). In agreement, these mice had a 2-fold reduction in hepatic lipid synthesis (Figure 3C). The capacity for hepatic lipid oxidation in SCFA-fed mice was 2-fold higher than that of controls (Figure 3D), further confirming the suggestion that hepatic lipid metabolism was shifted towards a more oxidative state.

Figure 2. 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) White adipose tissue mass of mice after the 6-week intervention with the indicated diets. (C) Insulin tolerance tests were done in mice after 5 weeks of intervention with the indicated diets and after a 4h fast. *p<0.05 acetate vs control, #p<0.05 propionate vs control, $p<0.05 butyrate vs control. (D-E) Blood glucose and insulin levels measured in animals after the 6-week intervention with the indicated diets and after a 4h fast. (F) HOMA-IR was calculated from fasting glucose and insulin levels in mice after 6 weeks intervention with the indicated diets. (G) RER was evaluated using indirect calorimetry data in mice after 5 weeks of intervention with the indicated diets. (H-I) Plasma NEFA concentrations and liver triglycerides in mice after the 6-week intervention with the indicated diets. All values in the figure are presented as mean ± SEM for n=6-8; *p<0.05, **p<0.01, ***p<0.001 vs control.
Since activation of AMPK shifts lipid metabolism from synthesis to oxidation (258) and butyrate and acetate have been shown to activate AMPK (17, 137), we wondered whether the metabolic effects of SCFAs might be mediated through activation of this kinase. We therefore measured the phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase (ACC) and observed increased phosphorylation in SCFA-fed mice (Figure 3E). Since phosphorylation inactivates ACC, this should lead to lower concentrations of its product malonyl-CoA. The latter is an endogenous inhibitor of carnitine palmitoyltransferase 1 (CPT-1), the first enzyme in the β-oxidation of fatty acids (259). Indeed, not only did SCFA treatment result in lower hepatic malonyl-CoA concentrations (Figure 3F), it also increased the enzymatic capacity ($V_{\text{max}}$) of CPT-1 (Figure 3G). Since the latter was measured in diluted liver homogenates, endogenous malonyl-CoA should be too low to affect the result. This implies that CPT-1 is stimulated via a dual mechanism: by raising its $V_{\text{max}}$ and by reducing the concentration of its inhibitor.

To identify the cause of SCFA-induced AMPK activation, we measured the AMP/ATP ratio, which is a sensitive reflection of the metabolic state of the cell and the direct activator of AMPK (260). SCFA-fed mice had increased hepatic AMP/ATP ratios (Figure 3H), which were mainly due to reduced ATP concentrations (Figure S2D). Such reduced ATP concentrations can be a result of increased mitochondrial proton leakage, leading to mitochondrial uncoupling and subsequently reduced ATP synthesis (261). 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). In state 4 the respiration is due to proton leakage across the inner mitochondrial membrane and the respiratory control ratio (RCR = state 3 rate / state 4 rate) is used as an index of mitochondrial coupling. We observed lower RCR values in liver mitochondria from SCFA-fed mice due to an increased state 4 respiration rate (Figure 3I), demonstrating that there is indeed 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 (Figure 3J), suggesting that proton leak via UCP2 is responsible for the uncoupling observed (261).

**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 to regulate UCP2 expression (262-264). When activated, PPARα enhances the expression of uncoupling proteins as well as proteins involved in fatty-acid oxidation. In contrast, PPARγ expression and activity need to be reduced to stimulate UCP2 expression (263, 265). In addition, both PPARα and PPARγ – expressed in liver, adipose and muscle – are known to regulate whole-body lipid metabolism (266-270). Clearly, however, SCFAs did not stimulate expression of PPARα – or its target genes – in liver, adipose or muscle tissue (Figures S3A-C). 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 (Figures S3D-G), suggesting that PPARγ may well be the mediating factor between SCFAs and the UCP2-AMPK-ACC pathway.

To find out whether PPARγ is 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). 24h treatment with SCFAs reduced mRNA and protein levels of PPARy and its target genes in HepG2 and 3T3-L1 cells, but not in C2C12 cells (Figures 4A-C). SCFAs also enhanced the activity of the UCP2-AMPK-ACC
Figure 4. 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. (G) 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). (H) 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. All values in the figure are presented as mean ± SEM for n=6; *p<0.05 vs control. This figure is complemented by Figure S3.
signaling pathway in HepG2 and 3T3L1 cells, but not in C2C12 cells (Figures 4D-F), in line with our *in vivo* results.

Next, we investigated whether interfering with PPARγ activity affected the effects of SCFAs. HepG2 and 3T3-L1 cells were incubated with either the full PPARγ agonist rosiglitazone or the PPARγ antagonist GW9662 and the effects of SCFAs on the UCP2-AMPK-ACC signaling pathway were studied. By offsetting the partial repression of PPARγ expression and activity by SCFAs, rosiglitazone abolished both the SCFA-induced reduction in PPARγ expression and the accompanying increase in the activity of the UCP2-AMPK-ACC pathway (Figure 4G). 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 (Figure 4H). Apparently, either activation or inhibition of the activity of PPARγ abolished the SCFA-induced increase of the activity of the UCP2-pAMPK-pACC pathway. Together, 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 deficiency impairs the SCFA-induced reduction in hepatic steatosis but not the protection against HFD-induced obesity and insulin resistance

To distinguish between the role of PPARγ in the liver and that in other tissues, we fed mice with a liver-specific knock-out (L-KO) of PPARγ an HFD supplemented with SCFAs. Body weight gain was still significantly attenuated by all three individual SCFAs (Figure 5A) and was accompanied by a reduced WAT mass (Figures 5B and S4A). The increased insulin sensitivity in SCFA-fed mice was not affected by disruption of hepatic PPARγ, since SCFA feeding of the L-KO mice still enhanced glucose disposal upon insulin injection and lowered insulin and HOMA-IR levels (Figures 5C-D and S4B-C). This is in line with our previous observation that insulin sensitivity was increased in peripheral tissues, but not in the livers of SCFA-fed wild-type mice (Figure 1N). In contrast to wild-type mice, SCFA-fed PPARγ L-KO mice had higher RER values and plasma NEFA concentrations than those of the non-SCFA-fed controls (Figures 5E-F). In addition, the reduction in hepatic triglycerides and increase in hepatic lipid oxidation capacity upon SCFA treatment were abolished in PPARγ L-KO mice (Figures 5G-H). In PPARγ L-KO mice, we were no longer able to detect any differences in protein levels of UCP2, pAMPK and pACC in the liver between the SCFA-fed and non-SCFA-fed groups (Figure 5I), nor did we see any effects on the target genes of PPARγ (Figure S4D).

Next we wondered whether the persisting decrease in body weight gain and insulin resistance in SCFA-fed L-KO PPARγ mice was due to an effect of SCFAs on PPARγ in white adipose tissue. Indeed, SCFA-fed L-KO PPARγ mice exhibited a 2-fold increase in lipid oxidation capacity in adipose tissue (Figure 5H). This increase was accompanied by the following effects in the adipose tissue of these mice: a reduction in PPARγ protein levels (Figure 5I) and in the transcripts of its target genes Cd36, Lpl, Fabp4 and Pltp (Figure S4E), and a concomitant increase of UCP2, pAMPK and pACC protein levels (Figure 5I). These findings were similar to our observations in the livers of SCFA-fed wild-type mice (Figure 3). Altogether, these results
Hepatic PPARγ deficiency impairs the SCFA-induced reduction in hepatic steatosis but not the protection against HFD-induced obesity and insulin resistance. Eight-week-old male liver-specific PPARγ knock-out mice were fed a high-fat diet 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) White adipose tissue mass after 10 weeks on the indicated diets. (C) Insulin tolerance tests were performed on mice after 9 weeks on 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. (D) HOMA-IR was calculated from fasting glucose and insulin levels in mice after 10 weeks on the indicated diets. (E) RER was evaluated using indirect calorimetry data in mice after 9 weeks on the indicated diets. (F-G) Plasma NEFA concentrations and liver triglycerides in mice after 10 weeks on the indicated diets. (H) Fatty-acid ß-oxidation in liver and white adipose tissue was measured in mice after 10 weeks on the indicated diets. (I) PPARγ, UCP2, pAMPK and pACC protein levels were assessed by western blot in liver and white adipose tissue lysates. Quantification is shown in the right panel. All values in the figure are presented as mean ± SEM for n=6-8; *p<0.05, **p<0.01, ***p<0.001 vs control. This figure is complemented by Figure S4.

clearly indicate that hepatic PPARγ deficiency impairs the SCFA-induced reduction in hepatic steatosis, but not the protection against HFD-induced obesity and insulin resistance.
Adipose PPARγ deficiency impairs the SCFA-induced protection against HFD-induced obesity and insulin resistance but not the reduction in hepatic steatosis

The persisting decrease in body weight gain and increase in insulin sensitivity in SCFA-fed L-KO PPARγ mice prompted us to test the role played by adipose PPARγ in the systemic effects of SCFAs. To this end, we fed adipose-specific PPARγ knock-out (A-KO) mice an HFD supplemented with SCFAs. The effects of SCFAs on body weight gain and WAT mass were completely abolished in A-KO PPARγ mice on an HFD (Figures 6A-B and S5A, respectively). The SCFA-induced increase in insulin sensitivity was also abolished by disruption of adipose PPARγ, as could be concluded from the SCFA-independent HOMA-IR levels, and glucose and insulin plasma concentrations (Figures 6C-D and S5B-C). In the A-KO PPARγ mice, SCFAs had no effect on RER and plasma NEFA concentrations (Figures 6E-F), in contrast to our observations in the L-KO PPARγ mice (Figures 5E-F). The strong reduction in liver triglycerides was, however, preserved in SCFA-fed A-KO PPARγ mice (Figure 6G). In addition, the increase in liver size that we observed when disrupting adipose PPARγ in mice fed a HFD was abolished by SCFA treatment (Figures S5D-E). These differences can be attributed to the SCFA-induced increase in hepatic lipid oxidation capacity that was still present in A-KO PPARγ mice (Figure 6H). Importantly, while disruption of adipose PPARγ did not influence the SCFA-induced effects on either lipid oxidation capacity, expression of PPARγ and its target genes, or the UCP2-AMPK-ACC pathway in liver tissue (Figures 6H-I and S5F), it did abolish these effects in adipose tissue (Figures 6H-I and S5G). Taken together, these results clearly indicated that SCFA-induced protection against HFD-induced obesity and insulin resistance is impaired by adipose PPARγ deficiency while the reduction in hepatic steatosis is not.

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. The subsequent chain of events comprises upregulation of UCP2, activation of AMPK, inhibition of ACC, enhanced fatty acid oxidation and mild uncoupling of respiration from ATP synthesis. Organ-specific ablation of the PPARγ gene revealed independent roles for the PPARγ protein in liver and adipose tissue. Our results are in line with other studies in which similar physiological effects of SCFAs have been found and which have also implicated AMPK signaling (17, 122, 123). 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-fatty-acid oxidation network. This cascade shifts metabolism in adipose tissue and liver from lipogenesis to fatty-acid oxidation. Here it should be noted that SCFA-induced activation of the enzyme CPT-1 – which exerts strong control over the mitochondrial fatty-acid oxidation flux (271) – took place via a dual mechanism, i.e. both by enhancing its catalytic capacity ($V_{max}$) and reducing the concentration of its inhibitor, malonyl-CoA. The latter is the product of the ACC enzyme, which is phosphorylated and inactivated by AMPK. The shift to fatty-acid oxidation is only sustainable, however,
Adipose PPARγ deficiency impairs the SCFA-induced protection against HFD-induced obesity and insulin resistance but not the reduction in hepatic steatosis. Eight-week-old male adipose-specific PPARγ knock-out mice were fed a high-fat diet 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) White adipose tissue mass of mice after 10 weeks on 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. (C) Insulin tolerance tests were performed on mice after 9 weeks on the indicated diets. (D) HOMA-IR was calculated from 4h fasting glucose and insulin levels in mice after 10 weeks on the indicated diets. (E) RER was evaluated using indirect calorimetry data in mice after 9 weeks on the indicated diets. (F-G) Plasma NEFA concentrations and liver triglycerides in mice after 10 weeks on the indicated diets. (H) Fatty-acid β-oxidation in liver and white adipose tissue was measured in mice after 10 weeks on the indicated diets. (I) PPARγ, UCP2, pAMPK and pACC protein levels were assessed by western blot in liver and white adipose tissue lysates. Quantification of the densitograms is shown in the right panel. All values in the figure are presented as mean ± SEM for n=6-8; *p<0.05, **p<0.01, ***p<0.001 vs control. This figure is complemented by Figure S5.

if the demand for its products is also stimulated (272). Indeed, we found increased state 4 respiration, pointing to partial uncoupling of oxidative phosphorylation from respiration, and
lower levels of ATP, which further stimulates respiration. 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 (273).

The observed mitochondrial uncoupling may – at least in part – be explained by the increased expression of UCP2 that we measured. UCP2 belongs to a family of mitochondrial uncoupling proteins comprising UCP1, 2 and 3, all three with proton leak activity (274, 275). 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 (276). This allows UCP2 to dissipate the proton gradient across the mitochondrial inner membrane in a substrate-dependent manner.

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-fatty-acid oxidation network, suggests that the organs respond directly to the SCFAs that have been taken up into the body. This idea is further corroborated by our recent finding in mice 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 (Chapter 2). Interestingly, it was recently shown that the intestinal gluconeogenesis pathway is an equally important mediator of SCFA-induced reduction of body weight and insulin resistance as PPARγ in our study. De Vadder et al. (277) showed - by capsaicin-induced periportal nervous deafferentation - that propionate exerts its effect via a gut-brain communication axis. Intestinal knockout of the gluconeogenic gene G6P abolishes 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.

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 (278). 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 (279) and our results suggest that SCFAs act as highly effective endogenous SPPARMs. To turn this finding into an application, human trials are needed. So far, however, only few human studies have investigated the effects of SCFA supplementation on the metabolic syndrome. Nevertheless, intravenous administration of acetate or propionate has been shown to reduce plasma free fatty acids in humans (131, 132). In another study, 12 weeks of dietary vinegar (acetate) supplementation in obese Japanese subjects led to lower body weight, body fat mass and

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serum triglycerides levels than in the placebo-controlled group (124). This is in agreement with our observation that SCFAs not only prevent but also reverse a HFD-induced body weight increase: SCFA supplementation to mice that were already obese not only increased insulin sensitivity but also had anti-adipogenic effects through a reduction in PPARγ expression and activity. Altogether, this makes the inexpensive SCFAs very attractive compounds to prevent and reverse HFD-induced obesity and insulin resistance.
Supplemental figures

Figure S1. SCFA treatment reduces WAT mass but does not affect the activity pattern. Eight-week-old male C57Bl/6J mice were fed a high-fat diet mixed either with acetate, propionate or butyrate (5% w/w). (A) WAT to body weight ratio of mice after 12 weeks on high-fat diet. (B) Locomotor activity was evaluated by beam breaks using indirect calorimetry cages after 10 weeks on high-fat diet. All values in the figure are presented as mean ± SEM for n=6-8; ***p<0.001 vs control. This figure is supplemental to Figure 1.

Figure S2. SCFA treatment reduces the expression of lipogenic genes in the liver. Eight-week-old male C57Bl/6J mice were fed a high-fat diet mixed either with acetate, propionate or butyrate (5% w/w). (A) Liver weight to body weight ratio of mice after 12 weeks on high-fat diet. (B) Hepatic mRNA expression of lipogenic genes was assessed via qPCR. (C) Hepatic FASN protein expression was assessed by western blot. Quantification is shown in the right panel. (D) Hepatic adenine nucleotides were determined by HPLC. All values in the figure are presented as mean ± SEM for n=6-8; *p<0.05, **p<0.01, ***p<0.001 vs control. This figure is supplemental to Figure 3.
Figure S3. 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. Quantification is shown in the right panel. All values in the figure are presented as mean ± SEM for n=6-8; *p<0.05 vs control. This figure is supplemental to Figure 4.
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Figure S4. SCFA treatment affects WAT mass 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) WAT to body weight ratio 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-E) mRNA expression of PPARγ and target genes was assessed via qPCR in liver and adipose tissue of mice after 10 weeks on diet. All values in the figure are presented as mean ± SEM for n=6-8; *p<0.05, **p<0.01, ***p<0.001 vs control. This figure is supplemental to Figure 5.
Figure S5. SCFA treatment affects liver tissue in A-KO PPARY mice. Eight-week-old male adipose-specific PPARY knock-out mice were fed a high-fat diet mixed either with acetate, propionate or butyrate (5% w/w). (A) WAT to body weight ratio of mice 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) Liver weight of mice fed for 10 weeks. (E) Liver weight to body weight ratio of mice fed for 10 weeks. (F-G) mRNA expression of PPARY and target genes was assessed via qPCR in liver and adipose tissue of mice after 10 weeks on diet. All values in the figure are presented as mean ± SEM for n=6; ***p<0.001 vs control. This figure is supplemental to Figure 6.
### Supplemental information

**Table S1.** Primer sequences used for qPCR.

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