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ORIGINAL ARTICLE

Metabolic responses to long-term pharmacological inhibition of CB$_1$-receptor activity in mice in relation to dietary fat composition

AH Koolman[1], VW Bloks[1], MH Oosterveer[1], I Jonas[2], F Kuipers[1], PJJ Sauer[1] and G van Dijk[2,3]

[1]Department of Pediatrics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands;
[2]Unit Neuroendocrinology, Center for Behavioral Neurosciences, University of Groningen, Haren, The Netherlands and
[3]Center for Isotope Research, University of Groningen, Groningen, The Netherlands

Background and objectives: The antiobesity effects of suppressed endocannabinoid signaling may rely, at least in part, on changes in lipid fluxes. As fatty acids exert specific effects depending on their level of saturation, we hypothesized that the dietary fatty acid composition would influence the outcome of treatment with a CB$_1$-receptor antagonist (rimonabant).
Methods: Mice were treated with rimonabant (10 mg kg^{-1} body weight per day) or vehicle while equicalorically fed either a low-fat diet (LF), a high-fat (HF) diet or an HF diet in which 10% of the saturated fatty acids (SFAs) were replaced by poly-unsaturated fatty acids (PUFA) from fish oil (FO). Food intake and body weight were registered daily. Indirect calorimetry was performed and feces were collected. After 3 weeks, mice were killed for blood and tissue collection.
Results: Relative to the LF diet, the HF diet caused anticipated metabolic derangements, which were partly reversed by the HF/FO diet. The HF/FO diet, however, was most obesity-promoting despite inhibiting lipogenesis as indicated by low gene expression levels of lipogenic enzymes. On all three diets, rimonabant treatment improved metabolic derangements and led to significantly lower body weight gain than their respective controls. This latter effect appeared largest in the HF/FO group, but occurred without major changes in nutrient absorption and energy expenditure.
Conclusion: The effects of chronic rimonabant treatment on body weight gain occurred irrespective of diet-induced changes in lipogenic activity, food intake and daily energy expenditure, and were, in fact, most pronounced in HF/FO mice. The effects of dietary PUFA replacement in an HF diet on expansion of adipose tissue might allow the favorable effects of dietary PUFA on dyslipidemia and hepatic steatosis. In light of other disadvantageous effects of weight gain, this might be a risky trade-off.
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Introduction

Detailed understanding of the mechanisms involved in regulation of energy balance is crucial for treatment and prevention of obesity and associated comorbidities. The endocannabinoid system (ECS) has recently been implicated in the regulation of energy balance.[1–3] The psychoactive substance, Δ^9-tetrahydrocannabinol, as well as endogenous ligands of the ECS, anandamide and 2-arachidonoylglycerol, stimulate food intake by activation of CB$_1$-receptors.[4–7] CB$_1$-receptor-null mice are lean and resistant to diet-induced obesity.[8–11] Consistent with these findings, the CB$_1$-receptor antagonist SR141716, also known as rimonabant, was shown to be able to reduce food intake and body weight in obese humans.[12,13] In humans as well as in rodents the effects of CB$_1$-receptor antagonism on food intake are generally transient, whereas changes in body fat content upon treatment are more persistent,[7,14–16] suggesting a role for the ECS in energy fluxes independent of food intake.

In line with this are several reports to indicate that CB$_1$-receptor activity affects lipid mobilization and utilization. With respect to fat tissue, for example, Matias *et al.*[17] observed direct effects of endocannabinoids on lipid droplet formation in mouse and human adipocytes, and these effects could be prevented by CB$_1$-receptor antagonism.[2] Furthermore, Cota *et al.*[10] found that stimulation of CB$_1$-receptors of primary adipocytes increases lipoprotein lipase activity, an effect that

could be blocked by co-administration of the CB_1-receptor antagonist rimonabant. Jbilo *et al.*[18] found that treatment with this CB_1-receptor antagonist increases gene expression of enzymes involved in lipolysis and β-oxidation in white and brown adipose tissue. Herling *et al.*[19] and Osei-Hyiaman *et al.*[19,20] independently reinforced these results by showing increased rates of lipid oxidation (by indirect calorimetry) following bolus administration of rimonabant, particularly during the post-ingestive phase. Finally, CB_1-receptor antagonist in obese patients consistently improved plasma lipid profiles.[12,13,21] With respect to the liver, Osei-Hyiaman *et al.*[11] observed that feeding a high-fat (HF) diet increases hepatic levels of anandamide and CB_1-receptor density, and showed that pharmacological activation of CB_1-receptors by HU210 in mice increases hepatic gene expression of *Srebp-1c* (*srebf1*), *Acc1* (*Acaca*) and *Fasn*, all indicative of increased lipogenesis. While CB_1-receptor-null mice have reduced hepatic lipogenesis,[20] which was suggested to explain their lean phenotype, data on the effects of chronic rimonabant-treatment on markers of hepatic lipogenesis are currently lacking. Taken together, these data indicate that CB_1-receptor antagonism is a useful tool against disturbances in lipid fluxes known to be underlying, or at least associated with, the 'metabolic syndrome'.

It is generally accepted that not only the quantity of dietary fat intake, but also its quality affects lipid fluxes in the body.[22] Thus, while a high-saturated-fat diet stimulates the expression of genes encoding lipogenic enzymes in the liver, such as *Srebp-1c* and *Fasn* expression,[23] a diet with a high content of poly-unsaturated fatty acids (PUFAs) has the opposite effects.[24,25] Since changes in lipid fluxes are suggested to be, at least in part, responsible for the antiobesity effect of rimonabant treatment, it can be hypothesized that efficacy of rimonabant treatment may depend on changes in lipid fluxes resulting from differences in dietary fat composition. Up till now, relatively little attention has been paid to the role of dietary fat composition in relation to the efficacy of rimonabant treatment. Humans consume food that varies considerably in fat content and in the ratio of saturated fatty acids (SFAs) to PUFAs. In fact, because of presumed favorable effects on lipid profiles and on the risk for atherosclerosis, PUFA supplementation is popular in those at risk of obesity-associated comorbidities.

As different fatty acids have specific effects on lipid fluxes and compartmentalization of triglycerides in the body, we hypothesized that differences in dietary fatty acid composition will influence the outcome of treatment with a CB_1-receptor antagonist like rimonabant. Therefore we performed a controlled study of mice in which we analyzed the effects of dietary fatty acid composition in relation to treatment with rimonabant on obesity-associated derangements.

Materials and methods

Animals
Eight-week-old male C57BL/6J mice were obtained from Harlan (Zeist, The Netherlands) and were individually

Table 1 Fatty acid profiles of experimental diets in $mg\,g^{-1}$

	LF	HF	HF/FO
C14:0	0.5	12.2	16.1
C16:0	8.4	92.5	79.5
C16:1	0.7	11.5	18.0
C18:0	3.7	76.3	50.5
C18:1	13.7	133.2	101.0
C18:2	16.9	11.5	9.7
C18:3	1.9	2.9	15.2
C20–22	0.4	4.0	53.3
C16 desaturation index	0.1	0.1	0.2
C18 desaturation index	3.7	1.7	2.0
Ratio n-6/n-3	12.0	ND[a]	0.4
Total dietary fat content	6%	36%	36%

Abbreviations: FO, fish oil; HF, high fat; LF, low fat; ND, not detectable.
[a]The level of n-6 PUFAs in the HF diet are ND. Composition of experimental diets.

housed in a light-controlled (lights on 8 AM–8 PM) and temperature-controlled (21 °C) facility. Mice were allowed tap water and food *ad libitum*. Experimental protocols were approved by the local Experimental Ethical Committee for Animal Experiments. We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research.

Experimental diets
All experimental diets were obtained from Abdiets BV (Woerden, The Netherlands). Mice received either a low-fat (LF) diet (standard laboratory chow RMH-B 2103), an HF diet containing 36 weight % fat consisting of bovine fat (custom synthesis, diet number 4031.45) or a HF fish-oil-enriched diet (HF/FO) containing 36 weight % fat consisting for 58% of bovine fat and for 42% of fish oil (FO), (custom synthesis, diet number 4031.54). After preparing pellets, diets were stored at −20 °C. The HF/FO diet was replaced every 2 days to prevent oxidation of fatty acid species. For diet composition, see Table 1.

Experimental procedures
The effect of dietary PUFA and fat intake, the effect of treatment with a CB_1-receptor antagonist and the interaction between diet and this treatment were studied in a Latin square design. On arrival, mice were divided into three dietary groups: LF, HF and HF/FO. Mice were matched for plasma lipids, glucose and body weight. Half of the animals in each dietary group were subjected to CB_1-receptor antagonist (rimonabant) treatment. Thus, six groups of eight mice were compared in this experiment.

Intake and body weight were registered daily. Rimonabant (reference compound kindly provided by Solvay) was administered orally at a dose of approximately $10\,mg\,kg^{-1}$ body weight per day by thoroughly mixing it through the diet. Oral rimonabant treatment was started after 3 weeks on the various diets. Indirect 24-h calorimetry was performed before start of treatment with the CB_1 antagonist and

2 weeks after start of treatment. Feces were collected before start of treatment and after 18 days of treatment. After 3 weeks of oral rimonabant treatment (and thus 6 weeks after the start of the diets), treated and control mice were killed, blood and tissue were collected.

Indirect calorimetry

Mice were placed in an open-circuit, indirect calorimetry system for 24 h with access to water and food. Gas-exchange measurements were performed with an eight-channel, open-flow system. Flow rates were measured and controlled with a mass flow controller. O_2 and CO_2 concentrations of dried inlet and outlet air from each chamber were measured every 10 min with a paramagnetic O_2 analyzer and an infrared CO_2 gas analyzer. Data were collected from each metabolic cage separately. The respiratory quotient (RQ) was defined as CO_2 production (l)/O_2 consumption (l). Energy expenditure was calculated according to Brouwer[26] using the following equation: $(16.8*VO_2*0.001) + (5.02*VCO_2*0.001)$. Lipid oxidation and carbohydrate oxidation were calculated according to Lusk using the following equations:[27]

$$\text{Lipid oxidation(g/hr)}: \quad 38.461 * (VO_2(\text{mol/hr}) - VCO_2(\text{mol/hr}))$$
$$\text{Carbohydrate oxidation(g/hr)}: \quad 94.017 * VCO_2(\text{mol/hr}) - 66.239 * (VO_2(\text{mol/hr}))$$

Feces

The energy content of dried, homogenized feces was determined using a bomb calorimeter (CBB 330, standard benzoic acid $6320 \, \text{cal} \, \text{g}^{-1}$, BCS-CRM no.90N).

Plasma lipids and adipokines

Plasma lipids were measured using commercially available kits from Roche (Mannheim, Germany) for triglycerides and cholesterol. Plasma leptin, resistin and tumor necrosis factor-α levels were determined using a commercially available adipokine lincoplex kit (Linco Research, St Charles, MO, USA). Adiponectin was measured using a commercially available RIA kit (Linco Research).

Hepatic lipids

Livers were removed and freeze-clamped. Before further analysis, livers were crushed on liquid nitrogen and stored at −80 °C. For hepatic lipid extraction, frozen crushed livers were homogenized in ice-cold saline. Hepatic lipids were extracted according to Bligh and Dyer[28] and hepatic triglycerides and total cholesterol were measured using commercially available kits from Roche; free cholesterol was measured using a commercially available kit from Wako Chemicals (Neuss, Germany). Phospholipid content of the liver was determined according to Böttcher *et al.*[29]

Gene expression in liver and epididymal adipose tissue

Total RNA was isolated from frozen liver and epididymal adipose tissue using the TRI-reagent method (Sigma, Zwijndrecht, The Netherlands). Using random primers, RNA was converted to cDNA with M-MuLV Reverse Transcriptase (Sigma) according to the manufacturer's protocol. For real-time PCR, cDNA was amplified using the appropriate primers and probes. The sequences of the primers and probes for β-*actin*, *Srebp-1c (Srebf1)*, *Fasn*, *Acc1 (Acaca)* and *Acc2 (Acacb)* have been published (http://www.labpediatricsRUG.nl Realtime Primers Datalist Pediatrics UMCG). Relative gene expression levels were normalized to β-*actin* expression.

Body composition

Carcasses were eviscerated and stored at −20 °C. Carcasses and organs were dried to constant weight at 103 °C, and fat was extracted by using petroleum ether (Boom BV, Meppel, The Netherlands) in a soxlet apparatus. Percentage fat of carcasses and organs were determined from weight differences before and after the fat extraction procedure.

Statistical analysis

All values in the figures and in the tables represent means ± standard errors of the means for the number of animals indicated in the figure and table legends. To evaluate effects of diet, rimonabant treatment and their interactions, data were statistically analyzed using a general linear model with Bonferroni *post hoc* analyses. Treatment effects were further analyzed by Student's *t*-test. In case of non-parametric distribution, Mann–Whitney *U*-test was used for statistical analysis. Statistical significance of differences was accepted at a *P*-value of less than 0.05. Analyses were performed using SPSS 16.0 for Windows software (SPSS, Chicago, IL, USA).

Results

Effects of dietary fat composition on body weight gain, adiposity and food efficiency in C57BL/6J mice

Mice were matched for serum levels of glucose, triglycerides and total cholesterol, as well as for body weight at the onset of the experiment. Figure 1a shows changes in body weight relative to initial weights from the start of the experimental diets. In the control groups on all the three experimental diets, body weights increased over the course of the experiment, but most pronounced in HF/FO-fed mice. At day 22, body weights of mice fed HF/FO were significantly increased compared with that of mice fed the LF and mice fed the HF diet (Figure 1b). Data on body composition shown in Table 2 illustrate that increased body weight in HF/FO-fed mice was associated with increased adiposity in these mice as compared with that in HF-fed mice and LF-fed mice. There was a significant increase in fat mass in mice fed the HF/FO diet as compared with that in mice fed LF as well as that in mice fed the HF diet. Dietary fat composition did not affect body fat distribution as is shown in Figure 1c.

Increased weight gain in the HF/FO group could not be explained by higher food intake. In fact, mice fed the HF/FO diet had slightly reduced caloric intake; however, this trend
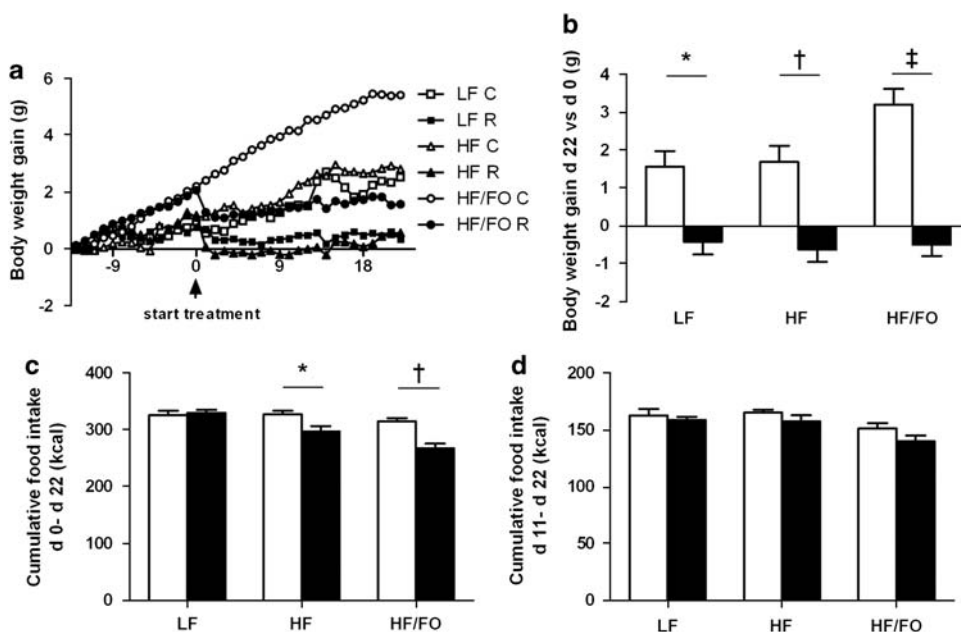
Figure 1 Body weight gain and food intake. (**a**) Body weight gain compared with body weight at the start of diet expressed in grams per day. On day 0, oral treatment with rimonabant was started as indicated by the arrow. Open squares, LF C (LF control group); black squares, LF R (LF treatment group); open triangles, HF C (HF control group); black triangles, HF R (HF treatment group); open circles, HF/FO C (HF/FO control group); black circles, HF/FO R (HF/FO treatment group). Values are means for $n = 8$ mice per group. Body weight at day 0 (means ± s.d. for $n = 16$ mice per diet group), LF group 25.6 ± 1.4, HF group 28.3 ± 2.0, HF/FO group 27.0 ± 2.2. (**b**). Body weight gain between days 0 and 22. Open bars, control groups; black bars, rimonabant treatment groups. Values are means ± s.e.m. for $n = 8$ mice per group. *, LF R versus LF C, $P = 0.002$; †, HF R versus HF C, $P = 0.001$; ‡, HF/FO R versus HF/FO C, $P = 0.000$. Trend towards significant interaction between treatment and diet, $P = 0.063$. (**c**). Cumulative intake day 0 until day 22 expressed in kilocalories. Open bars, control groups; black bars, treatment groups. Values are means ± s.e.m. for $n = 8$ mice per group, *, HF R versus HF C, $P = 0.020$; †, HF/FO R versus HF/FO C, $P = 0.000$. Significant interaction between treatment and diet, $P = 0.003$. (**d**). Cumulative intake day 11 until day 22 expressed in calories. Open bars, control groups; black bars, treatment groups. Values are means ± s.e.m. for $n = 8$ mice per group. No significant differences. FO, fish oil; HF, high fat; LF, low fat.

Table 2 Body composition of mice fed LF, HF and HF/FO diets either with or without adding a CB_1-receptor antagonist

	LF		HF		HF/FO	
	Control	Rimonabant	Control	Rimonabant	Control	Rimonabant
Body mass (g)	29.09 ± 0.62	26.05 ± 0.63§	30.56 ± 0.55	28.84 ± 0.82	33.63 ± 1.14†,‡	29.19 ± 1.38**
Lean carcass (g)	4.00 ± 0.08	4.03 ± 0.15	3.76 ± 0.04*	3.73 ± 0.06	4.21 ± 0.18 ‡	3.89 ± 0.13
Fat mass (g)	3.65 ± 0.44	2.56 ± 0.25 §	6.53 ± 0.51*	4.86 ± 0.51#	9.15 ± 0.79†,‡	6.09 ± 0.88**
Muscular fat (g)	1.07 ± 0.14	0.84 ± 0.07	1.26 ± 0.09	1.13 ± 0.14	2.32 ± 0.20†,‡	1.40 ± 0.24**
Visceral fat (g)	1.19 ± 0.11	0.81 ± 0.09 §	2.26 ± 0.17*	1.77 ± 0.14	2.84 ± 0.25†	1.99 ± 0.24**
Epididymal	0.69 ± 0.05	0.51 ± 0.04 §	1.49 ± 0.10*	1.21 ± 0.09#	1.81 ± 0.21†	1.22 ± 0.15**
Retroperitoneal	0.29 ± 0.03	0.19 ± 0.03 §	0.45 ± 0.04*	0.28 ± 0.03#	0.71 ± 0.06†,‡	0.46 ± 0.07**
Mesenteric	0.20 ± 0.05	0.12 ± 0.04	0.32 ± 0.04	0.29 ± 0.05	0.32 ± 0.09	0.31 ± 0.08
Subcutaneous fat (g)	1.33 ± 0.16	0.78 ± 0.11§	2.89 ± 0.26*	2.18 ± 0.18	3.81 ± 0.45†	2.50 ± 0.39**

Abbreviations: FO, fish oil; HF, high fat; LF, low fat. Values are mean ± s.e.m. for $n = 7$–8 mice; *, $P < 0.05$ HF control versus LF control; †, $P < 0.05$ HF/FO control versus LF control; ‡, $P < 0.05$ HF/FO control versus HF control; §, $P < 0.05$ LF control versus LF rimonabant treatment; #, $P < 0.05$ HF control versus HF rimonabant treatment; **, $P < 0.05$ HF/FO control versus HF/FO rimonabant treatment.

did not reach statistical significance (Figures 1d and e). To assess whether increased absorption could contribute to increased body weight gain in mice fed HF/FO, residual energy contents in feces collected over a period of 48 h were determined using bomb calorimetry (Figure 2a). Fecal energy excretion was significantly lower in the HF/FO control group (1.58 kcal 24 h^{-1} ± 0.45) as compared with that in the LF control group (3.11 kcal/24 h ± 0.47; $P = 0.000$) and the HF control group (2.22 kcal 24 h^{-1} ± 0.37; $P = 0.037$). Reduction

of fecal energy excretion in the HF control group as compared with that in the LF control group was also significant ($P = 0.008$). However, calculated as absorbed energy (food intake in kcal 24 h^{-1} minus residual fecal energy in kcal 24 h^{-1}), the amount of absorbed energy was not affected by diet as shown in Figure 2b.

Food efficiency (body weight gain in gram per kilocalorie intake) calculated for the treatment period is shown in Figure 2c. Food efficiency of mice in the HF/FO control

group was significantly higher as compared with that in food efficiency in the LF control group (113.7% higher in the HF/FO control group compared with levels calculated in the LF control group; $P = 0.006$) and the HF control group (99.4% higher in the HF/FO control group compared with levels calculated in the HF control group; $P = 0.010$).

Effects of dietary fat composition on lipid profile and lipogenic markers in C57BL/6J mice

Plasma concentrations of triglycerides were significantly lower in the HF/FO control group as compared with that in the LF and HF control groups (Table 3). In the HF control group as well as HF/FO control group, plasma cholesterol concentrations were significantly elevated as compared with that in the LF-fed control group. Hepatic triglycerides, hepatic total cholesterol, free cholesterol and cholesterol esters were significantly elevated in the HF group as compared with that in the LF group (Table 3). The hepatic levels of total cholesterol, free cholesterol and cholesterol esters normalized in mice fed HF/FO as compared with that in the HF group, and became indistinguishable from those in LF controls. Hepatic triglyceride levels were decreased in the HF/FO control group as compared with that in the HF

control group, but remained significantly elevated compared with that in the LF control group. Both plasma leptin and adiponectin levels were significantly increased in the HF/FO control group as compared with that in the other control groups (Table 3).

Consistent with previous data from our laboratory, hepatic expression of genes encoding lipogenic enzymes (shown in Figure 3) were significantly elevated in the HF control group as compared with that in the LF control group, and in the HF/FO group expression levels were similar to the LF control group or even lower.

Effects of dietary fat composition on RQs and oxygen consumption in C57BL/6J mice

As expected, calorimetry data showed higher RQs for mice in the LF group as compared with those in the HF group and the HF/FO group (Table 4). In the dark phase and in the light phase, oxygen consumption and calculated energy expenditure were significantly increased in HF-fed mice as compared with that in the LF control group and the HF/FO control group. There were no significant differences between the HF/FO-fed and the LF-fed mice regarding oxygen consumption or energy expenditure. There was a significant reduction in
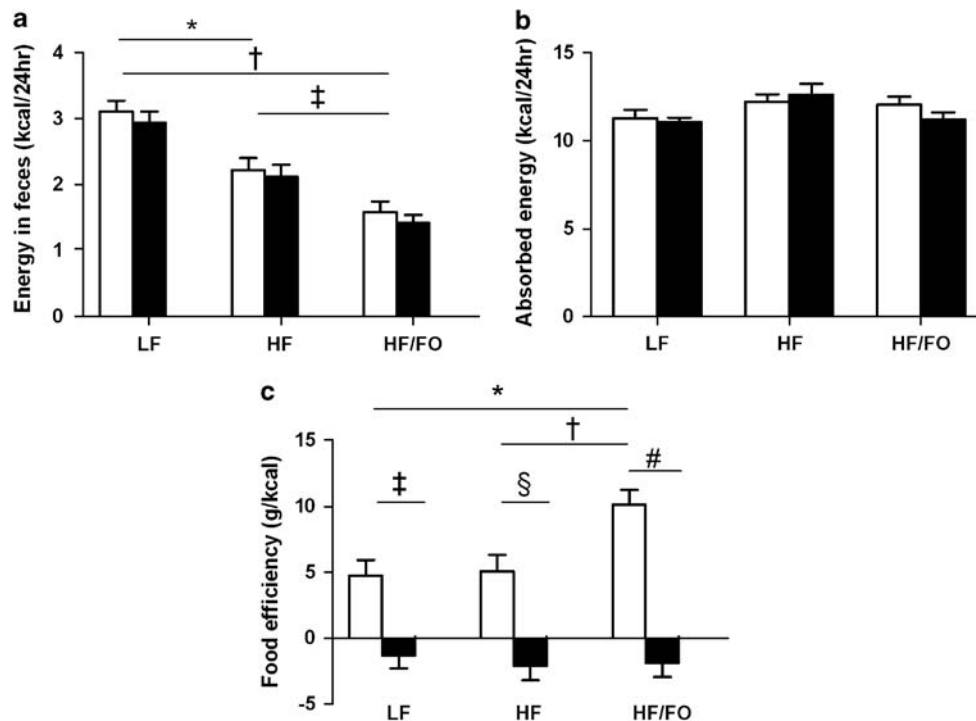

Figure 2 (**a**) Fecal energy excretion. Residual energy in feces collected during 48 h, measured by bomb calorimetry. Open bars, control groups; black bars, rimonabant treatment groups. Values are means ± s.e.m. for $n = 4$–8 mice per group. HF C versus LF C, *$P = 0.008$; HF/FO C versus LF C, †$P = 0.000$; HF C versus HF/FO C, ‡$P = 0.037$. (**b**) Energy absorption. Absorbed energy calculated from food intake in kilocalories per day minus residual fecal energy in kilocalories per day. Open bars, control groups; black bars, rimonabant treatment groups. Values are means ± s.e.m. for $n = 4$–8 mice per group. No significant differences. (**c**) Food efficiency: Food efficiency over day 0–day 22 expressed in gram body weight gain per kilocalorie intake. Open bars, control groups; black bars, treatment groups. Values are means ± s.e.m. for $n = 8$ mice per group. *, HF/FO C versus LF C, $P = 0.006$; †, HF/FO C versus HF C, $P = 0.010$; ‡, LF R versus LF C, $P = 0.002$; §, HF R versus HF C, $P = 0.001$; ‖, HF/FO R versus HF/FO C, $P = 0.000$. Significant interaction between treatment and diet, $P = 0.027$. FO, fish oil; HF, high fat; HF C, high fat control; LF, low fat; LF C, low fat control.

Table 3 Hepatic and plasma parameters in mice fed LF, HF and HF/FO diets either with or without adding a CB_1-receptor antagonist

	LF		HF		HF/FO	
	Control	Rimonabant	Control	Rimonabant	Control	Rimonabant
Plasma						
Triglycerides (mmol l^{-1})	0.92 ± 0.14	0.68 ± 0.10	1.07 ± 0.12	1.03 ± 0.17	0.70 ± 0.06	0.48 ± 0.10
Cholesterol (mmol l^{-1})	3.59 ± 0.18	3.09 ± 0.21	5.93 ± 0.26*	5.94 ± 0.19	6.16 ± 0.22†,‡	6.14 ± 0.36
Leptin (ng ml^{-1})	5.38 ± 2.59	3.27 ± 0.68	10.25 ± 1.93	9.35 ± 1.40	32.75 ± 5.17†	11.68 ± 3.44**
Resistin (ng ml^{-1})	3.43 ± 0.55	3.03 ± 0.16	5.87 ± 0.59*	6.69 ± 0.57	7.21 ± 0.72†,‡	4.13 ± 0.75**
TNF-α (pg ml^{-1})	2.51 ± 0.62	2.20 ± 0.29	1.68 ± 0.44	1.33 ± 0.27	2.75 ± 0.32	2.28 ± 0.43
Adiponectin (μg ml^{-1})	10.72 ± 0.97	12.36 ± 2.42	9.03 ± 0.35	10.04 ± 0.53	23.07 ± 2.29†	15.94 ± 1.55**
Liver						
Triglycerides (nmol mg^{-1} liver)	17.29 ± 1.30	14.61 ± 0.67	40.79 ± 0.88*	38.03 ± 1.28	24.82 ± 1.41†,‡	26.32 ± 1.99
Total cholesterol (nmol mg^{-1} of liver)	4.88 ± 0.27	5.24 ± 0.35	7.23 ± 0.21*	7.32 ± 0.29	5.07 ± 0.20‡	5.72 ± 0.14**
Free cholesterol (nmol mg^{-1} of liver)	4.35 ± 0.29	4.61 ± 0.35	5.66 ± 0.15*	5.47 ± 0.26	4.41 ± 0.14‡	4.90 ± 0.11**
Cholesterolesters (nmol mg^{-1} of liver)	0.54 ± 0.10	0.64 ± 0.13	1.57 ± 0.09*	1.86 ± 0.12	0.66 ± 0.09‡	0.82 ± 0.06
Phospholipids (nmol mg^{-1} of liver)	24.98 ± 1.10	24.40 ± 0.66	24.48 ± 0.34	25.39 ± 0.23#	26.65 ± 0.57	27.13 ± 0.43

Abbreviations: FO, fish oil; HF, high fat; LF, low fat; TNF, tumor necrosis factor. Blood samples taken on the day the mice were killed. Values are mean ± s.e.m. for $n = 6$–8 mice; *, $P < 0.05$ HF control versus LF control; †, $P < 0.05$ HF/FO control versus LF control; ‡, $P < 0.05$ HF/FO control versus HF control; §, $P < 0.05$ LF control versus LF rimonabant treatment; #, $P < 0.05$ HF control versus HF rimonabant treatment; **, $P < 0.05$ HF/FO control versus HF/FO rimonabant treatment.
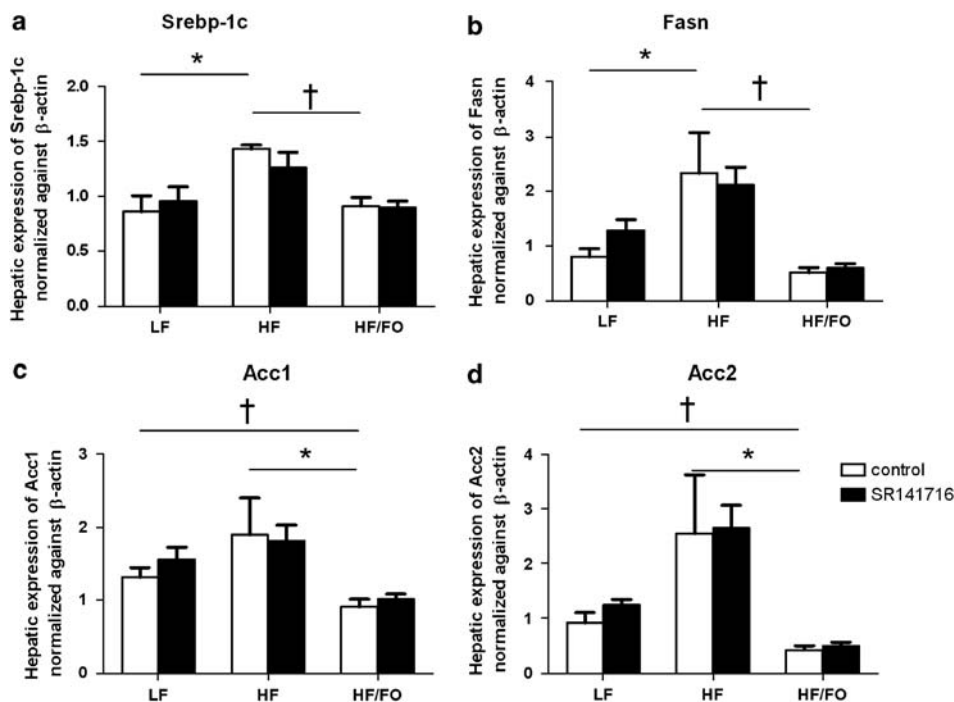

Figure 3 Hepatic lipogenic gene expression. (a). Relative hepatic mRNA expression of *Srebp-1c* (*srebf1*) in control mice (white bars) and mice treated with rimonabant (black bars) on either LF, HF or HF/FO. Results are normalized to β-*actin*. Values are means ± s.e.m. for $n = 3$–8 mice per group. HF C versus LF C, *$P = 0.027$; HF/FO C versus HF C, †$P = 0.003$. (b). Relative hepatic mRNA expression of *Fasn* in control mice (white bars) and mice treated with rimonabant (black bars). Results are normalized to β-*actin*. Values are means ± s.e.m. for $n = 3$–8 mice per group. HF C versus LF C, *$P = 0.019$; HF/FO C versus HF C, †$P = 0.001$. (c). Relative hepatic mRNA expression of *Acc1* (*Acaca*) in control mice (white bars) and mice treated with rimonabant (black bars). Results are normalized to β-*actin*. Values are means ± s.e.m. for $n = 3$–8 mice per group. HF C versus HF/FO C, *$P = 0.012$; HF/FO C versus LF C, †$P = 0.034$. (d). Relative hepatic mRNA expression of *Acc2* (*Acacb*) in control mice (white bars) and mice treated with rimonabant (black bars). Results are normalized to β-*actin*. Values are means ± s.e.m. for $n = 3$–8 mice per group. HF C versus HF/FO C, *$P = 0.007$; HF/FO C versus LF C, †$P = 0.013$. FO, fish oil; HF, high fat; HF C, high fat control; LF, low fat; LF C, low fat control.

CO_2 production in HF/FO-fed mice as compared with that in LF-fed and HF-fed mice.

Lipid oxidation was significantly increased in the HF control group as compared with that in the LF control group in the dark as well as light phase. Lipid oxidation was even more pronounced in the HF/FO control group. HF/FO fed mice had significantly elevated lipid oxidation compared with that in HF-fed mice. In the light and in the dark phase,

Table 4 Indirect calorimetry data from mice fed LF, HF and HF/FO diets either with or without adding a CB_1-receptor antagonist

	LF		HF		HF/FO	
	Control	Rimonabant	Control	Rimonabant	Control	Rimonabant
O_2 consumption (ml h^{-1})						
Dark phase	22.73 ± 0.76	22.08 ± 0.72	26.51 ± 0.54*	26.02 ± 0.39	23.04 ± 0.77‡	24.36 ± 0.27
Light phase	19.92 ± 0.74	17.82 ± 0.76	22.84 ± 0.53*	22.08 ± 0.39	19.71 ± 0.53‡	20.36 ± 0.45
CO_2 production (ml h^{-1})						
Dark phase	22.39 ± 0.86	20.29 ± 0.65	21.70 ± 0.41	20.95 ± 0.26	17.72 ± 0.62†,‡	18.47 ± 0.22
Light phase	18.32 ± 0.95	15.22 ± 0.66§	18.80 ± 0.42	17.87 ± 0.31	15.08 ± 0.41†, ‡	15.47 ± 0.34
RQ						
Dark phase	0.98 ± 0.01	0.92 ± 0.01§	0.82 ± 0.00*	0.81 ± 0.00#	0.77 ± 0.00†,‡	0.76 ± 0.00**
Light phase	0.92 ± 0.02	0.85 ± 0.01§	0.82 ± 0.00*	0.81 ± 0.00#	0.77 ± 0.00†,‡	0.76 ± 0.00
EE per gram lean mass (kJ h^{-} g^{-1})						
Dark phase	0.48 ± 0.02	0.46 ± 0.01	0.54 ± 0.01*	0.53 ± 0.01	0.46 ± 0.02‡	0.49 ± 0.01
Light phase	0.41 ± 0.02	0.36 ± 0.02	0.46 ± 0.01*	0.45 ± 0.01	0.39 ± 0.01‡	0.41 ± 0.01
Lipid oxidation (mg h^{-1})						
Dark phase	1.3 ± 1.1	10.4 ± 0.9§	28.1 ± 1.0*	29.4 ± 0.7	35.2 ± 0.8†,‡	36.1 ± 1.3
Light phase	9.2 ± 1.9	15.8 ± 0.8§	23.6 ± 0.6*	24.4 ± 0.6	30.8 ± 0.9†,‡	30.0 ± 0.8
Carbohydrate oxidation (mg h^{-1})						
Dark phase	101.5 ± 4.2	76.6 ± 4.3§	46.4 ± 1.7*	40.2 ± 1.2#	26.4 ± 1.3†,‡	21.7 ± 1.4**
Light phase	68.9 ± 6.8	43.4 ± 2.9§	41.5 ± 1.0*	35.5 ± 0.9#	21.4 ± 1.5†,‡	18.5 ± 1.2

Abbreviations: EE, energy expenditure; FO, fish oil; HF, high fat; LF, low fat; RQ, respiratory quotient. Values are mean ± s.e.m. for $n = 7–8$ mice; *, $P < 0.05$ HF control versus LF control; †, $P < 0.05$ HF/FO control versus LF control; ‡, $P < 0.05$ HF/FO control versus HF control; §, $P < 0.05$ LF control versus LF rimonabant treatment; #, $P < 0.05$ HF control versus HF rimonabant treatment; **, $P < 0.05$ HF/FO control versus HF/FO rimonabant treatment.

HF/FO-fed mice had significantly decreased carbohydrate oxidation compared with both the HF control group and the LF control group. In HF-fed mice carbohydrate oxidation was also significantly decreased in the light phase and in the dark phase as compared with that in the LF control group.

Effects of rimonabant treatment on body weight and body fat mass in C57BL/6J mice on different diets
Treatment with rimonabant decreased body weight in all three diet groups in the first week of treatment as compared with the respective controls (Figure 1a). Approximately 1 week after start of treatment, mice in the treatment groups gained weight again. Yet, throughout the experiment, body weights of mice in the treatment groups remained significantly lower than that in the control groups. At day 22, on all three diets, mice treated with rimonabant had gained significantly less weight than non-treated mice (Figure 1b). The reduction in body weight gain in treated mice appeared to be most prominent in the HF/FO treatment group as compared with that in the HF/FO control group. This was not caused by diminished weight gain in the treated mice fed HF/FO compared with treated mice fed LF or HF, but rather by a higher weight gain in the untreated HF/FO group as compared with that in the LF control group and the HF control group.

The effect of rimonabant treatment on body weight is reflected by changes in adiposity (Table 2). This effect was most prominent in mice fed the HF/FO diet. The reduction in fat mass in all three treatment groups is due to reduction of both abdominal fat mass and subcutaneous fat mass. Treatment with rimonabant did not have any significant effect on lean mass of mice in the three diet groups as compared with their controls.

Effects of rimonabant treatment on lipid profile and hepatic lipogenic gene expression in C57BL/6J mice on different diets
Treatment with rimonabant did not affect plasma triglyceride and plasma cholesterol (Table 3). Hepatic triglyceride concentrations were also not affected by treatment with rimonabant. In the HF/FO-fed mice treated with rimonabant, plasma levels of leptin and adiponectin were decreased as compared with those in the respective controls. There was a significant interaction between treatment and diet in leptin levels ($P = 0.002$) as well as in adiponectin levels ($P = 0.013$), meaning that *post hoc* analysis revealed no treatment effect in LF-fed and in HF-fed mice, whereas rimonabant treatment significantly reduced plasma levels of leptin and adiponectin in HF/FO-fed mice.

Notably, we did not observe any decrease in hepatic lipogenic gene expression in the rimonabant-treated groups as compared with the respective control groups.

Effects of rimonabant treatment on food intake and 'food efficiency' and fecal energy excretion in C57BL/6J mice on different diets
We observed no effect of administering CB_1-receptor antagonist on food intake in the LF-fed treatment group

as compared with the LF-fed control group. However, after start of treatment, food intake was significantly lower in the HF treatment group and the HF/FO treatment group. The effects on food intake diminished during the first week of treatment. At day 4, there was no difference in food intake between the HF treatment group and the HF control group anymore, and after 10 days the difference in food intake had also disappeared between the HF/FO treatment group and the HF/FO control group (data not shown). The cumulative caloric intake calculated for the treatment period was significantly reduced in the HF/FO treatment group as compared with that in the HF/FO control group (HF/FO treatment versus HF/FO control $P<0.001$), and in the HF treatment group compared with that in the HF control group (HF treatment versus HF control, $P=0.02$), as is illustrated in Figure 1c. However, there were no significant differences in cumulative caloric intake calculated from day 11 until day 22 (Figure 1d), illustrating the observation that the effect of treatment with a CB_1-receptor antagonist on food intake is transient.

To assess if decreased absorption could contribute to decreased weight gain in treated mice, we measured residual energy contents in feces by bomb calorimetric analysis of feces collected over a period of 48 h. Treatment with CB_1-receptor antagonist had no significant effect on fecal energy excretion on either diet (Figure 2a) and calculated as absorbed energy (food intake in kcal $24 h^{-1}$ minus residual fecal energy in kcal $24 h^{-1}$) the amount of absorbed energy was not affected by treatment (Figure 2b).

On all three diets, rimonabant treatment led to significant reduction in food efficiency (Figure 2c; LF treatment versus LF control, $P=0.002$; HF treatment versus HF control, $P=0.001$; HF/FO treatment versus HF/FO control, $P<0.001$). There was a significant interaction between diet and treatment ($P=0.027$). Indeed, effect of treatment was most prominent in the HF/FO treatment group as compared with that in the HF/FO control group. Again, this was a consequence of higher food efficiency in the HF/FO control group rather than of lower food efficiency in the HF/FO treatment group.

Effects of rimonabant treatment on RQ and oxygen consumption in C57BL/6J mice on different diets
Table 4 shows that treatment with rimonabant significantly reduced the RQ during both the light phase and the dark phase in LF-fed mice (light-phase LF treatment versus LF control, $P=0.004$, dark-phase LF treatment versus LF control $P<0.001$) and in HF-fed mice (light-phase HF treatment versus HF control, $P=0.003$, dark-phase HF treatment versus HF control, $P=0.033$). In the HF/FO treatment group, a significant reduction in RQ as compared with that in the HF/FO control group was only observed in the dark phase (HF/FO treatment versus HF/FO control, $P=0.011$). A significant increase in lipid oxidation and a significant decrease in carbohydrate oxidation were observed in LF-fed mice upon rimonabant treatment in the light phase as well as in the dark phase. There was no treatment effect on lipid

oxidation in the HF groups or in the HF/FO groups. Yet, there was a significant decrease in carbohydrate oxidation in the HF treatment group as compared with that in the HF control group in the light phase and in the dark phase, and in the HF/FO treatment group compared with that in the HF/FO control group only in the dark phase.

Rimonabant treatment did not affect oxygen consumption or energy expenditure as calculated from indirect calorimetry data, neither in the light phase nor in the dark phase, in any of the groups (Table 4).

Discussion

In the present study, we compared the effects of treatment with rimonabant, a selective endocannabinoid CB_1-receptor antagonist, on food intake and on hormonal and metabolic characteristics, in young adult mice fed diets with differences in the total amount and ratio of SFAs versus PUFAs. Despite notable differences in hormonal and metabolic profiles among mice fed the different experimental diets, we found that rimonabant treatment prevented weight gain and improved metabolic derangements, without major differences in energy intake and expenditure. This highlights a role for the ECS in triglyceride deposition in adipose tissue as well as in the liver irrespective of dietary fat content or composition.

Compared with mice fed a fibered LF, carbohydrate-rich diet, feeding a diet with HF content caused increase in body fat mass and hyperleptinemia, and a massive increase in hepatic triglyceride content in mice. These well-known derangements are major risk factors for development of hepatosteatitis and type-2 diabetes mellitus.[30–32] The increased hepatic fat deposition—being a hallmark of the metabolic syndrome—appeared to be abolished when mice were fed a diet with a fat content similar as the aforementioned HF diet, but in which 10% of the SFA were replaced by PUFA derived from FO (HF/FO diet). We observed, however, that lipid deposition inside adipose tissue as well as plasma leptin levels were greatly augmented in HF/FO-fed mice as compared with that in those fed the HF diet.

The finding that a 10% replacement of SFA by PUFA caused reduction of hepatic triglyceride deposition almost back to the level observed in the LF-fed mice fits well with the documented effects of PUFA to stimulate hepatic lipid utilization,[33] and to reduce hepatic lipogenesis.[24] Indeed, mice in the present study fed the HF/FO diet showed reduced hepatic gene expression levels of lipogenic enzymes *Srebp-1c*, *Acc1, Acc2* and *Fasn* compared with those observed in the HF group. In addition, the rate of lipid oxidation assessed by indirect calorimetry was clearly highest in the HF/FO group. For these reasons, it is counterintuitive that ingestion of an HF/FO-diet potentiated the level of body adiposity well above the levels observed in the HF and LF group. Mice fed the HF/FO diet showed significantly elevated plasma levels of adiponectin, which could have, in part, prevented fat

deposition in extra-adipose tissues, and at the same time, mediated the increased expansion of adipose tissue in this group. Such an effect has been proposed by Kim et al.[34] who showed that overexpression of adiponectin leading to elevated plasma adiponectin levels, also led to increased adipose tissue mass. Like in our study, the increased plasma adiponectin levels together with expanded fat mass were associated with normalization of hepatic and plasma lipid profiles. Another issue relevant to the beneficial effect of the HF/FO diet on metabolic endpoints while enhancing fat mass (that is, relative to those observed in the HF diet condition), is the observation that the HF/FO diet seems to augment, percent-wise, the subcutaneous fat depot (subcutaneous adipose tissue) more than the visceral fat depot (visceral adipose tissue) as compared with the HF diet, and with respect to the corresponding values at day 22 in the LF diet. While both diets augment subcutaneous adipose tissue more than visceral adipose tissue, the difference is approximately 20% higher with the HF/FO diet. In view of the well-established strong connections between visceral adipose tissue and some metabolic disorders,[35] this perhaps may have contributed to the higher adiponectin levels (despite the stronger increase in body weight) and also the beneficial effects on hepatic lipogenesis observed with the HF/FO diet.

The exaggerated expansion of adipose tissue of HF/FO-fed mice as compared with the other groups could not be attributed to an increase in food intake or to an increased gastrointestinal absorption rate, which indicates that these animals were more 'food efficient' than those fed the HF and LF diet. Indeed, calculation of body weight gain per absorbed energy content revealed a doubling of food efficiency in the HF/FO group as compared with that in the HF and LF groups. Food efficiency depends on energy expenditure (EE), which in turn is comprised of resting metabolic rate, activity thermogenesis, and thermic effects of food. Resting metabolic rate comprises thermogenesis, detoxification, maintenance of membrane potentials and tissue differentiation and tissue maintenance. EE during the light and the dark phase and resting metabolic rate calculated from indirect calorimetry were indeed lower in the HF/FO group as compared with that in the HF group, which may have underlied the increased food efficiency and adipose tissue expansion in the HF/FO group relative to the HF group. Compared with LF-fed mice, however, this lowering of EE and resting metabolic rate in the HF/FO group was not significant. This discrepancy of increased food efficiency and weight gain in the HF/FO group versus the LF group without alterations in EE may be explained by a shift between the various components of energy expenditure without affecting total energy expenditure. For example, a decrease in the thermic effects of food could make more energy available for storage and tissue expansion. We are currently performing experiments to specifically address these possibilities.

As pointed out above, rimonabant treatment prevented weight gain and ameliorated adiposity in all three experimental diet groups despite marked differences in adiposity and metabolic profiles between the groups. After start of treatment with the CB_1-receptor antagonist, there was a slight and temporal reduction of food intake in the HF and HF/FO group, which explains the reduction in body weight by rimonabant-treatment only to a limited degree. Since total absorbed energy (as assessed by bomb calorimetry of feces) was not different among groups, this indicates that body weight loss induced by treatment with rimonabant was mediated by changes in metabolism. In fact, rimonabant treatment rendered mice remarkably food inefficient, such that the diet-induced differences in food efficiency were entirely lost upon rimonabant treatment. However, the effects of rimonabant could not be explained by differences in metabolic rate since EE measured by indirect calorimetry was similar in treated versus non-treated mice in the respective diet groups. It is tempting to speculate that metabolic coupling was affected by rimonabant treatment in the different diet groups, which contributed to prevent weight gain. While efficiency of metabolic coupling as a potential mechanism has to be addressed, our results regarding discrepancies between weight loss by blockade of CB_1 signaling, without changes in energy intake and expenditure corresponds with findings in several other recent studies.[10,36] Reports that, in contrast to these results, do demonstrate an increase in EE in animals and humans treated with a CB_1-receptor antagonist[20,37–39] frequently employ an acute rather than continuous treatment. Kunz et al.[37] clearly demonstrated rapid development of tolerance to rimonabant towards induction of oxygen consumption, stressing differences in effects of acute versus chronic treatment. Moreover, investigators frequently normalize EE and oxygen consumption to total body weight, thereby ignoring fat-free versus fat mass, which causes an over-estimation of EE when fat mass is lost without changes in lean mass.[20,38]

The effect of CB_1-receptor antagonism to prevent weight gain and to ameliorate adiposity levels in the LF, HF as well as HF/FO-fed mice corresponds with other studies showing treatment effects irrespective of diet composition.[14–16,18,21] It is, however, rather surprising in light of the findings by others showing that HF diets given to mice cause dysregulation of the ECS, either via alterations in the expression of CB_1 receptors, via alterations in the levels of endogenous ligands, or both.[17,40–42] Also the fatty acid composition of dietary fat may influence endocannabinoid action.[43] For example, the presence or absence of n-3 PUFAs in the diet affects endocannabinoid levels in the brain, as shown by Berger et al.[44] and Watanabe et al.[45] Batetta et al.[46] recently found that dietary n-3 PUFAs can reduce inflammatory markers and liver triglyceride levels, and these effects were associated with lower levels of endocannabinoid ligands in peripheral organs. Finally, there is an in vitro study showing that n-3 PUFA can reduce the levels of both anandamide and 2-AG in differentiated mouse adipocytes, whereas n-6 PUFAs (that is, arachidonic acid) were found to increase endocannabinoid levels.[47] The efficacy of an antagonist such as rimonabant

should depend on the extent of tonic activation of CB_1 receptors by, among other things, endocannabinoids. Since n-6 PUFA content is relatively high in FO (that is, with a ratio of n-6/n-3 of 0.4 in the HF/FO diet, and no n-6 at all in the HF diet), this may provide an explanation for the finding that rimonabant is more efficacious in mice fed the HF/FO diet to induce weight loss in the present study.

Associated with a reversal of HF-diet-induced obesity by rimonabant, studies generally reported lowering of hepatic triglyceride accumulation and plasma leptin levels, and increased plasma adiponectin levels by rimonabant treatment.[11,15,18,21] The reversal of these comorbidities by rimonabant was not observed in the present study. It is possible that the discrepancy between the results of our study and others is due to differences in the age of mice and experimental duration. In our study, endpoint measurements were performed at 14 weeks of age, whereas in all other studies mice used were 6 months or older, and mice in those studies were subjected to experimental diets for months.[11,15,18,21] Nevertheless, we did find profound inhibitory effects of the HF/FO diet on markers for hepatic lipogenesis relative to the HF diet condition. It was previously suggested by Osei-Hyiaman et al.[11] that HF-diet-induced obesity was a consequence of increased lipogenesis (by increased hepatic Srebp-1c expression), with a permissive role for hepatic CB_1 signaling in these effects. This is in contrast with our results that showed reduction of body weight gain in mice treated with rimonabant on all three diets, and, in fact, reduction of body weight gain was most pronounced in treated mice on the HF/FO diet in which lipogenesis in liver was suppressed. Thus, our results suggest that suppression of hepatic lipogenesis does not play a role in the effect of rimonabant on body weight, at least in young adult mice. This fits with the most recent observation of Osei-Hyiaman et al.[20] that liver-specific $CB_1^{-/-}$ mice are not resistant to diet-induced obesity, whereas total $CB_1^{-/-}$ mice are.

In conclusion, our results show that treatment with a CB_1-receptor antagonist is not only effective in reducing body weight gain in mice fed an HF diet based on SFAs, but that a significant reduction in body weight is achieved irrespective of dietary fat quality and quantity. Furthermore, the effect of chronic rimonabant treatment to limit body weight gain occurs independent of basic lipogenic activity and, in fact, appears to be most pronounced in mice fed a diet with a mixed SFA/PUFA composition, by which lipogenic activity was lowest. Finally, we demonstrated that SFA replacement in an HF diet by PUFAs not only results in improvement of HF-diet-induced metabolic derangements, but also in augmentation of adipose tissue stores. While the effects of dietary PUFA replacement in an HF diet on expansion of adipose tissue might allow the favorable effects of dietary PUFAs on dyslipidemia and hepatic steatosis, this might be a risky trade-off in light of other disadvantageous effects of weight gain. Thus, if FO supplements are used to prevent the adverse metabolic effects of a Western-type diet in children

and adolescents, based on the findings in the present study, the outcome will be an exacerbation of the development of obesity in young people. If other preventive measures (for example, exercise programs, avoiding Western-type diets, and so on) turn out to be fruitless, improvement of pharmacotherapy might remain the last resort for the treatment of obesity at exceedingly young age.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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