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ARTICLE

n-3 Fatty acids and rosiglitazone improve insulin sensitivity through additive stimulatory effects on muscle glycogen synthesis in mice fed a high-fat diet

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Abstract
Aims/hypothesis Fatty acids of marine origin, i.e. docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) act as hypolipidaemics, but they do not improve glycaemic control in obese and diabetic patients. Thiazolidinediones like rosiglitazone are specific activators of peroxisome proliferator-activated receptor γ, which improve whole-body insulin sensitivity. We hypothesised that a combined treatment with a DHA and EPA concentrate (DHA/EPA) and rosiglitazone would correct, by complementary additive mechanisms, impairments of lipid and glucose homeostasis in obesity.

Methods Male C57BL/6 mice were fed a corn oil-based high-fat diet. The effects of DHA/EPA (replacing 15% dietary lipids), rosiglitazone (10 mg/kg diet) or a combination of both on body weight, adiposity, metabolic markers and adiponectin in plasma, as well as on liver and muscle gene expression and metabolism were analysed. Euglycaemic–hyperinsulinaemic clamps were used to characterise the changes in insulin sensitivity. The effects of the treatments were also analysed in dietary obese mice with impaired glucose tolerance (IGT).
Results DHA/EPA and rosiglitazone exerted additive effects in prevention of obesity, adipocyte hypertrophy,

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low-grade adipose tissue inflammation, dyslipidaemia and insulin resistance, while inducing adiponectin, suppressing hepatic lipogenesis and decreasing muscle ceramide concentration. The improvement in glucose tolerance reflected a synergistic stimulatory effect of the combined treatment on muscle glycogen synthesis and its sensitivity to insulin. The combination treatment also reversed dietary obesity, dyslipidaemia and IGT.

Conclusions/interpretation DHA/EPA and rosiglitazone can be used as complementary therapies to counteract dyslipidaemia and insulin resistance. The combination treatment may reduce dose requirements and hence the incidence of adverse side effects of thiazolidinedione therapy.

Keywords Adiponectin · Diabetes · Docosahexaenoic acid · Eicosapentaenoic acid · Fish oil · Obesity · PPAR · Rosiglitazone · Thiazolidinedione

Abbreviations

Akt/PKB	Protein kinase B
cHF	Corn oil-based high-fat diet
cHF+F	cHF diet supplemented with eicosapentaenoic and docosahexaenoic acids
cHF+F+TZD	cHF diet supplemented with eicosapentaenoic and docosahexaenoic acids and rosiglitazone
cHF+TZD	cHF diet supplemented with rosiglitazone
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
HMW	High molecular weight
IGT	Impaired glucose tolerance
n-3 LC-PUFA	*n*-3 Long-chain polyunsaturated fatty acids
PPAR	Peroxisome proliferator-activated receptor
TZD	Thiazolidinedione

Introduction

Both dietary and pharmacological interventions are required for therapy of type 2 diabetic patients. Naturally occurring *n*-3 long-chain polyunsaturated fatty acids (LC-PUFA), namely eicosapentaenoic acid (EPA; 20:5 *n*-3) and docosahexaenoic acid (DHA; 22:6 *n*-3), which are abundant in sea fish, act as hypolipidaemics, reduce cardiac events and decrease progression of atherosclerosis, as reviewed by Ruxton et al. [1]. Therefore, *n*-3 LC-PUFA are now regarded as healthy constituents of diets for diabetic patients [2, 3]. Several studies in obese humans even demonstrated reductions of adiposity after *n*-3 LC-PUFA supplementation [4, 5]. However, *n*-3 LC-PUFA appear to have little effect on glycaemic control in diabetic patients [4, 6, 7]. In rodents fed a high-fat diet, *n*-3 LC-PUFA efficiently prevented development of obesity [8–10] and of impaired glucose tolerance (IGT) [11, 12].

The effects of *n*-3 LC-PUFA are largely mediated by peroxisome proliferator-activated receptors (PPAR), with PPAR-α and PPAR-δ (-β) representing the main targets [13]. However, PPAR-γ, liver X receptor-α, hepatic nuclear factor-4 and sterol regulatory element binding protein-1 are also involved [12, 14, 15]. Besides acting directly as regulatory ligands, *n*-3 LC-PUFA also act through their active metabolites, eicosanoids and other lipid molecules [16]. The hypolipidaemic and anti-obesity effects of *n*-3 LC-PUFA probably depend on the in situ suppression of lipogenesis and increase of fatty acid oxidation in several tissues [10, 14, 17]. This metabolic switch might reduce accumulation of toxic fatty acid-derivatives, while protecting insulin signalling in liver and muscle [11, 12, 18].

On the other hand, PPAR-γ ligands [19] such as thiazolidinediones (TZDs) are the preferred therapeutic agents for insulin resistance in type 2 diabetic patients. However, TZDs like rosiglitazone and pioglitazone are also associated with unwanted side effects, such as oedema and weight gain [20], possible risk of heart failure [21] and bone loss [22]. These compounds are likely to improve glycaemic control mostly by repartitioning fat away from skeletal muscle [23], while augmenting insulin action in various tissues [24–29].

In spite of the differences in the ability of TZDs and *n*-3 LC-PUFA to reverse IGT, both types of compounds reduce adipose tissue inflammation [24, 30], one of the key factors contributing to the development of insulin resistance in obesity, while also increasing adiponectin [12, 15, 24, 25, 31, 32].

We hypothesised that partially overlapping mechanisms of action of *n*-3 LC-PUFA and TZDs, namely the activation of metabolic switch by *n*-3 LC-PUFA and the induction of lipid repartitioning by TZDs, could have synergistic effects in a combination treatment, leading to an improvement of glycaemic profile so far not described for these strategies when considered separately. We therefore evaluated the effects of: (1) partial replacement of dietary lipids by *n*-3 LC-PUFA; (2) a low non obesogenic dose of rosiglitazone; and (3) a combination of both on whole-body parameters of glucose and lipid metabolism in mice fed high-fat diet. To further elucidate the effects of the treatments, a detailed analysis of liver and muscle metabolism was also performed.

Methods

Animals and treatments Male mice were maintained (two animals per cage) at 22°C on a 12 h light–dark cycle (light on from 06:00 hours) and allowed free access to standard laboratory chow (lipid content ~3.4% wt/wt; extruded R/M-H diet; Ssniff Spezialdiäten, Soest, Germany). Two types of studies were used.

First, a 'prevention study' was performed to characterise the effects of *n*-3 LC-PUFA, rosiglitazone and their combination, on developing obesity and IGT in mice fed high-fat diet. At 3 months of age, male C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) were randomly assigned to a corn oil-based high-fat diet (cHF; lipid content ~35.2% wt/wt, mainly corn oil) or to the following treatments: (1) cHF diet supplemented with EPA and DHA (cHF+F) as concentrate of *n*-3 LC-PUFA (46% DHA, 14% EPA; 1050TG; EPAX, Lysaker, Norway) replacing 15% of dietary lipids; (2) cHF diet supplemented with rosiglitazone (cHF+TZD) (10 mg/kg diet); and (3) cHF diet supplemented with EPA, DHA and rosiglitazone (cHF+F+TZD) (see Electronic supplementary material [ESM] Tables 1 and 2). Some mice were maintained on the standard chow diet. Various analyses (described below) were performed at 5 to 20 weeks after initiation of treatment. Mice were killed under anaesthesia (09:00–11:00 hours) by cervical dislocation in ad libitum fed state, unless stated otherwise.

Second, in a 'reversal study', obesity and IGT were induced by feeding male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) the cHF diet between 3 and 7 months of age, prior to the subsequent 8-week-long treatment as above; i.e. with cHF, cHF+F, cHF+TZD and cHF+F+TZD, respectively.

During treatments, fresh rations of food were distributed daily. Food consumption and body weights were recorded once a week. Experiments were conducted under the guidelines for the use and care of laboratory animals of the Institute of Physiology and followed the 'Principles of laboratory animal care' (NIH publication no. 85-23, revised 1985).

Metabolites, hormones and enzymes Non-esterified fatty acids, triacylglycerols, total cholesterol and insulin were determined in EDTA-plasma [9]. Blood glucose was measured by calibrated glucometers (LifeScan, Milpitas, CA, USA). Total adiponectin was measured by ELISA [31] (R&D Systems, Abingdon, UK). Distribution of adiponectin multimeric complexes was determined using western blotting [33].

Body composition See ESM for details.

Tissue lipid content Liver and muscle triacylglycerol content was estimated in ethanolic KOH tissue solubilisates [32]. Ceramide content was estimated in lipid extracts from soleus muscle homogenates using HPLC [34].

Quantitative RT-PCR-based gene expression analysis Levels of various transcripts were evaluated in total RNA isolated from liver or gastrocnemius muscle [10]. For primers, see ESM Table 3.

Glycogen synthesis in diaphragm Left and right hemidiaphragms were dissected and separately incubated ex vivo to measure incorporation of glucose into glycogen, as previously described [35] (see ESM).

In vivo hepatic VLDL-triacylglycerol production The rate of liver triacylglycerol synthesis was evaluated using Triton WR1339 [36] and calculated from the slope of the curve of plasma triacylglycerol levels [37] (see ESM).

Light microscopy and immunohistochemical analysis Epididymal fat samples were processed to detect MAC-2/galectin and perilipin, using specific antibodies [32, 38] (see ESM).

Glucose tolerance test Intraperitoneal glucose tolerance test was performed in fasted mice as described [32].

Euglycaemic–hyperinsulinaemic clamp This was performed as described (ESM) [39–41].

Activation of protein kinase B Phosphorylation of protein kinase B (Akt/PKB) was measured in soleus muscles using western blots (ESM Fig. 1).

Statistics Data were analysed by ANOVA and *t* test, as described in ESM. All values are presented as means±SE. Comparisons were judged to be significant at $p \leq 0.05$.

Results

Prevention of body weight gain and lipid accumulation Body weight was increased by cHF diet with significant differences between chow and cHF becoming apparent at 4 weeks (Fig. 1a). Treatment by cHF+F or by cHF+TZD diets tended to prevent body weight gain (Fig. 1a). The combination treatment, cHF+F+TZD, reduced body weight gain significantly (Fig. 1a). The same pattern of body weight gain continued throughout the duration of the study, which lasted for 20 weeks. None of the diets affected mean food consumption over the course of the study (Fig. 1b). Body composition was analysed at 15 weeks (Fig. 1c), i.e. when the effect of cHF+F+TZD diet on

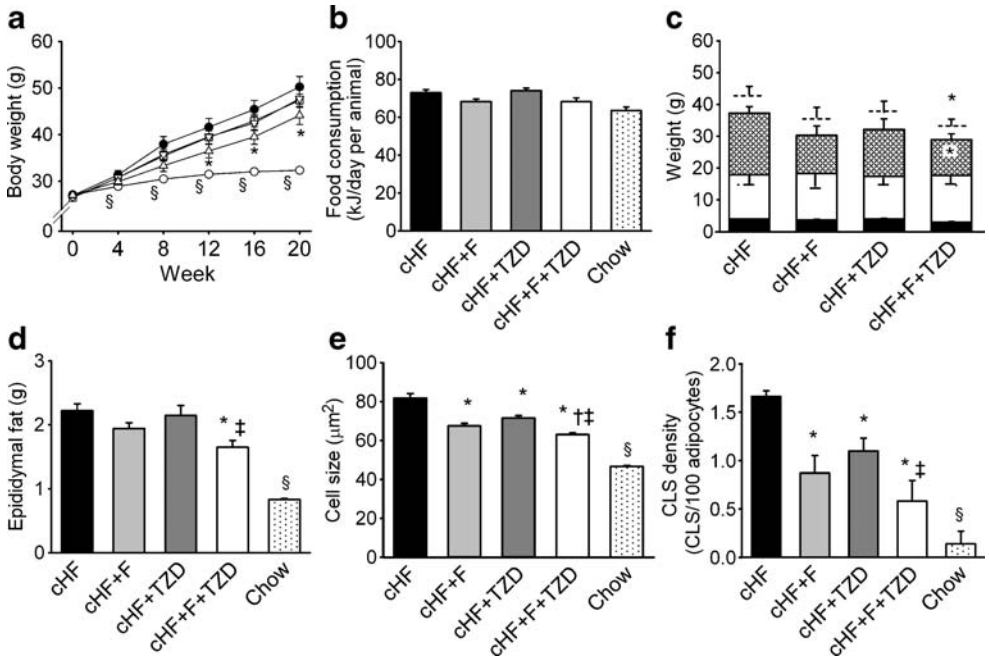

Fig. 1 Body weight (**a**), food consumption (**b**), body composition and size of adipocytes (**c–e**), and macrophage infiltration of adipose tissue (**f**). Three-month-old mice were placed on cHF diet or various cHF-based diets (cHF+F, cHF+TZD and cHF+F+TZD), or maintained on a chow diet; this treatment lasted for up to 20 weeks. **a** Body weights during 20-week treatment by cHF (black circles), cHF+F (white squares), cHF+TZD (white inverted triangles), cHF+F+TZD (white triangles) or chow (white circles) diet (n=16). **b** Mean food consumption during 20 week treatment (n=8). **c** Body composition at 15 weeks. Bar height, weight of eviscerated carcass; black section, protein; white + black sections, lean body mass; cross-hatched section, fat; dashed horizontal lines above bars, body weight of mice before killing. Error bars, SE (n=5–6). **d** Epididymal fat at 8 weeks, weight of fat depot. **e** Size of adipocytes. **f** Relative count of crown-like structures (CLS), formed by MAC-2-positive macrophages surrounding adipocytes. The morphometry data are based on measurements of more than 1,000 cells taken randomly from six different areas per animal. **d–f** Data are means±SE (n=7–8). *$p\leq0.05$ for difference from cHF (ANOVA); $^{\dagger}p\leq0.05$ for difference from cHF+F (ANOVA); $^{\ddagger}p\leq0.05$ for difference from cHF+TZD (ANOVA); $^{\S}p\leq0.05$ for difference from cHF (t test)

body weight became significant (Fig. 1a). Protein and lean body mass were similar in mice fed various cHF diets. On the other hand, total weight of eviscerated carcass and total lipid content reflected changes in body weight. Changes in body weight correlated with the changes in adipose tissue mass, with a significant reduction of the content of subcutaneous (not shown) and epididymal (Fig. 1d) fat in the animals treated for 8 weeks by cHF+F+TZD. Histological analysis combined with immunodetection of macrophages revealed cHF diet-induced hypertrophy of adipocytes in epididymal fat at 8 weeks (Fig. 1e), accompanied by increased content of macrophages aggregated in crown-like structures surrounding individual adipocytes (Fig. 1f), indicating induction of low-grade inflammation of adipose tissue in cHF-mice [38]. The induction of adipocyte hypertrophy and macrophage infiltration was significantly counteracted by cHF+F and cHF+TZD diets, with cHF+F+TZD diet exerting the strongest effect.

Indirect calorimetry at 8 weeks did not reveal any effect of the treatments on whole-body oxygen consumption or respiratory exchange ratio in mice with free access to their respective diets. No differences in physical activity between the groups were detected (not shown).

Given the induction of weight gain by rosiglitazone in previous studies on rodents, the induction of a lean phenotype by the cHF+TZD, and especially by the cHF+F+TZD diets was surprising and is, perhaps, related to higher doses of rosiglitazone used in other studies [24, 27]. Indeed, when rosiglitazone content in the diet was increased tenfold (i.e. from 10 to 100 mg rosiglitazone per kg diet), this increase was associated with higher body weight gain as early as 4 weeks after initiation of the feeding (26.3± 0.8 g vs 28.5±0.5 g; n=8; p=0.04).

Compared with standard chow, the cHF diet induced accumulation of triacylglycerols in liver and gastrocnemius, a mixed-fibre muscle, with a stronger effect observed at 20 than at 8 weeks (Table 1). Compared with cHF, none of the treatments significantly affected the triacylglycerol accumulation, except for cHF+TZD, which increased liver triacylglycerols at 8 weeks. The cHF+F+TZD diet also increased liver triacylglycerol content at 8 weeks, albeit to a smaller extent (Table 1). No differences in the triacylglycerol content between subgroups were observed in soleus,

Table 1 Lipid accumulation in liver and skeletal muscle

Tissue	cHF	cHF+F	cHF+TZD	cHF+F+TZD	Chow
Liver triacylglycerol (mg/g)					
8 weeks	80±15	70±8	197±23[ab]	123±15[b]	34±3[d]
20 weeks	159±16	153±11	171±10	153±15	36±2[d]
Gastrocnemius muscle triacylglycerol (mg/g)					
8 weeks	39±4	41±5	41±6	37±5	18±2[d]
20 weeks	56±3	61±3	50±4	60±4	25±3[d]
Soleus muscle triacylglycerol (mg/g)					
20 weeks	47±5	51±3	48±7	50±2	34±3
Soleus muscle ceramide (pmol/g)					
20 weeks	17.9±0.8	16.0±0.6	17.2±0.5	14.2±0.3[ac]	16.6±0.5

Data are means±SE (n=7–8)

Mice (3 months old) were placed on various diets and killed in ad libitum fed state, after 8 or 20 weeks on the diets.

[a]$p \leq 0.05$ for difference from cHF (ANOVA); [b]$p \leq 0.05$ for difference from cHF+F (ANOVA); [c]$p \leq 0.05$ for difference from cHF+TZD (ANOVA); [d]$p \leq 0.05$ for difference from cHF (t test)

an oxidative muscle, after the prolonged treatment. However, the cHF+F+TZD diet significantly lowered ceramide content in the soleus muscle as compared with cHF-fed mice (Table 1).

Prevention of dyslipidaemia and reduction of hepatic VLDL-triacylglycerol synthesis In ad libitum fed mice, triacylglycerol levels increased while NEFA and cholesterol levels remained unchanged between 8 and 20 weeks of cHF feeding (Table 2). Triacylglycerol and NEFA levels were significantly suppressed by cHF+F+TZD, even below the levels observed in chow diet-fed mice. Cholesterol levels were reduced at 8 but not at 20 weeks. The other treatments (cHF+F and cHF+TZD) induced smaller or no reduction of plasma lipid levels (Table 2).

We also investigated the effect of 8 weeks of treatment on liver VLDL-triacylglycerol synthesis (Fig. 2). Plasma triacylglycerol levels were measured in fasted mice just prior to and for 3 h after injection of Triton WR-1339. Under these conditions, the nonionic detergent blocks lipolytic degradation of triacylglycerol-rich lipoproteins in the peripheral tissues, with the rise in plasma triacylglycerols proportionate to production of VLDL in the liver. All the treatments significantly decreased the rate of VLDL-triacylglycerol synthesis, with the strongest reduction (approximately twofold) by cHF+F+TZD.

Prevention of IGT and insulin resistance Insulinaemia increased between 8 and 20 weeks of high-fat feeding, suggesting development of insulin resistance (Table 2). The rise in insulin levels was prevented to a similar extent by cHF+TZD and cHF+F+TZD diets, while the cHF+F diet exhibited a significant but smaller effect.

Both at 8 and 20 weeks, the cHF+F and cHF+TZD diets increased total immunoreactive adiponectin in plasma. An even stronger induction was observed with a combination of the two treatments (Table 2). Multimeric adiponectin complexes in plasma were also analysed. Although the ratio between high molecular weight (HMW) and total adiponectin was similar in the cHF- and chow-fed mice, it was increased by all the other treatments, with the highest additive effect observed in cHF+F+TZD-fed animals, irrespective of treatment duration (Table 2).

To characterise insulin sensitivity and glycaemic control, a glucose tolerance test was performed at 8 weeks (Fig. 3a). Fasted glycaemia was ~1.4-fold higher in the cHF- as compared with the chow-fed mice and was not significantly affected by any of the treatments (Fig. 3a, b). In contrast, feeding cHF resulted in IGT, as revealed by ~1.4-fold higher AUC values (glucose tolerance test) in the cHF- compared with the chow-fed mice (Fig. 3c). Both cHF+TZD and cHF+F+TZD diets prevented development of IGT, while cHF+F+TZD displayed the largest improvements in glucose tolerance.

At 8 weeks, a euglycaemic–hyperinsulinaemic clamp was also performed to evaluate precisely the changes in whole-body insulin sensitivity brought about by various treatments (Fig. 3d–i). In the hyperinsulinaemic conditions, the amount of exogenous glucose required to maintain euglycaemia, i.e. the glucose infusion rate, was significantly (~1.7-fold) higher in mice treated by cHF+F or cHF+TZD than in cHF-fed mice (Fig. 3e), suggesting improvements in insulin sensitivity. Importantly, cHF+F+TZD resulted in a ~2.4-fold higher glucose infusion rate than that in cHF-fed mice, representing the strongest effect among all the treatments. Hepatic glucose production in the hyperinsulinaemic conditions was decreased in the cHF+F mice to a significantly lower level than in the other subgroups (Fig. 3f), suggesting that DHA/EPA improves hepatic insulin sensitivity. The cHF+F+TZD and cHF+TZD (but not cHF+F) treatments significantly improved whole-body glucose turnover, with cHF+F+TZD showing the most dramatic effect (Fig. 3g). The rate of whole-body glycolysis was not significantly affected by the treatments (Fig. 3h). Whole-body glycogen synthesis was strongly stimulated by cHF+F+TZD, while cHF+F and cHF+TZD diets had no effect (Fig. 3i). No

Table 2 Prevention of dyslipidaemia and hyperinsulinaemia, and induction of adiponectin

Variable	cHF	cHF+F	cHF+TZD	cHF+F+TZD	Chow
8 weeks					
Triacylglycerol (mmol/l)	1.3±0.1	1.1±0.1	1.0±0.1	0.8±0.1[ab]	
NEFA (mmol/l)	0.49±0.05	0.43±0.04	0.38±0.03[a]	0.27±0.02[abc]	
Cholesterol (mmol/l)	3.7±0.2	3.2±0.2[a]	3.8±0.1[b]	3.1±0.1[ab]	
Insulin (pmol/l)	330±60	210±30	270±30	165±45[a]	
Adiponectin (μg/ml)	8.6±0.4	11.0±0.5	10.3±0.2	12.3±0.7[a]	
HMW:total	0.27±0.02	0.30±0.03	0.35±0.02[a]	0.41±0.01[abc]	
20 weeks					
Triacylglycerol (mmol/l)	2.1±0.1	1.8±0.2	1.5±0.1[a]	1.1±0.1[ab]	1.3±0.1[d]
NEFA (mmol/l)	0.50±0.03	0.35±0.07[a]	0.41±0.03	0.29±0.02[ac]	0.49±0.03
Cholesterol (mmol/l)	4.4±0.2	3.7±0.1[a]	3.8±0.2	3.7±0.2	2.1±0.1[d]
Insulin (pmol/l)	1125±165	630±120[a]	360±45[ab]	270±30[ab]	90±15[d]
Adiponectin (μg/ml)	5.3±0.3	8.2±0.6[a]	8.6±0.4[a]	10.1±0.4[abc]	4.4±0.3
HMW:total	0.33±0.03	0.46±0.01[a]	0.49±0.02[a]	0.55±0.02[ab]	0.34±0.02

Mice (3 months old) were placed on various diets, and after 8 or 20 weeks of treatment lipid metabolites and hormones were measured in plasma of mice killed in ad libitum fed state. Data are means±SE (n=8)
[a]$p\leq0.05$ for difference from cHF (ANOVA); [b]$p\leq0.05$ for difference from cHF+F (ANOVA); [c]$p\leq0.05$ for difference from cHF+TZD (ANOVA); [d]$p\leq0.05$ for difference from cHF (t test)
HMW:total; ratio of HMW:total adiponectin

differences in muscle glycogen content between groups were observed (not shown).

Muscle- and liver-specific effects of the treatments To confirm the results on whole-body glycogen synthesis, measurements were also performed ex vivo, in dissected diaphragms from mice subjected to various treatments (Fig. 4). Diaphragm contains glycolytic and oxidative muscle fibres and is suitable for this type of analysis due to its small diffusion barrier for oxygen [42]. Compared with chow, cHF reduced the rate of basal (~2.5-fold) and insulin-stimulated (~2.8-fold) glycogen synthesis. This deleterious effect of cHF feeding was completely prevented by cHF+F+TZD (Fig. 4). Neither cHF+F nor cHF+TZD had any significant effect on basal or insulin-stimulated glycogen synthesis. Improvements in insulin-stimulated glycogen synthesis also correlated with the induction of Akt/PKB phosphorylation at Ser473 in soleus muscle of mice fed cHF+F+TZD (ESM Fig. 1).

Analysis of gene expression from gastrocnemius, a mixed-fibre muscle, revealed a trend (Fig. 5a) for induction by the combination treatment of *Pdk4*, *Cpt1a* and *Cpt1b*, supporting the notion that the combination treatment induced a switch augmenting lipid over glucose catabolism. Interestingly, the cHF+TZD diet strongly induced expression of *Scd1*, while cHF+F and cHF+F+TZD treatments had an opposite effect.

Analysis of hepatic gene expression (Fig. 5b) revealed a downregulation of gluconeogenic genes *Pck2* and *G6pc* by cHF+F+TZD, while cHF+F and cHF+TZD diets exerted less pronounced effects. The cHF+F+TZD diet strongly induced expression of *Acot2*, in correlation with upregulation of *Cpt1b*, suggesting increased turnover of acyl-CoA and fatty acid oxidation in mitochondria. A similar pattern of induction of *Acox1*, a marker of peroxisomal fatty acid oxidation, was observed. Both cHF+TZD and cHF+F+TZD strongly induced expression of *Scd1*, while cHF+F had no effect. In contrast, *Fas* was downregulated by cHF+F and cHF+F+TZD, while cHF+TZD had no effect.

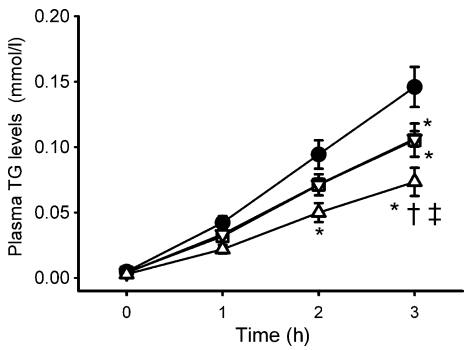

Fig. 2 Hepatic VLDL-triacylglycerol production. At 3 months of age, mice were randomly assigned to various diets. After 8 weeks of treatment, mice were fasted for 6 h, anaesthetised, injected with Triton WR1339 and plasma triacylglycerol (TG) levels were measured before (time 0) and at 1, 2 and 3 h after injection in mice fed cHF (black circles), cHF+F (white squares), cHF+TZD (white inverted triangles) and cHF+F+TZD (white triangles). Data are means±SE (n=5–8). *$p\leq0.05$ for difference from cHF; †$p\leq0.05$ for difference from cHF+F; ‡$p\leq0.05$ for difference from cHF+TZD (repeated measures ANOVA)

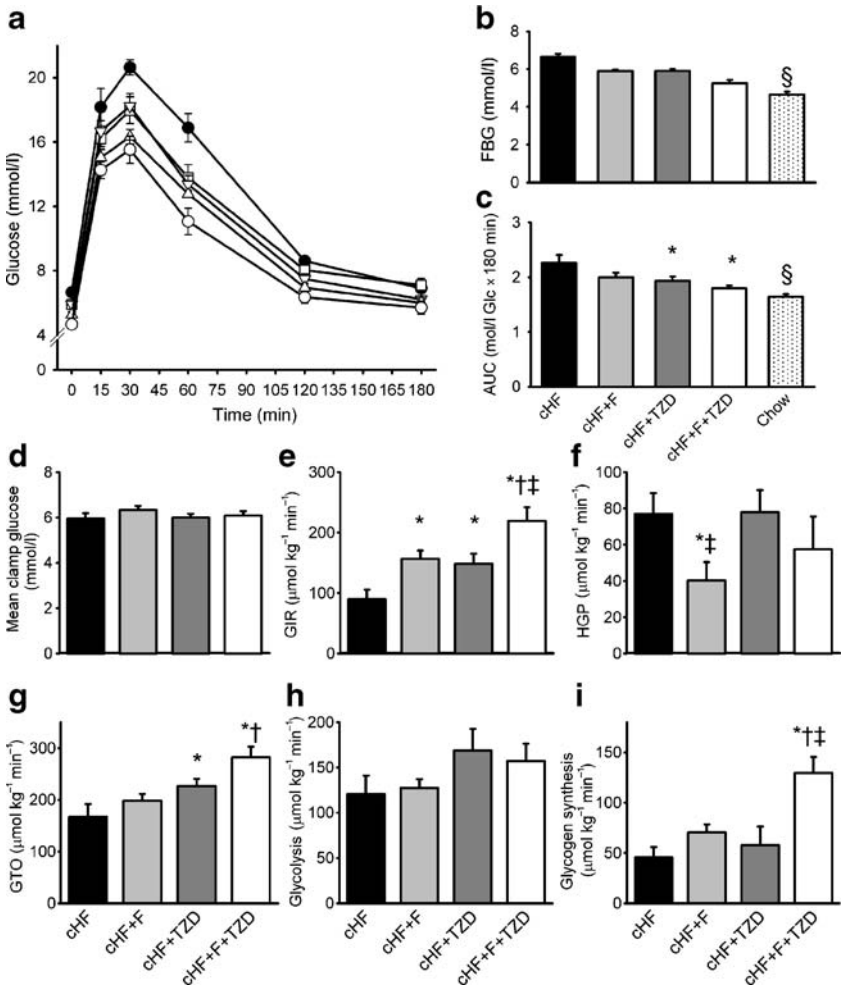

Fig. 3 Glucose homeostasis. At 8 weeks after treatment as described, glucose tolerance test (**a–c**) or euglycaemic–hyperinsulinaemic clamp (**d–i**) were performed in two separate experiments. **a** Plasma glucose levels following intraperitoneal glucose injection (time 0) to fasted mice treated by cHF (black circles), cHF+F (white squares), cHF+TZD (white inverted triangles), cHF+F+TZD (white triangles) or chow (white circles). **b** Fasted blood glucose (FBG) levels at time 0 as above (**a**). **c** Area under the glycaemic curve values as above (**a**). **d** Mean plasma glucose levels during euglycaemic–hyperinsulinaemic clamp. **e–h** Variables, as indicated, that were evaluated during stimulation by insulin. Data are means±SE (n=6–9). *$p\leq0.05$ for difference from cHF; $^{\dagger}p\leq0.05$ for difference from cHF+F; $^{\ddagger}p\leq0.05$ for difference from cHF+TZD (ANOVA); $^{\S}p\leq0.05$ for difference from cHF (t test). GIR, glucose infusion rate; GTO, glucose turnover: HGP, hepatic glucose production

Interestingly, no adverse effects of the combination treatment on enzymatic markers of muscle and liver damage were observed (ESM Table 4).

Reversal of obese phenotype Given both the high efficacy of the cHF+F+TZD treatment in prevention of the high-fat diet-induced disorders in mice and the failure of n-3 LC-PUFA to reverse insulin resistance in humans (see Introduction), the effects of cHF+F and cHF+TZD and their combination were also studied in obese mice with IGT (Table 3). Compared with cHF-fed animals, body weight gain, triacylglycerols and NEFA levels were suppressed by cHF+F, while cHF+TZD decreased plasma triacylglycerols and insulin levels. Only cHF+F+TZD affected all the variables studied, showing additive effects of DHA/EPA and rosiglitazone in the reversal of obesity, while decreasing plasma NEFA and cholesterol levels. The cHF+F+ TZD diet also lowered plasma triacylglycerol and insulin concentrations, to a similar extent to that achieved by cHF+TZD (Table 3).

To evaluate the capacity of treatments to reverse IGT in dietary obese mice, a glucose tolerance test was performed 1 week before the end of the study. Fasting glycaemia was decreased to a similar extent by all treatments (Table 3). However, glucose tolerance measured as AUC was markedly improved by cHF+F+TZD, indicating additive effects of DHA/EPA and rosiglitazone in the reversal of IGT. Both cHF+F and cHF+TZD improved glucose tolerance, but to a smaller extent than cHF+F+TZD (Table 3).

Discussion

Combination of life style changes with pharmacological interventions is required for treatment of diabetes and other metabolic diseases associated with obesity. However, safer, more efficacious and less expensive strategies are still needed. We show here that long-term treatment combining partial replacement of dietary lipids by DHA/EPA and a low dose of rosiglitazone markedly and in an additive manner prevented development of dyslipidaemia and insulin resistance, reduced accumulation of body fat and adipocyte hypertrophy, and also induced adiponectin in mice fed a high-fat diet. Importantly, this treatment also reversed IGT in obese mice.

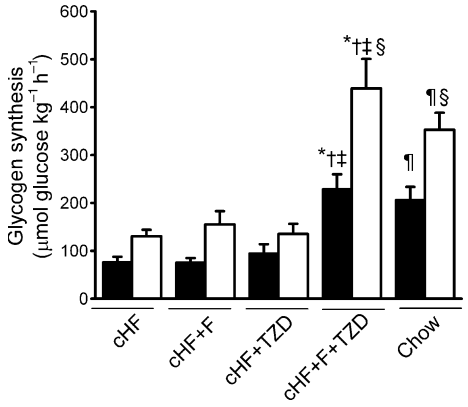

Fig. 4 Glycogen synthesis in diaphragm muscle. At 5 weeks after initiation of treatment as described, left and right hemidiaphragms of mice were dissected and incubated with [U ^{14}C]glucose in the absence (black bars) or presence (white bars) of 250 μU/ml insulin to measure the rate of glycogen synthesis. Data are means±SE (n=7–8). *p≤0.05 for difference from cHF; †p≤0.05 for difference from cHF+F; ‡p≤0.05 for difference from cHF+TZD (ANOVA); §p≤0.05 for difference from non-insulin-stimulated value within the diet (ANOVA); ¶p≤0.05 for difference from cHF (t test)

As described before [9, 10], DHA/EPA reduced fat accumulation, without affecting food consumption. Unexpectedly, a relatively low dose of rosiglitazone (10 mg rosiglitazone per kg diet) was sufficient to augment the induction of lean phenotype by DHA/EPA. In contrast, at a tenfold higher dose, rosiglitazone promoted obesity, as observed in most other studies in mice [24, 27]. In humans, rosiglitazone therapy reduced abdominal and increased subcutaneous fat weight [20]. However, weight of both fat depots in mice was decreased by the combination treatment of DHA/EPA with rosiglitazone, suggesting that treatment by a low dose of rosiglitazone combined with dietary n-3 LC-PUFA intake may also reduce adiposity in humans.

Indirect calorimetry in mice did not reveal any differences in fuel partitioning or energy expenditure between groups. In addition, no difference in physical activity was detected. Therefore, the mechanism for reduced body weight gain in the cHF+F+TZD-treated mice remains unclear. However, even small undetectable changes in the above variables (or in food intake) or their combination, lasting for several weeks, could result in a substantial difference in body weight.

It has been observed previously that treatment by DHA/EPA or rosiglitazone ameliorated adipocyte hypertrophy [9, 43]. In line with these observations, the greatest reduction of adipocyte hypertrophy was observed with the combination of DHA/EPA and rosiglitazone. Compared with large adipocytes, small cells are more insulin-sensitive and less lipolytic, while releasing fewer inflammatory cytokines, as reviewed [20], and more adiponectin [44]. While treatment with DHA/EPA [15, 31] or TZD [15, 25] has previously been shown to induce adiponectin in mice, the current study showed for the first time that the combination of the two increased plasma adiponectin more potently. Importantly, the stimulatory effect of the combination treatment was specific for HMW adiponectin, the form of adiponectin associated with insulin sensitivity [33, 45]. Importantly, the combination treatment also reduced most potently the low-grade adipose tissue inflammation associated with obesity

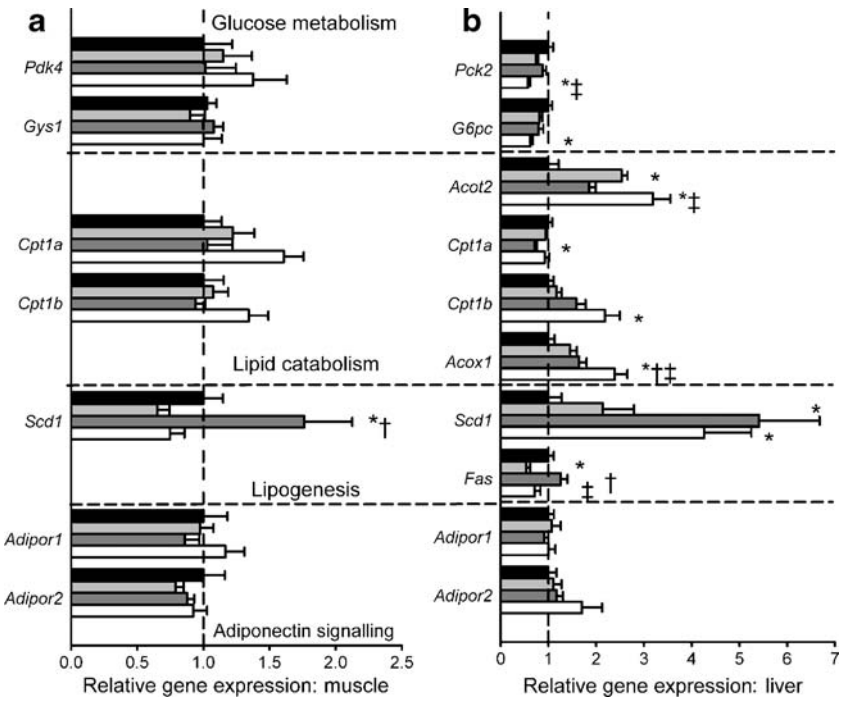

Fig. 5 Quantitative RT-PCR data showing gene expression in mouse gastrocnemius muscle (**a**) and liver (**b**) after 8 weeks of dietary treatments. Black bars, cHF; light grey bars, cHF+F; dark grey bars, cHF+TZD; white bars, cHF+F+TZD. Data are means±SE (n=6–7). *p≤0.05 for difference from cHF (ANOVA); †p≤0.05 for difference from cHF+F (ANOVA); ‡p≤0.05 for difference from cHF+TZD (ANOVA)

Table 3 Reversal of obesity, dyslipidaemia and impaired glucose homeostasis

Variables	cHF	cHF+F	cHF+TZD	cHF+F+TZD
Body weight				
Initial (g)	43.2±2.4	43.3±2.2	45.0±2.0	44.9±1.7
Final (g)	47.3±2.4	43.0±2.2	47.5±1.7	41.8±1.3
Gain (g)	4.1±1.0	−0.3±1.0[a]	2.5±1.1	−3.1±1.8[abc]
Plasma lipids and insulin				
Triacylglycerols (mmol/l)	1.34±0.16	0.90±0.10[a]	0.94±0.03[a]	0.84±0.08[a]
NEFA (mmol/l)	0.46±0.04	0.36±0.03[a]	0.40±0.02	0.27±0.03[abc]
Cholesterol (mmol/l)	5.10±0.36	3.94±0.15	4.02±0.48	3.24±0.34[a]
Insulin (pmol/l)	510±54	356±80	285±74[a]	215±33[a]
Glucose tolerance test				
FBG (mmol/l)	7.49±0.55	5.94±0.22[a]	5.88±0.55[a]	5.22±0.22[a]
AUC (mmol l^{-1} × 180 min)	3,404±201	2,739±136[a]	2,592±161[a]	2,109±124[abc]

Data are means±SE (n=8)

To induce obesity and IGT, mice were fed cHF diet between 3 and 7 months of age, and then treated by different diets for 8 more weeks. Body weight was recorded just before and at the end of the 8-week-treatment. After the treatment, plasma lipids and insulin were measured in ad libitum fed mice. Glucose tolerance test was performed in fasted mice 7 weeks after initiation of treatment

[a]$p \leq 0.05$ for difference from cHF; [b]$p \leq 0.05$ for difference from cHF+F; [c]$p \leq 0.05$ for difference from cHF+TZD (ANOVA)

FBG, fasting blood glucose

[24, 38]. Thus, the additive effects of DHA/EPA and rosiglitazone in the reduction of fat cell hypertrophy, induction of HMW adiponectin and decrease of adipose tissue inflammation might contribute to the beneficial effects on glucose homeostasis.

In a previous study [24], dual pharmacological activation of PPAR-α (by Wy-14,643) and PPAR-γ (by rosiglitazone) in mice improved insulin sensitivity with increased efficacy compared with treatment by either PPAR agonist alone, while increasing both adiponectin and adiponectin receptors in adipose tissue [24]. In our experiments, the treatments did not significantly affect expression of adiponectin receptor genes *Adipor1* and *Adipor2*, either in muscle or in liver, while DHA/EPA and rosiglitazone still improved insulin sensitivity in an additive manner. Importantly, in our experiments, naturally occurring *n*-3 LC-PUFA rather than synthetic PPAR-α agonists were used in the combination with a relatively low dose of rosiglitazone.

Euglycaemic–hyperinsulinaemic clamps in mice and ex vivo measurements in diaphragm muscle showed synergistic induction of glycogen synthesis at the basal and insulin-stimulated conditions by the combination treatment, indicating that skeletal muscle was the major organ responsible for the additive effects of DHA/EPA and rosiglitazone on whole-body glycaemic control. Interestingly, and in accordance with previous studies, neither treatment by DHA/EPA [11, 12, 18], nor rosiglitazone alone (at the relatively low dose used) [23], significantly affected the rate of glycogen synthesis [34]. Increased insulin sensitivity of skeletal muscle is further supported by changes in Akt/PKB phosphorylation. A putative mechanism behind the synergistic effect of the combination treatment is reduction of muscle ceramide concentration. Interestingly, improvements in glucose homeostasis by the treatments became more pronounced with a longer duration of cHF feeding, despite the absence of any effect on triacylglycerol accumulation in the liver and muscle. This observation is not without precedent [12].

In accordance with previous studies, DHA/EPA [11, 12, 18], but not the low dose of rosiglitazone [23], were able to depress hepatic glucose production under hyperinsulinaemic conditions, suggesting improvement of hepatic insulin sensitivity by the former treatment. Given the ability of *n*-3 LC-PUFA to prevent development of IGT, the above data also suggest that the effect on hepatic glucose production mentioned above may be required for this protection to occur. On the other hand, the reversal of IGT and insulin resistance by TZDs may be more dependent on the enhancing of insulin action in skeletal muscle. The latter mechanism could be mediated either by TZDs, or even more potently, according to our results, by the combination of DHA/EPA and TZDs.

The additive improvement of glycaemic control correlated with the hypolipidaemic effect of the treatments. Plasma levels of NEFA and triacylglycerols were even lower in mice treated by cHF+F+TZD than in mice fed chow diet, in spite of the higher dietary supply of lipids and the adipose tissue content of the former mice. These results are in accordance with the known hypolipidaemic effects of *n*-3 LC-PUFA [1] and rosiglitazone [25] in mice. Interest-

ingly, in humans, only pioglitazone but not rosiglitazone exerted a hypolipidaemic effect [19], documenting that the hypolipidaemic effect of TZDs may be dissociated from their effect on insulin sensitivity [25].

The lowering of NEFA levels in mice subjected to various treatments could reflect several mechanisms, including adipose tissue lipolysis, re-esterification of NEFA in liver and adipose tissue, and stimulation of lipid oxidation in liver [14] and other organs [10] by *n*-3 LC-PUFA, as well as by TZDs [26, 27]. The fact that *n*-3 LC-PUFAs are able to counteract the inhibition of hepatic fatty acid oxidation by insulin [46] may contribute to the additive effect of the DHA/EPA and rosiglitazone treatment on lipid catabolism. The suppression of plasma triacylglycerol levels possibly resulted from an increased triacylglycerol uptake by muscle and other tissues induced by TZDs [47] and by *n*-3 LC-PUFA [48], or from decreased hepatic VLDL-triacylglycerol production. The former mechanism was not studied in our experiments; however, we did demonstrate the inhibition of hepatic VLDL-triacylglycerol production by either DHA/EPA or rosiglitazone, with the strongest effect being observed in the combination treatment. The decrease of hepatic triacylglycerol production by DHA/EPA may represent a functional outcome of the coordinated suppression of lipogenic genes by *n*-3 LC-PUFA [17]. In addition, stimulation of AMP-activated protein kinase by *n*-3 LC-PUFA, resulting in a metabolic switch from lipogenesis to lipid catabolism, may also be involved [49].

In contrast, the mechanism of suppression of VLDL-triacylglycerol formation by rosiglitazone must be different, since rosiglitazone increased *Scd1* expression and triacylglycerol content in the liver. The mechanism may reflect an increased rate of fatty acid re-esterification induced by rosiglitazone [50], rather than suppression of de novo lipogenesis in hepatocytes. Our data, in accordance with the effects of the treatments on muscle *Scd1*, document differential modulation of the genes involved in de novo fatty acid synthesis (*Fas*) and desaturation (*Scd1*) by DHA/EPA and rosiglitazone. The induction of *SCD1* expression and activity in association with insulin sensitisation by rosiglitazone has been observed before [29], but the underlying mechanism remains unknown.

In conclusion, an original combined treatment using *n*-3 LC-PUFA with low-dose rosiglitazone generated additive effects in the prevention as well as reversal of adipose tissue hypertrophy, hyperlipidaemia and impaired glycaemic control in mice fed an obesogenic diet. Multiple mechanisms underlined the beneficial whole-body effects of the combination treatment with a prominent synergistic stimulation of insulin-sensitive muscle glycogen synthesis. The combined use of *n*-3 LC-PUFA and TZDs thus represents a potential strategy for treatment of type 2 diabetes and other obesity-associated metabolic disorders. The inclusion of *n*-3 PUFA in treatment with TZDs may reduce the dose requirement and the incidence of adverse side effects associated with the TZD-based therapy.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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Electronic supplementary material

Methods

Body composition At 15 weeks of a separate 'prevention study', mice were killed by exsanguination under diethyl ether anaesthesia. Carcass without intestinal tract and urinary bladder emptied was dissolved in ethanolic KOH, protein content of the solubilisate was determined by Kjeldahl digestion and micro-Conway method, and triacylglycerol content was measured by enzymatic estimation (enzymatic assay of free glycerol F6428; Sigma, St Louis, MO, USA).

Glycogen synthesis in diaphragm Left and right hemidiaphragm were dissected and separately incubated ex vivo to measure incorporation of glucose into glycogen, as previously described for soleus muscle. Reaction proceeded for 2 h at 37ºC in continuously gassed buffer (see above), containing, in addition, 5.5 mmol/l unlabelled glucose, 18.5 kBq/ml of [U-^{14}C]glucose (UJV, Rez, Czech Republic) and 3 mg/ml BSA (Fraction V, ICN, OH, USA) in the absence or presence of 250 μU insulin /ml (using the two hemidiaphragms from each mouse).

In vivo hepatic VLDL-triacylglycerol production The rate of liver triacylglycerol synthesis was evaluated using Triton WR1339, an inhibitor of plasma VLDL clearance. Mice were fasted from 08:00 to 14:00 hours, before induction of anaesthesia (100 mg pentobarbital per kg of body weight). Triton WR1339 (Sigma) was injected intravenously (500 mg/kg of body weight) as a 150 g/l solution in 0.9% (wt/wt) NaCl. Blood samples were taken before the injection and at 1, 2 and 3 h after injection. The level of triacylglycerols in plasma was determined using enzymatic kit (BioLaTest; Lachema, Brno, Czech Republic). The rate of hepatic VLDL-triacylglycerol production was calculated from the slope of the curve of plasma triacylglycerol levels.

Light microscopy and immunohistochemical analysis Epididymal fat samples were fixed in 4% (vol./vol.) formaldehyde, embedded in paraffin and processed to detect MAC-2/galectin-3 and perilipin, using specific antibodies. Digital images were captured using light microscope (AX70) and a camera (DP 70; both from Olympus, Tokyo, Japan). Morphometry was performed using a morphometric programme (Lucia IMAGE, version 4.81; Laboratory Imaging, Prague, Czech Republic).

Euglycaemic–hyperinsulinaemic clamp Five days before the experiment an indwelling catheter was placed into the left femoral vein under anaesthesia [5]. The mice were allowed to recover for 5 to 7 days, followed by fasting for 6 h prior to the experiment. Whole-body

glucose turnover was determined in euglycaemic–hyperinsulinaemic conditions. Insulin was infused at a constant rate of 4.8 mU kg^{-1} min^{-1} for 3 h, while D-[3-^{3}H]glucose was infused at a rate 15.9 kBq/min. Throughout the infusion, glucose concentration and D-[3-^{3}H]glucose specific activity were determined in 5 µl aliquots of tail blood. Euglycaemia (~5.55 mmol/l) was maintained by periodically adjusting a variable infusion of 16.5% (wt/wt) glucose. For more details, see previous publications [5-7].

Analysis of blood samples from euglycaemic–hyperinsulinaemic clamps In order to assess D-[3-^{3}H]glucose, ^3H_2O and total glucose concentrations, blood samples were firstly deproteinised by precipitation with $ZnSO_4$/$Ba(OH)_2$, followed by centrifugation [5]. The first aliquot of the supernatant fraction was evaporated to dryness to determine the radioactivity corresponding to D-[3-^{3}H]glucose. The second aliquot was used to determine radioactivity of D-[3-^{3}H]glucose and ^3H_2O. Plasma ^3H_2O radioactivity was then calculated as the difference between radioactivity in the second and first samples. In the third aliquot of the supernatant fraction, total glucose concentration was assessed by the glucose oxidase method (Glukosa God 1500; PLIVA-Lachema, Brno, Czech Republic). D-[3-^{3}H]Glucose-specific activity was calculated by dividing the D-[3-^{3}H]glucose enrichment of plasma (dpm/ml) by the total plasma glucose concentration (mg/ml). Time points in which steady-state D-[3-^{3}H]glucose specific activity varied more than ±15% were not taken into account [5]. The rates of insulin-stimulated glucose turnover were determined as the ratio of the [3-^{3}H]glucose infusion rate (dpm/min) to the specific activity of plasma glucose [6]. The rate of hepatic glucose production in hyperinsulinaemic conditions was calculated as the difference between glucose turnover and the glucose infusion rate. Whole-body glycolysis was calculated from the increment per min in plasma ^3H_2O radioactivity during the last 60 min multiplied by the body water space and divided by D-[3-^{3}H]glucose specific activity [7]. Whole-body glycogen synthesis was estimated by subtracting whole-body glycolysis from whole-body glucose turnover, provided that glycolysis and glycogen synthesis accounted for most of maximal insulin-stimulated glucose turnover.

Statistics Data were analysed by one-way ANOVA (comparison of cHF, cHF+F, cHF+TZD, cHF+F+TZD diets), two-way ANOVA, repeated measures ANOVA and *t* test using SigmaStat statistical software. The Tukey test for all pairwise multiple comparisons was used. All values are presented as means±SE. Comparisons were judged to be significant at $p \leq 0.05$.

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Electronic supplementary material

ESM Table 1 Macronutrient composition and energy content of diets, and supplementation by DHA/EPA and rosiglitazone

Variable	cHF	cHF+F	cHF+TZD	cHF+F+TZD	Chow
Macronutrient composition					
Lipid (% diet, wt/wt)	35.2	35.2	35.2	35.2	3.4
Carbohydrate (% diet, wt/wt)	35.4	35.4	35.4	35.4	55.3
Protein (% diet, wt/wt)	20.5	20.5	20.5	20.5	19.3
Energy density (kJ/g)	22.8	22.8	22.8	22.8	16.3
Supplement					
DHA/EPA concentrate (%)[a]	0	15	0	15	0
Rosiglitazone (mg/kg diet)[b]	0	0	10	10	0

cHF diet was prepared as described [1], except that extruded Ssniff R/M-H diet and corn oil were used instead of chow ST-1 diet and sunflower oil. For detailed fatty acid composition of the diets, see ESM Table 2. cHF+F diet corresponds to cHF-F1 diet in our previous studies [1-3]. All the cHF-based diets contained 210 mg α-tocopherol/kg diet

[a]Percentage of dietary lipids replaced by DHA/EPA concentrate (containing 46% DHA and 14% EPA, in a form of triacylglycerol, and 4 mg/g α-tocopherol as antioxidant; 1050TG; EPAX, Lysaker, Norway)

[b]Rosiglitazone maleate (Avandia; GlaxoSmithKline) was admixed to cHF diet

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid

Electronic supplementary material

ESM Table 2 Fatty acid composition of dietary lipids

Fatty acid (g/100 g)	Chow	cHF	cHF+F
8:0	–	0.33	0.40
10:0	–	0.56	0.59
12:0	0.04	2.15	2.22
14:0	0.15	2.06	2.15
14:1 *n*-5	–	0.11	0.11
16:0	15.18	13.67	12.77
16:1 *n*-9	0.05	0.03	0.04
16:1 *n*-7	0.18	0.27	0.40
18:0	2.54	2.97	3.10
18:1 *n*-9	16.18	27.10	24.00
18:1 *n*-7	1.00	0.83	0.85
18:2 *n*-6	57.78	47.63	40.44
18:3 *n*-6	–	0.01	0.01
18:3 *n*-3	6.01	1.71	1.65
18:4 *n*-3	–	0.05	0.18
20:0	0.17	0.16	0.30
20:1 *n*-9	0.30	0.12	0.31
20:2 *n*-6	0.07	0.06	0.11
20:3 *n*-6	–	–	0.02
20:4 *n*-6	–	–	0.19
20:3 *n*-3	–	–	0.03
20:4 *n*-3	–	–	0.10
20:5 *n*-3 (EPA)	–	0.02	2.10
22:0	0.18	0.09	0.14
22:1 *n*-9	0.02	–	0.04
22:4 *n*-6	–	–	0.03
22:5 *n*-6	–	–	0.19
22:5 *n*-3	–	–	0.52
22:6 *n*-3 (DHA)	–	–	7.11
24:0	0.09	0.05	–
Sum SFA	18.4	22.1	21.6
Sum MUFA	17.8	28.5	25.7
Sum *n*-6 PUFA	57.9	47.7	41.0
Sum *n*-3 PUFA	6.0	1.8	11.7

Fatty acid composition of the diets was determined using gas chromatography

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; – , <0.1%

Electronic supplementary material

ESM Table 3 Oligonucleotide primers for quantitative real-time RT-PCR

Gene	Forward primers	Reverse primers
Acot2	TGGGAACACCATCTCCTACAA	CCACGACATCCAAGAGACCA
Adipor1	ACTCCCAAGCACCGGCAGACAAGA	CCAGGGAAGAAGCGCTCAGGAATC
Adipor2	GTTCCTGAAGGCTGCCACCATAG	TGAACAAAGGCACCAGCAACCAC
Acox1	GCTGGGCTGAAGGCTTTTACTACC	CACCTGCTGCGGCTGGATAC
Cpt1a	GCAGCTCGCACATTACAAGGACAT	ACTATGTGTCCTGTGGCGGGGGCT
Cpt1b	GGATGATGGCTACGGGGTCTCTTA	AGGGCAGCTGGGGTATCTCTTTTC
Eef1a	TGACAGCAAAAACGACCCACCAAT	GGGCCATCTTCCAGCTTCTTACCA
Fas	GGCTGCCTCCGTGGACCTTATC	GTCTAGCCCTCCCGTACACTCACTCGT
G6pc	CATGGGCGCAGCAGGTGTAT	TGGGGAAAGTGAGCAGCAAGGTA
Gys1	CCCGGTCCCCTTTCCTGATACAA	CTGCCCTCCCCCTCCCAAATG
Pck2	GGCAGCATGGGGTGTTTGTAGGA	TTTGCCGAAGTTGTAGCCGAAGAAG
Pdk4	GGCTTGCCAATTTCTCGTCTCTA	TTCGCCAGGTTCTTCGGTTCC
Ppib	ACTACGGGCCTGGCTGGGTGAG	TGCCGGAGTCGACAATGATGA
Scd1	ACTGGGGCTGCTAATCTCTGGGTGTA	TAACAAACCCACCCAGAGATAAAGCC

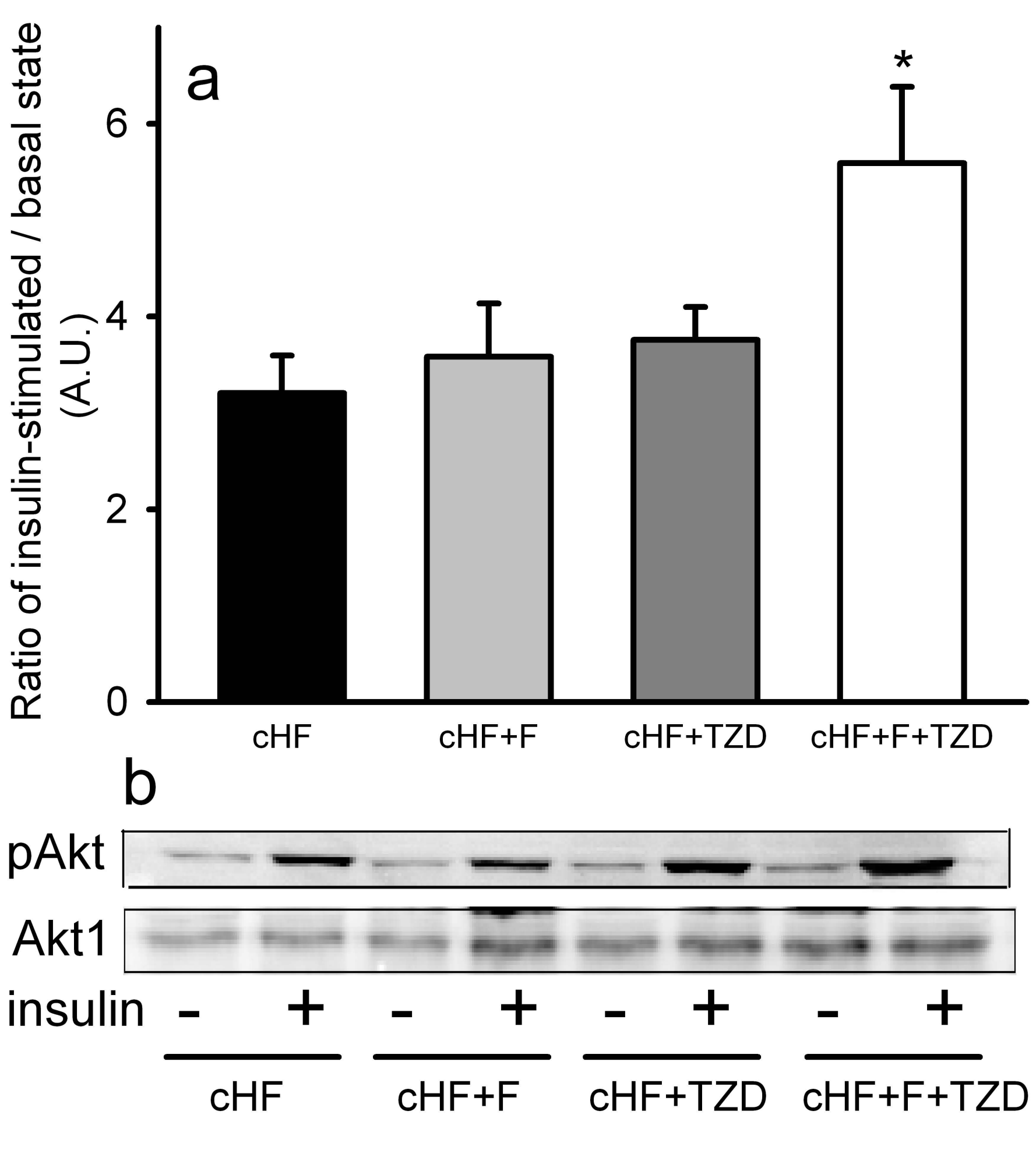
Primers were designed using Lasergene software (DNASTAR, Madison, WI, USA)
In addition to the elongation factor-1α, cyclophilin-β was also used to normalise transcript levels with similar results (not shown)

Electronic supplementary material

ESM Table 4 Plasma markers of hepatic and muscle damage

Enzyme	cHF	cHF+F	cHF+TZD	cHF+F+TZD
AST	9.9±2.0	11.0±1.7	11.7±1.5	11.0±0.5
ALT	1.4±0.3	1.4±0.4	1.8±0.3	1.3±0.1
CK	222±22	197±20	226±16	219±8

Data are means±SE (*n*=8)
Three-month-old mice were placed on cHF diet or treated by various cHF-based diets (cHF+F, cHF+TZD and cHF+F+TZD). After 8 weeks on the diets, mice were killed in an ad libitum fed state, and activities (μmol/s) of enzyme markers of liver (AST, aspartate aminotransferase; ALT, alanine aminotransferase) and muscle (CK, creatinine kinase) damage were measured


a
Ratio of insulin-stimulated / basal state (A.U.)
0
2
4
6
*
cHF
cHF+F
cHF+TZD
cHF+F+TZD
b
pAkt
Akt1
insulin
-
+
-
+
-
+
-
+
cHF
cHF+F
cHF+TZD
cHF+F+TZD

ESM Fig. 1 Phosphorylation of Akt/PKB in soleus muscle. At 5 weeks after initiation of treatment as described, soleus muscles from anaesthetised mice (6 mg pentobarbital sodium per 100 g body weight) were carefully dissected and immediately transferred into pre-warmed 1.5 ml of freshly oxygenated (95% O_2, 5% CO_2) Krebs–Ringer bicarbonate buffer (pH 7.4), containing 8 mmol/l mannitol and 2 mmol/l pyruvate (30°C). After 10 min, pairs of muscles from each mouse were transferred into fresh medium, in the absence or presence of 2 mU/ml insulin and incubated for 1 h at 30°C. After incubation, muscles were quickly frozen in liquid nitrogen to preserve Akt/PKB phosphorylation. Homogenates from frozen muscles were prepared as described before [4] and total and phosphorylated Akt/PKB were evaluated by western blot analysis using Akt1 mouse antibody (Cell Signaling, Danvers, MA, USA) and Phospho-Akt (Ser473) rabbit antibody (Cell Signaling) on an imaging system (Odyssey Infrared; LI-COR, Bad Homburg, Germany). a Ratio of phosphorylated Akt signal intensity in insulin-stimulated and non-stimulated state. b Representative immunoblot of phosphoAkt (Ser473) and total Akt1. Data are means±SE; n=5–7. *$p \leq 0.05$ for difference from cHF diet (PDF 23.5 KB)

Induction of lipid oxidation by polyunsaturated fatty acids of marine origin in small intestine of mice fed a high-fat diet

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Abstract

Background and Approach: Dietary polyunsaturated fatty acids (PUFA), in particular the long chain marine fatty acids docosahexaenoic (DHA) and eicosapentaenoic (EPA), are linked to many health benefits in humans and in animal models. Little is known of the molecular response to DHA and EPA of the small intestine, and the potential contribution of this organ to the beneficial effects of these fatty acids. Here, we assessed gene expression changes induced by DHA and EPA in the wildtype C57BL/6J murine small intestine using whole genome microarrays and functionally characterized the most prominent biological process.

Results: The main biological process affected based on gene expression analysis was lipid metabolism. Fatty acid uptake, peroxisomal and mitochondrial beta-oxidation, and omega-oxidation of fatty acids were all increased. Quantitative real time PCR, and -in a second animal experiment- intestinal fatty acid oxidation measurements confirmed significant gene expression differences and showed in a dose-dependent manner significant changes at biological functional level. Furthermore, no major changes in the expression of lipid metabolism genes were observed in the colon.

Conclusions: We show that marine n-3 fatty acids regulate small intestinal gene expression and increase fatty acid oxidation. Since this organ contributes significantly to whole organism energy use, this effect on the small intestine may well contribute to the beneficial physiological effects of marine PUFAs under conditions that will normally lead to development of obesity, insulin resistance and diabetes.

Background

Diets rich in polyunsaturated fatty acids (PUFA) of n-3 series show many beneficial health effects, both in animal models and humans. These include effects on cardiovascular and immune systems, on glucose homeostasis, as well as on the accumulation of body fat (e.g. reviewed by [1-3]). However, recent epidemiological studies started a debate on the possible health benefits of n-3 PUFA [4, 5]. To resolve the potential health benefits of these fatty acids, knowledge of the underlying mechanisms is needed.

To elucidate molecular effects of n-3 PUFA *in vivo*, gene expression analyses have been undertaken in animal models using a variety of dietary fatty acids in several tissues, including brain, liver, heart, and adipose [6-16]. The majority of those studies focused on liver and white adipose tissue (WAT), which is not surprising given the fact that these are considered the main target organs in a dietary intervention with fatty acids. Since the intestine contributes to a significant extend to the resting metabolic rate and daily energy expenditure [17], it is of relevance to also understand the effects on this organ. Recent studies [18, 19] also showed a clear and significant difference of intestinal gene expression between diets high in diacylglycerol versus triacylglycerol, indicating a profound contribution of the small intestine to fatty acid metabolism. Moreover, induction of lipid catabolism genes in the intestine may be involved in the anti-obesity effect of diacylglycerols as compared with triacylglycerols [18, 19] and it may even contribute to a differential sensitivity of two inbred mice strains to an obesogenic high-fat diet [20].

Since the most prominent health benefits have been associated with the long-chain n-3 PUFA of marine origin (for references see [21, 22]), we have investigated the molecular effects of eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic

acid (DHA; 22:6 n-3) in n-3 high-fat diets. These diets, which do not differ in the total amount of fat relative to control, will be further referred to as EPA&DHA. In our previous studies using similar diets, we showed an anti-adipogenic effect of EPA&DHA [8, 23], which was associated with induction of mitochondrial biogenesis and beta-oxidation of fatty acids in WAT, but not in the liver [8].

We hypothesized that, using long-term dietary intervention studies, dietary fatty acid composition may modulate gene expression and lipid metabolism in the intestine, and that especially EPA and DHA may stimulate expression of genes involved in lipid catabolism. To examine this, we performed gene expression analysis of the mouse small intestine and colon, using whole genome oligonucleotide arrays and validation experiments using quantitative real time PCR (qRT-PCR). Results were confirmed in an additional animal experiment by both qRT-PCR and functional intestinal fatty acid beta oxidation measurements.

Results

Phenotypic effects of EPA&DHA

Dietary intervention with EPA&DHA in wildtype mice resulted in anti-adipogenic and anti-diabetic effects as described before: significantly lower body weight and epididymal adipose tissue weight, while food intake was non-significantly different [8, 23]. Furthermore, the intake of EPA&DHA increased adiponectin expression and secretion from WAT, and protected the mice against induction of insulin resistance by the high-fat diet [23]. Indeed, glucose tolerance tests showed significantly increased glucose tolerance (decreased area under the curves) by increasing amounts of EPA&DHA in the diets, correlating with decreased fasting plasma insulin levels (data not shown). This was associated with induction of mitochondrial biogenesis and beta-oxidation of fatty acids in WAT based on gene and protein expression, but not in the liver [8]. However, the role of the intestine in the improved whole-body metabolic phenotype has not yet been analysed in detail.

Effects of EPA&DHA on small intestinal gene expression; analysis of genes expressed in both control and intervention group.

To investigate the effects of EPA&DHA on gene expression in the intestinal tract, we isolated RNA from scrapings from small intestine of mice following a 4-week dietary intervention. We compared, using whole genome microarray analysis as initial step, the control sHF diet, which was rich in ALA and free of EPA or DHA, with the isocaloric sHFf-F2 diet, in which 44 % of lipids were replaced by an EPA and DHA concentrate.

In total, 155 probesets, corresponding to 134 well-annotated genes, showed a significant regulation with an absolute fold change (FC) ≥ 2.0 (Supplementary Table 1). This list of 134 genes comprised 110 unique genes, and increased expression was observed for 80 (102 probesets) unique genes, while 30 (32 probesets) unique genes showed decreased expression (Table 1). Functional interpretation using gene ontology and pathway analysis showed metabolism as highly regulated, which included changes in fatty acid uptake, fatty acid oxidation and cholesterol biosynthesis, amongst others. Of note, most, if not all, pathways selected were linked together by the energy molecule acetyl-CoA. The approach using a FC ≥ 1.5 as threshold resulted in a similar list of regulated pathways, of which metabolism, and especially lipid metabolism ranked highest (a more detailed description of these approaches and detailed results can be found in Supplemental Analysis). Since it is known that a dietary intervention at physiological levels generally gives small effects on gene expression changes ([24, 25], own unpublished observations), and the sufficiently large group of genes with FC ≥ 2.0, we focussed on those 100 unique, differentially expressed genes (see Table 1).

Detailed inspection of the expression data (Supplementary Table 1; see also for full names of the genes) revealed that EPA&DHA induced expression of the genes involved in branched chain and/or straight chain fatty acid β-oxidation occurring in peroxisomes and mitochondria, and fatty acid ω-oxidation, the latter is indicated by the increased expression of *Cyp4a10* (FC=5.6). Furthermore, induction of the peroxisomal biogenesis factor gene *Pex11a* (FC=2.2) might imply an increase in the number of peroxisomes.

In mitochondria, β-oxidation downstream of peroxisomal branched chain oxidation was upregulated by EPA&DHA, as suggested by increased expression of

the genes *CPT1a* (FC=2.9), and *Hadhb* (FC=2.2) amongst others. Activity of carnitine palmitoyltransferase 1 (encoded by *Cpt1a*) is rate-limiting in mitochondrial fatty acid uptake for β-oxidation. The gene encoding 3-hydroxy-3-methyglutaryl-CoA synthase *(Hmgcs2)* was also very strongly upregulated (FC=4.6), in accordance with the fact that in liver this enzyme is the rate limiting enzyme in the synthesis of ketone bodies from the acetyl-CoA generated by fatty acid β-oxidation. Importantly, also expression of *Pdk4* was increased by EPA&DHA (FC=3.0), which strongly suggested a switch from glycolysis to fatty acid oxidation [26]. Many of the aforementioned genes are targets of peroxisome proliferator activated receptor (*PPAR)* alpha [27, 28], which itself was also upregulated by EPA&DHA (FC=2.0).

Cholesterol uptake from the lumen into intestinal tissue was increased as observed by increased gene expression of *Cd36* (FC=2.3) and *Scarb1* (FC=2.2) [29]. Simultaneously, of the 21 genes included in the cholesterol biosynthesis pathway from acetyl-CoA to cholesterol, 2 were not expressed, while the majority showed an inhibition by EPA&DHA, including the rate-limiting enzyme squalene epoxidase (*Sqle*, FC=-2.1). Clearly, such a cooperative inhibition of the majority of the genes in this pathway suggests an orchestrated function within the small intestine. However, the main transcription factor regulating this pathway, *Srebf1* (SREBP), did not show differential expression. Regulation by n-3 PUFAs was likely given the fact that we could not detect a difference in the cholesterol content between the two diets (data not shown), but the exact mechanisms involved remain unexplained. Finally, downstream steroid hormone biosynthesis was upregulated as shown by a few family members of *Hsd3b* (*Hsd3b2*: FC=2.3; *Hsd3b3* FC=2.0) and *Hsd17b* (*Hsd17b4* two probesets: FC=1.6 and 2.0; *Hsd17b13* FC=2.4).

Transcription factor identification by promoter analysis

Literature data and annotation analysis was used to filter the set of regulated genes (see Methods). From the resulting 50 unique genes the promoter region was retrieved, which was used for enrichment analysis of transcription factor binding sites (TFBS). This resulted in a network of 47 genes and linked transcription factors (TFs) that comprised 42 unique input genes, including two TFs (PPARalpha and Dbp), and another 5 non-regulated TFs (NF-κB, Stat3, Sp1, Ahr, and Arnt1; Supplementary Table 4). The majority of differentially regulated genes contained binding sites for two transcription factors: PPARalpha (27 genes), which itself was significantly differentially regulated (see above), and NF-κB (20 genes). These data are in line with the well-known capacity of fatty acids to activate PPARalpha [30], as well as their known effects on NF-κB and Stat3 [3, 31].

Effects of EPA&DHA on small intestinal gene expression; analysis of genes expressed exclusively in either control or intervention group.

In addition to the genes that showed expression above the threshold in both groups, we also analysed the 159 [s1]probesets being expressed in only one diet group and showing differential expression. We focused on those genes showing a FC ≥ 2.0. In this way, fidelity of the dataset is within acceptable limits as most of these genes are expressed at low abundance. This resulted in 23 unique genes expressed only in the mice fed the EPA&DHA diet, while 47 unique genes showed only expression in the control group. All genes were classified into biological processes (Table 2, Supplementary Table 2), which largely overlapped with the pathways identified using the first subset. Of note, metabolism was also ranked highest, among which are the lipid metabolism genes acetyl-Coenzyme A carboxylase-β (*Acacb*), *Cpa2*, and the

peroxisomal protein encoded by *Acot3*, which all showed an induction from being absent in the control group to clear expression in EPA&DHA dietary group.

Inter-individual variation analysis and confirmation by real time qRT-PCR

Confirmation of differentially expressed genes was first performed using qRT-PCR. We selected a large panel of target genes covering multiple pathways and absolute FC values ranging from 1.3 to > 4. Expression changes (up- or downregulation) were confirmed for all genes (Table 3) and the FC was quite similar in all cases when compared to the microarray data.

To investigate in more detail inter-individual variation in gene expression and significance of the induced gene expression changes by nutritional treatment, we selected the following 6 genes representing the major pathways being influenced by the diet intervention (see above): fatty acid ß-oxidation in peroxisomes (*Acaa1a*) and mitochondria (*Cpt1a* and *Acacb*), the switch between glycolysis and fatty acid oxidation (*Pdk4*), biosynthesis of steroid hormones (*Hsd3b*), and biosynthesis of cholesterol (*Sqle*). For all genes we observed that the mean gene expression ratios (n=10-11 per dietary group) were similar to the observed ratios by microarray analysis of pooled samples. More importantly, gene expression changes using individual samples were statistically significant for all genes (Figure 1**,** Table 3).

Colon

The genes *Acaa1a*, *Cpt1a*, and *Sqle*, representing three pathways differentially regulated in small intestine, were analyzed by qRT-PCR in individual colon samples

(n=10). In none of the cases a significant difference in gene expression was observed (Figure 2). Exclusion of the two animals from the control group which showed lowest expression for these three genes (10-fold or more) did not influence the outcome of this analysis (maximum FC < 1.15, all non-significant; data not shown). This underscores the different function of the small intestine (mainly uptake and transfer of nutrients) and the colon (mainly water absorption).

Biological and physiological validation in 2nd animal experiment

To further validate the observed significant gene expression changes, we performed an independent second animal experiment and analysed small intestinal gene expression by qRT-PCR. Moreover, the increased gene expression suggesting increased intestinal fatty acid beta oxidation was functionally analysed, and finally, we used two EPA&DHA diets in order to investigate a dose-response relationship. In this experiment, cHF-based diets were used. Although the micronutrient composition of the control cHF diet is not defined as precisely as the semisynthetic sHFf diet used in the first experiment, and it is based on (n-6 PUFA rich) corn oil rather than on (ALA-rich) flaxseed, the control cHF diet promotes accumulation of fat much better compared with the sHFf diet and hence it allows for the detection of the anti-obesity effect of n-3 PUFA [22]. In addition to the control cHF diet, also two other cHF-based diets with 15 and 44 % of its lipids replaced by EPA&DHA (cHF-F1 and cHF-F2, respectively) were used. Gene expression analysis of genes involved in mitochondrial beta oxidation (*Cpt1a* and *Acacb*) independently and significantly confirmed the observed significant differences (Figure 3a,b). Moreover, these results showed a significant dose-response effect: increased gene expression upon increased EPA&DHA content in the diets (Figure 3a,b). Furthermore, functional analysis indeed

showed a significant dose-dependent increase in intestinal fatty acid beta oxidation upon increased EPA&DHA content in the diet compared to control diet: lowest levels in the control group, intermediate increased levels in the cHF-F1 group and highest levels in the cHF-F2 group (Figure 3c).

Discussion

Dietary intake of EPA&DHA induced changes in gene expression in the small intestine, including many metabolic genes. Especially genes involved in lipid catabolism were upregulated. In contrast, a large cluster of the genes engaged in cholesterol biosynthesis was downregulated. Importantly, all these effects were specifically induced by long-chain n-3 PUFA, EPA and DHA, as compared with their precursor ALA, and could not be detected in the colon for a selected set of genes.

It is tempting to suggest that increased catabolism of lipids induced by EPA&DHA in the small intestine contributes to the complex and beneficial effects of n-3 PUFA of marine origin. The small intestine mediates the entry of nutritional lipids and is one of the main sites of β-oxidation [32-34]. Therefore, an increase in lipid oxidation in the intestine may exert a hypolipidemic effect, i.e. one of the most pronounced effects of EPA and DHA in mammals (reviewed in [1, 3]). This effect of EPA&DHA in the intestine is surprisingly similar to the enhanced lipid oxidation induced by diacylglycerols versus triacylglycerols [18, 19]. These two types of treatments both have relatively little effects in liver and muscle ([20]; own unpublished data). In contrast, in white adipose tissue, intake of EPA&DHA induced genes of fatty acid oxidation, as well as quite specifically, mitochondrial biogenesis [8]. Taken together, EPA and DHA orchestrate gene expression adaptations in many tissues, including the intestine.

Recent studies addressing the gene expression changes of intestinal tissue upon fish oil or fatty acids, focussed on barrier genes only [16] or a focussed limited number of genes by qRT-PCR [34], while here a whole genome approach was used. This allows for detection of changes not only in the most likely pathways, but also in pathways not foreseen. Our study supports the findings by Mori et al. [34] for the

majority of their selected genes analyzed (*Cpt1a, Mod1, Pdk4, Hmgcs2, Cyp4a10*, and *Acadm*), as well as for barrier gene expression ([16], data not shown). Unexpectedly, we observed intestinal downregulation of cholesterol biosynthesis due to EPA&DHA, although this is in agreement with a similar effect observed in murine livers after tuna fish oil feeding [11]. In addition, this might coincide with a possible increase in cholesterol absorption as observed from *ScarB1*, *Cd36*, *Abca1* and *Ela3B* gene expression, even with identical cholesterol content of the diets. Intracellular homeostasis may decrease cholesterol biosynthesis to counteract increased influx. Maintenance of homeostasis is supported by non differential expression of *Soat2*/ACAT, involved in cholesterol esterification and of other genes in cholesterol metabolism (HMG-CoA reductase, *Npc1l1*, *Mttp, Abcg5, Abcg8, Nr1h2* (*LXRβ*) and *Nr1h3* (*LXRα*)). The intestinal lack of regulation of *Srebf1*/SREBP further strengthen the observations that PUFA regulation of SREBP that accounts for PUFA-mediated suppression of gene expression seems to be liver-specific [13]. Furthermore, of the regulatory machine known to be induced by DHA and EPA (PPARs, LXRs, HNF4A, and SREBPs), only PPARα showed differential expression in murine small intestine. This is further supported by our promoter-analysis of the differentially regulated genes, which showed PPARα as the major transcription factor involved.

Moreover, most if not all tissues analysed thus far show an increased energy metabolism upon n-3 FFA, and our results support the notion of the beneficial effects of fish oils independent of its n-3 effect.

Genes engaged in lipid oxidation and ketogenesis are in general upregulated in small intestine [35], liver [20], and skeletal muscle [36] by an increase in dietary fat content. When activated in the muscle, ketogenesis marks a metabolic disconnection between β−oxidation and tricarboxylic acid cycle and could lead to insulin resistance

[36]. In our study however, we compared diets with equal fat content in control and intervention groups, which only differed in their fatty acid composition. This implies that marine PUFAs specifically induce lipid catabolism in intestine. Furthermore, in comparison with another high fat Western diet [35], we observed similar (up or down) gene expression regulation by EPA&DHA diet (e.g. *Angplt4*, *Gsn*, and *Smpdl3*), as well as an inverse regulation (e.g. *ApoC2* and *H2Q10* are upregulated by a high-fat diet [35], but downregulated by EPA&DHA). Differences might be explained by the fatty acid content in the diets used.

Despite decreased adiposity due to EPA&DHA in the diet, these fatty acids have not affected total energy intake [8, 22], and also content of lipids in faeces was unaffected [22]. This strongly suggests a higher energy expenditure in the animals exposed to EPA&DHA. The results presented here indicate that one of the organs that physiologically contribute to increased oxidation of fatty acids is the small intestine.

Conclusions

In conclusion, we present data showing the involvement of small intestine in the complex changes of lipid metabolism exerted by long term dietary intake of EPA and DHA by gene expression analysis and functional *ex-vivo* beta oxidation analysis. Furthermore, we show that these effects are regulated in a dose-dependent manner. In view of its large contribution to overall energy metabolism, modulation of gene expression and metabolism in the intestine by dietary lipids, and especially long-chain n-3 PUFA of marine origin, represents a promising target for the prevention of obesity and associated co-morbidities.

Methods

Animals and diets

In the first experiment, male 4-month-old C57BL/6J mice were maintained for 4 weeks on semisynthetic high-fat (20% wt/wt) diets differing in the composition of n-3 PUFA. These mice were already used in our previous study [8]. Two isocaloric diets [8, 22] were used (n=12): control sHFf diet which contained flax-seed oil (rich in ALA) as the only lipid source, or the sHFf-F2 diet, which had the same composition except that 44 % of the lipids were replaced by a n-3 PUFA concentrate containing 6 % EPA and 51 % DHA (EPAX 1050TG; EPAX AS, Lysaker, Norway). This diet is denoted as EPA&DHA throughout the study. At the end of the experiment, mice were killed by cervical dislocation and small intestine from 3 cm under the stomach to caecum was isolated and cut lengthwise. The intestine was washed in 154 mM KCl and scraped. The epithelial cells were collected, frozen in liquid nitrogen and stored at −80°C. This procedure was also performed for 5 cm of colon.

In the second experiment, subgroups (n=9) of male 4-month-old C57BL/6J mice were fed either (i) control obesity-promoting high-fat (35 % lipids wt/wt; cHF) diet derived from standard chow diet based on corn oil, or (ii) and (iii) cHF diet with 15 and 44% of lipids, respectively, replaced by EPAX 1050TG (cHF-F1 and cHF-F2, respectively; see [22]). After 6 weeks, intestinal tissue was isolated for fatty acid beta oxidation and gene expression analysis by quantitative RT-PCR (qRT-PCR) only. Macronutrient composition, energy density, and fatty acid composition of dietary lipids of all the diets used in this study is well characterised [22]. The experiments were conducted

under the guidelines for the use and care of laboratory animals of the Institute of Physiology, Academy of Sciences of the Czech Republic.

RNA isolation

Extraction of total RNA was performed with the use of TRIzol (Invitrogen, Breda, The Netherlands). RNeasy columns (Qiagen, Venlo, The Netherlands) were used to purify the RNA. Quantitative and qualitative measures were performed using Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA). For the first animal experiment, four samples did not pass quality thresholds and were excluded from further analyses (two control small intestine samples and one EPA&DHA small intestine sample and one EPA&DHA colon sample). The remaining RNA samples were pooled per tissue per diet and integrity was analyzed after keeping the samples for 1 hour at 37°C using an Agilent bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands) with a RNA 6000 Nano LabChip kit, according to the manufacturer's instructions. The two pooled colon samples were not used further due to their apparent partial degradation (RNA Integrity Number (RIN) < 7.0). All individual RNA samples from the second animal experiment passed quality control criteria, and were used for subsequent qRT-PCR analysis.

Array hybridization and scanning

The pools of small intestine RNA samples (control (n=10) and EPA&DHA (n=11)) were hybridized on separate Affymetrix MOE430_2 GeneChip mouse arrays (Santa Clara, CA, USA). This array contains 45,102 probesets, detecting over 39,000 transcripts that represent 16,579 unique genes. Detailed methods for labelling and

subsequent hybridizations to the arrays are described in the eukaryotic section in the GeneChip Expression Analysis Technical Manual Rev. 3 from Affymetrix, and are available upon request. Arrays were scanned on a GeneChip Scanner 3000 (Affymetrix).

Microarray data analysis

Quality of the data was assessed on diagnostic plots generated from the raw, non-processed data, as described [37]. All arrays passed these strict criteria and were included in the analyses.

The Affymetrix default algorithm (MAS 5.0) was used to summarize data and significance of observed gene expression changes (present/absent call per probeset per array, fold change (FC) between normalized arrays, and its significance by the p-value). In total, 24270 probesets (54%) showed expression at least in either one of the arrays. Significant differentially expressed probesets were identified by direct comparison between the two dietary groups for all probesets called present on both arrays. Probesets that satisfied the general Affymetrix criterion of t-test probability < 0.27% (p-value < 0.0027) were considered to be significantly regulated, and these were further investigated. Probesets were annotated using information provided by Affymetrix (release of July 12th, 2006) and all gene symbols are presented throughout the article according to Mouse Genome Informatics [38].

Pathway analysis, including ranking, was done using Metacore (GeneGo, St. Joseph, MI, USA). The data of functionally annotated genes only were analyzed in two subsets. In order to successfully rank the most prominent differentially regulated pathway(s), a large set of genes was analyzed. In this way, those pathways showing a high number of significant regulated genes, given the (high) number of genes

expressed within the pathway, will rank highest. The first subset comprised those genes of which expression was present in both dietary conditions. Pathway analysis was done using a cut-off of absolute FC ≥ 1.5 or ≥ 2.0 and the p-value per gene as provided by MAS5.0, in order to increase detection of biological relevant regulated pathways. The second subset comprised probesets expressed in only one dietary group, and therefore absent in the other (FC ≥ 2.0). The given FCs as generated by MAS 5.0 were used, although in view of the absence of expression under one dietary condition these may not be reliable, hence the choice of separate analysis. Array data have been submitted to the Gene Expression Omnibus, accession number GSE11936.

Promoter analysis to identify transcription factor regulation

All differentially regulated probesets, as shown in Table 1, were analyzed using Genomatix BiblioSphere Pathway Edition software, without any pre-selection in up- or down-regulation. Unique genes were identified and those genes plus gene-gene interactions were filtered [39] for organ-specificity using MeSH [Medical Subject Heading] "Digestive System" [A03]. This resulted in a set of 50 unique input genes. Promoter regions of around 650 bp upstream of transcription start site per gene were analysed for known transcription factor binding sites (TFBS) combined with prior knowledge in literature. Criteria for this analysis were: only direct interactions were considered, a minimum of 3 published articles should describe the functional interaction (so called "function word level B2"), and for inclusion of a transcription factor (TF) showing TF-gene interaction, the additional criterion of at least two different input genes having this specific TFBS was compulsory. In this way we analyzed specifically only TF-gene interactions relevant for the gastrointestinal tract.

Quantitative real time PCR

qRT-PCR was performed according to Van Schothorst et al. [40]. Briefly, 1 µg of total RNA was used for cDNA synthesis. Primers were designed using Beacon Designer (Biosoft International, Palo Alto, USA) and ordered by Biolegio (Malden, The Netherlands). Gene symbols, names, accession numbers and primer sequences are listed in Supplementary Table 3. Analysis of the reaction efficiency [41] was performed with a dilution series of pooled cDNAs serving as standard curve. Briefly, after a 3 minute denaturation at 94°C, 40 cycles of 15 seconds at 94°C and 30 seconds at 59°C, were followed by a melting curve gradient. Calnexin (*Canx*) and hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*) were used as reference genes based on least variation observed in microarray data analysis. *Canx* showed most stable expression and was used and shown in all cases. Endorsable data were obtained using *Hprt1*. C_t-values of the two pools of dietary groups were measured for 17 genes in triplo and averaged, followed by calculation of the relative gene expression using the $2^{-\Delta\Delta CT}$ method [42].

Relative target gene expression was measured in more detail using individual samples for the genes *Cpt1a, Acaa1a, Sqle, Acacb, Pdk4* and *Hsd3b*, of which the latter three were not analysed using pooled RNA samples. Samples were run in duplicate, averaged, and relative gene expression was calculated using the standard curve method. qRT-PCR analyses for the second animal experiment were performed using individual RNA samples for target genes *Cpt1a* and *Acacb*, as described above. All values are presented as mean ± SE. In case of only 2 groups (first experiment), differences between groups were analyzed using Student t-tests with two-tailed, unequal variances and significance expressed at $p<0.05$, or lower as indicated, while differences between three groups (second experiment) were analysed using one-way

ANOVA and Tuckey's posthoc tests and significance expressed at $p<0.05$, or lower as indicated.

Fatty acid beta oxidation

Intestinal fatty acid beta oxidation was measured as published [43]. Briefly, samples were incubated in Krebs-Ringer bicarbonate buffer with [^{14}C(U)]-palmitate, $^{14}CO_2$ was trapped in hyamine hydroxide, quantified by liquid scintillation counting, and oxidation rate was normalized to tissue DNA content [44]. All data are presented as mean ± SE. Differences between groups were analyzed using one-way ANOVA and Tuckey's post hoc tests and considered statistically significance at $p<0.05$.

Authors' contributions

EvS, PF, NFvH, JKo and JKe conceived the studies, supervised the execution and drafted the mansucript. EvS, PF, OK, AB, JM, CV, and GH performed animal studies, RNA isolation, qRT-PCRs, microarray hybridisation and analyses, or fatty acid beta oxidation measurements. All authors have read and approved the final manuscript.

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Figures

Figure 1. Gene expression in small intestine.

Quantatative real-time PCR (qRT-PCR) was used to determine normalized gene expression levels in small intestine of individual wildtype mice that received the control diet (n=10, white bars) or EPA&DHA diet (n=11, black bars). Gene expression levels were normalized using calnexin and averaged per group; the mean expression level of the control group was arbitrarily set at 1. Bars are presented as mean ± standard error.

Genes shown (with the mean ratio between the groups shown in parenthesis) are **a** Acaa1 (2.52), **b** Acacb (3.91), **c** Cpt1a (2.79), **d** Hsd3b (1.91), **e** Pdk4 (3.24), **f** Sqle (-1.55). Statistical significance was analyzed using Student's *t*-test: * $p<0.05$, ** $p<0.01$. AU, arbitrary units.

Figure. 2. Gene expression in colon.

qRT-PCR was used to determine normalized gene expression levels in colon of individual wildtype mice that received the control diet (n=12, white bars) or EPA&DHA diet (n=11, black bars). Gene expression levels were normalized using calnexin and averaged per group; the mean expression level of the control group was arbitrarily set at 1. Bars are presented as mean ± standard error.

Genes and fold changes in parenthesis shown are **a** Acaa1 (1.29), **b** Cpt1a (1.37), **c** Sqle (1.08). All changes are non–significant ($p>0.2$, Student's *t*-test). AU, arbitrary units.

Figure 3. Dose-dependent intestinal gene expression and fatty acid beta oxidation.

In a second animal experiment, intestinal gene expression analysis was used to confirm and biologically validate observations in a dose-dependent manner. Gene expression analysis was performed using qRT-PCR of small intestinal samples of individual wildtype mice that received the control diet (n=9, white bars), EPA&DHA diet (n=9, black bars), and intermediate levels of dietary EPA&DHA (n=9, hatched bars). The triangle shows increasing dietary EPA&DHA content. Gene expression levels were normalized using calnexin and averaged per group; the mean expression level of the control group was arbitrarily set at 1. Bars represent mean ± standard error. Genes shown are **a:** Cpt1a, and **b:** Acacb. Functional fatty acid beta oxidation was used for biological validation (**c**). Intestinal tissue fatty acid beta oxidation (n=7–9) is shown for mice that received control diet (white bars), EPA&DHA diet (black bars), and intermediate levels of dietary EPA-DHA (hatched bars). The triangle shows increasing dietary EPA&DHA content. Statistical significance was analyzed using one-way ANOVA and Tuckey's post hoc tests: * $p<0.05$, *** $p<0.01$. AU, arbitrary units.

Table legends

Table 1. Differentially expressed, unique genes classified in biological processes.

Process	Upregulated	Downregulated	Total
Metabolism	40	9	49
Transport	4	3	7
Apoptosis/cell cycle	4	2	6
Cell adhesion/homeostasis/structure	4	1	5
Digestion	4	0	4
Immune system	1	3	4
Hemopoiesis	1	2	3
Transcription/translation	3	0	3
Signal transduction	2	0	2
Steroid hormone metabolism	2	0	2
Miscellaneous/unknown	8	7	15
Total:	73	27	100

Unique genes (expressed in both dietary groups, FC $\geq$ 2.0) are classified in 11 processes, and ranked on total number of genes per process. Number of genes upregulated or downregulated by EPA&DHA in small intestine is as indicated.

Table 2. Differentially expressed genes with exclusive expression in only EPA&DHA or control dietary group.

Process	Expressed in EPA & DHA diet	Expressed in control diet	Total
Metabolism	11	4	15
Transcription/translation/ splicing	3	6	9
Cell adhesion/ proliferation/ differentiation/ structure	-	7	7
Signal transduction	-	7	7
Transport	1	5	6
Protein folding/modification	1	4	5
Cell cycle	1	1	2
Growth factor	1	1	2
Miscellaneous/unknown	5	12	17
Total:	23	47	70

Annotated, unique genes (FC $\geq$ 2) were classified in biological processes. Processes were ranked on total number of genes being differentially expressed in small intestine.

Table 3. Microarray data validation by qRT-PCR

		Microarray ratio	qRT-PCR ratio	
Genes	Probeset ID	Pool	Pool	mean ratio (n=10-11)
Acaa1a	1456011_x_at	1.67	2.00	2.52 *
Acaa1b	1424451_at	2.19	1.75	
Acaa1a/Acaa1b	1416946_a_at	1.87		
	1416947_s_at	1.59		
Acacb	1427052_at	6.9 [a]	n.d.[c]	3.91 *
Acox1	1416409_at	1.24	1.34	
	1416408_at	1.35		
Acox2	1420673_a_at	2.91	1.97	
Acox3	1420684_at	n.c.[b]	-1.23	
Cpt1a	1434866_x_at	2.87	1.87	2.79 **
	1438156_x_at	2.60		
Cpt2	1416772_at	1.39	1.44	
Ela2	1448281_a_at	2.04	4.26	
Ela3b	1415884_at	2,64		
	1435611_x_at	4,20		
	1415883_a_at	4,38	7.89	
	1437326_x_at	4,72		
	1435012_x_at	5,17		
H2-Q10	1425137_a_at	-8.22	-2.39	
Hmgcs1	1433443_a_at	-1.82	-2.00	
	1433446_at	-1.57		
Hmgcs2	1431833_a_at	3.61	3.97	
	1423858_a_at	4.56		
Hsd3b2/6	1460232_s_at	2.50	n.d.	1.91 *
Hsd3b3	1427377_x_at	2.00		
Mod1	1416632_at	2.16	2.60	
	1430307_a_at	2.13		
Pck1	1423439_at	2.17	2.21	
	1439617_s_at	2.46		
Pdk4	1417273_at	3.01	n.d.	3.24 *
Sqle	1415993_at	-2.14	-2.35	-1.55 *

Genes are sorted alphabetically. In several cases gene family members were included for validation, although they did not show differential microarray gene expression. Data is represented by ratio of EPA&DHA/control diet. For qRT-PCR, data shown is obtained using a pool of samples (pool), and/or individual samples (EPA/DHA: n=11, control diet: n=10) and the mean ratio was calculated. [a] this probeset is non-expressed (absent) in control diet; ratio is 6.9; [b] n.c.: not changed. [c] n.d.: not analyzed; statistical significance was calculated using Student's *t*-test: * $p<0.05$, ** $p<0.01$.

Supplementary Material:

All files are in PDF format.

1. Supplementary Table 1.

Differentially regulated probesets expressed in both dietary groups.

All significant differentially expressed probesets of the microarrays which are shared between the two dietary groups (Fold change ≥ 2.0).

2. Supplementary Table 2.

Differentially regulated probesets expressed in only one dietary group.

All differentially expressed probesets of the microarrays which are unique for either one of the dietary groups.

3. Supplementary Table 3.

Real-time quantitative PCR: genes and primers.

Gene Symbols, Names, Annotation (RefSeq), and primer sequences for qRT-PCR are shown.

4. Supplementary Table 4.

Transcription factors enriched for involvement with differentially expressed genes.

Transcription factors were identified on the basis of an enriched presence of TFBS in the promoter region of the set of significantly differentially regulated genes, combined with data from scientific publications.

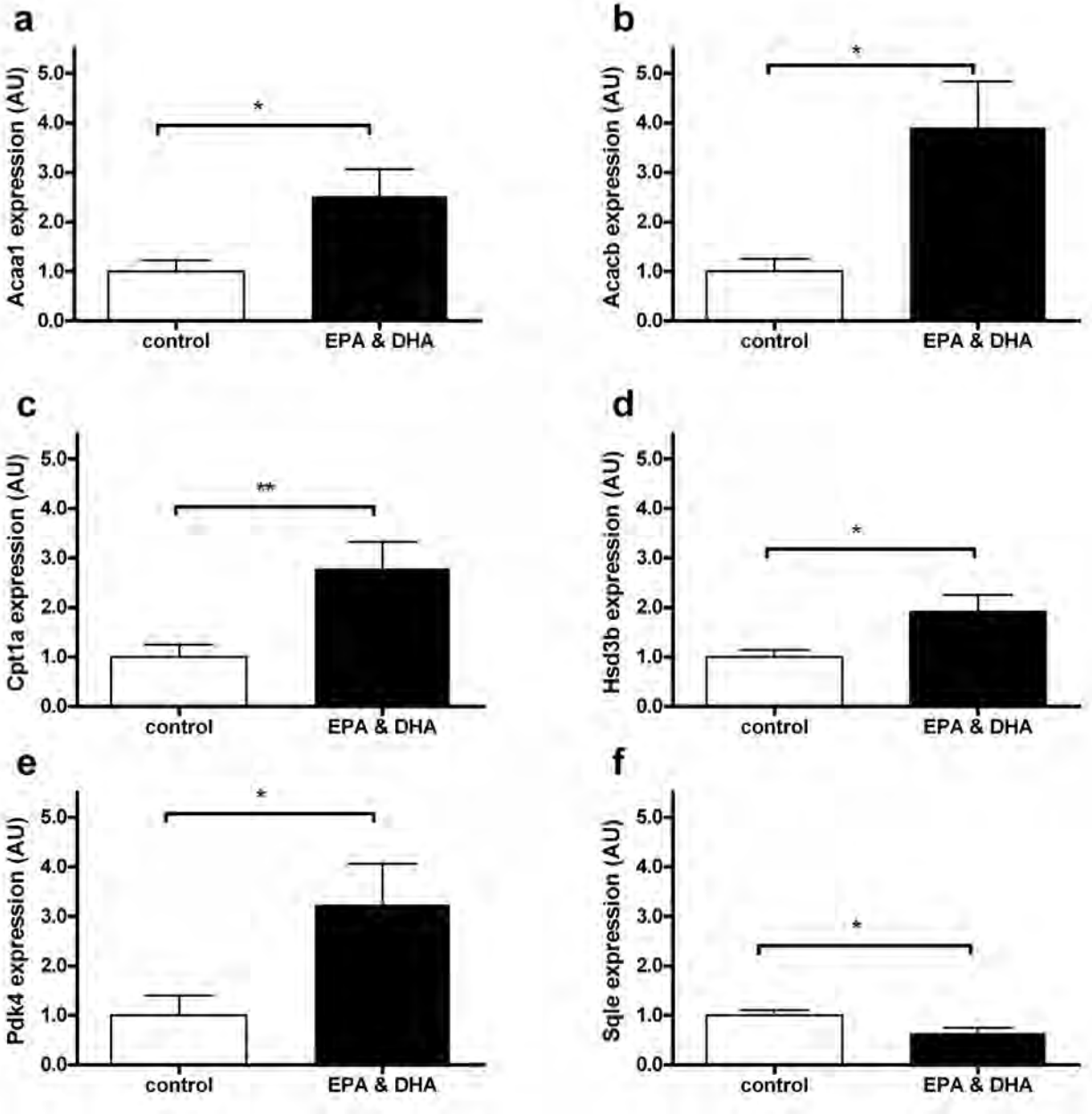
a
Acaa1 expression (AU)
5.0
4.0
3.0
2.0
1.0
0.0
*
control
EPA & DHA
b
Acacb expression (AU)
*
control
EPA & DHA
c
Cpt1a expression (AU)
**
control
EPA & DHA
d
Hsd3b expression (AU)
*
control
EPA & DHA
e
Pdk4 expression (AU)
*
control
EPA & DHA
f
Sqle expression (AU)
*
control
EPA & DHA

Figure 1

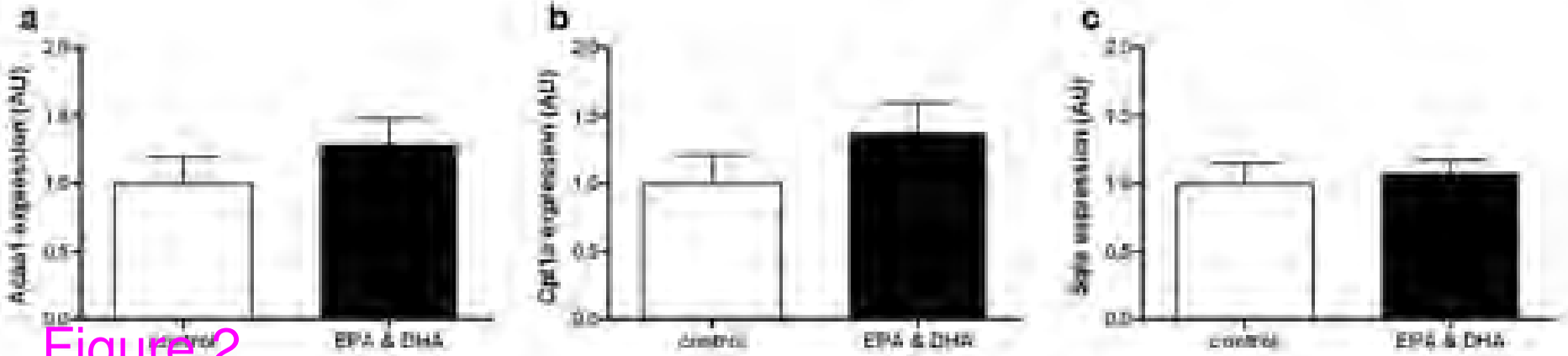

Figure 2

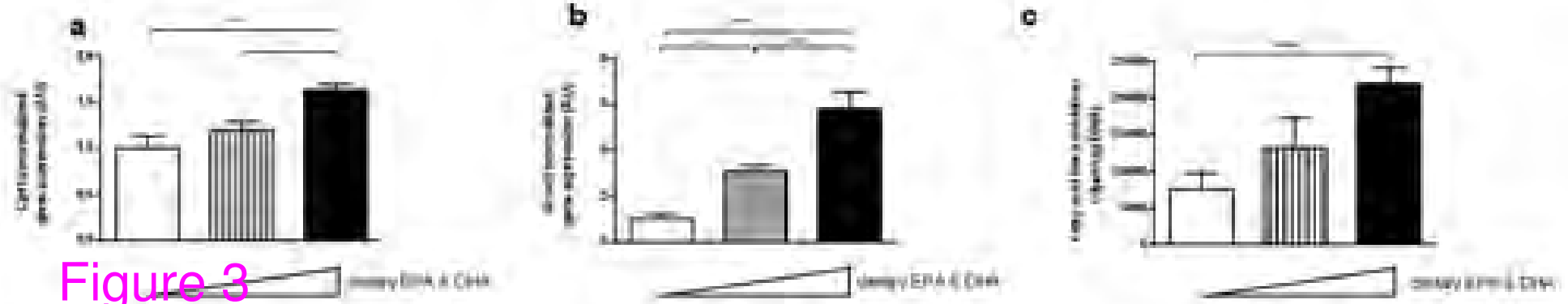

Figure 3

An increase in plasma adiponectin multimeric complexes follows hypocaloric diet-induced weight loss in obese and overweight pre-menopausal women

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ABSTRACT

Adiponectin is involved in the regulation of glucose and fatty acid metabolism, influences whole-body insulin sensitivity and protects arterial walls against the development of atherosclerosis. Plasma adiponectin is decreased in obese, insulin-resistant and Type 2 diabetic patients. Adiponectin circulates in plasma as high-, medium- and low-molecular mass forms (HMW, MMW and LMW respectively). The HMW form is believed to be closely associated with insulin sensitivity. The aim of the present study was to investigate whether diet-induced changes in body weight and insulin sensitivity were associated with changes in the quantity of adiponectin multimeric complexes. A total of 20 overweight or obese women (age, 39.4 ± 9.5 years; body mass index, 32.2 ± 6.4 kg/m^2) underwent 12 weeks of low caloric diet (600 kcal/day less than energy requirements; where 1 kcal $\approx$ 4.184 kJ). Plasma samples were drawn before and after the study for biochemical analysis and Western blot detection of adiponectin multimeric complexes. The hypocaloric diet resulted in a weight reduction (89.8 ± 16.4 kg compared with 83.1 ± 15.6 kg; $P < 0.001$) and an improvement in whole-body insulin sensitivity, as measured by HOMA (homoeostasis model assessment index; 1.9 ± 0.8 compared with 1.5 ± 0.7; $P = 0.013$). Increases in the quantities of HMW, MMW and LMW by 5.5, 8.5 and 18.1 % respectively, were observed ($P < 0.05$ for all of the forms). Total plasma adiponectin was increased by 36 % with borderline significance ($P = 0.08$). No correlations between changes in adiponectin complexes and changes in indices of insulin sensitivity were observed. In conclusion, diet-induced weight loss improved insulin sensitivity as well as increased the amount of HMW, MMW and LMW adiponectin complexes in plasma.

Key words: adiponectin, adipose tissue, dietary intervention, insulin resistance, multimeric complex, obesity, weight loss.

Abbreviations: AMPK, AMP-activated kinase; BMI, body mass index; CV, coefficient of variability; HDL-cholesterol, high-density lipoprotein cholesterol; HMW, high-molecular mass; HOMA, homoeostasis model assessment index; LCD, low-calorie diet; LDL-cholesterol, low-density lipoprotein cholesterol; LMW, low-molecular mass; MMW, medium-molecular mass; PBS-T, PBS containing 0.5 % Tween 20; TZD, thiazolidinedione; WHR, waist/hip ratio.

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INTRODUCTION

Obesity is known to be associated with a whole-body pro-inflammatory state and a number of metabolic disturbances included in the metabolic syndrome. In the search for the mechanisms that explain the link between obesity and metabolic syndrome, it has been suggested that endocrine substances produced by adipose tissue (adipocytokines) might play a role [1,2]. Adiponectin is a 30 kDa plasma protein secreted by mature adipocytes [3], representing 0.01 % of total plasma proteins [4]. Plasma adiponectin levels have been shown to be reduced in patients with Type 2 diabetes [5], insulin-resistant subjects [6] and obese individuals [4], as well as in patients with coronary heart disease [5]. These findings suggest that adiponectin has insulin-sensitizing and anti-atherosclerotic effects [7]. In human plasma, adiponectin has been shown to circulate in distinct multimeric complexes forming trimeric LMW (low-molecular mass), hexameric MMW (medium-molecular mass) and oligomeric HMW (high-molecular mass) complexes. However, some investigators, using different analytical methods, have distinguished only two adiponectin isoforms: LMW and HMW [6,8–12]. Adiponectin cell-surface receptors (AdipoR1 and AdipoR2) are expressed in muscle, liver and adipose tissue [13,14]. In myocytes and hepatocytes (mouse model), adiponectin stimulates phosphorylation and the activation of AMPK (AMP-activated protein kinase) [15], a key regulatory enzyme in glucose and lipid metabolism, inducing glucose uptake and fatty acid oxidation in muscle [15,16] and reducing hepatic gluconeogenesis [17]. Activation of different cellular transduction pathways appears to be specific for different multimeric complexes in different organs [18,19].

Previous investigations revealed that plasma HMW adiponectin levels are positively associated with insulin sensitivity indices [10,12,20,21] and plasma HDL-cholesterol (high-density lipoprotein-cholesterol), whereas a negative association with BMI (body mass index) and central body fat mass has been observed [10]. Mutations in the adiponectin gene associated with impaired formation of HMW complexes have been phenotypically connected with hypoadiponectinaemia and Type 2 diabetes in humans [18].

Weight loss achieved through caloric restriction (accompanied by physical activity in some studies) is an important and cost-effective measure in the treatment of obese individuals who are at a high risk of developing Type 2 diabetes and atherosclerosis [22–24]. In this context, the role of adiponectin and the distribution of its multimeric complexes have attracted interest because of their insulin-sensitizing and anti-atherosclerotic effects. Recently published studies of changes in adiponectin multimeric complexes following dietary intervention have yielded contradictory results; some showing no changes in their distribution [25], whereas others found increased quantities of HMW and MMW complexes [8]. These conflicting results might be partially attributable to small sample sizes and/or the inclusion of both men and women in the above-mentioned studies, as marked gender differences in total plasma adiponectin and in the distribution of multimeric complexes have been demonstrated [5].

The aim of the present study was to investigate the effect of a 12-week LCD (low-calorie diet) on plasma adiponectin multimeric complexes in relation to biochemical and anthropometrical parameters in a cohort of obese pre-menopausal women.

MATERIALS AND METHODS

Subjects

A total of 20 pre-menopausal women (age, 39.4 ± 9.5 years; body weight, 89.8 ± 16.4 kg; BMI, 32.2 ± 6.4 kg/m^2) participated in the study. Subjects were recruited by a referral from collaborating obesity units and through an advertisement in the local media. Obesity was defined as BMI $\geqslant$ 30 kg/m^2, and 13 women were obese and seven women were overweight, with a BMI of 26–30 kg/m^2. Elevated plasma total cholesterol (> 5.0 mmol/l) was present in 16 women (range, 4.1–8.1 mmol/l), elevated plasma LDL-cholesterol (low-density lipoprotein cholesterol; > 3 mmol/l) was present in 17 women (range, 2.5–5.6 mmol/l), and hypertriglyceridaemia (> 1.7 mmol/l) was found in two women (range, 0.8–2.1 mmol/l). All of the participants had a blood pressure below 135/80 mmHg. Two women had impaired fasting glucose levels at baseline, but none of the participants were found to have diabetes. None of the women had any other chronic disease and all were medication free. Pregnancy was excluded at the beginning of the study. The subjects had a stable body weight for at least 3 months prior to the beginning of the study. Each participant gave her written informed consent before starting the study. All aspects of the study were performed in accordance with the Declaration of Helsinki and were approved by the Ethical Committee of the Third Faculty of Medicine, Charles University, Prague, Czech Republic.

Clinical protocol

The subjects were investigated at 08.00 hours after an overnight fast before and again at the end of the 12-week LCD (see the description of the dietary intervention below). Body weight, waist circumferenc and hip circumference were measured. Body composition was assessed using the multi-frequency bioimpedance method (Quad scan 4000; Bodystat). CVs (coefficients of variability) of fat mass and fat-free mass were 1.7 and 0.8 % respectively. Blood samples for plasma analyses were collected from an indwelling polyethylene catheter inserted into the antecubital vein. After collection, blood was processed

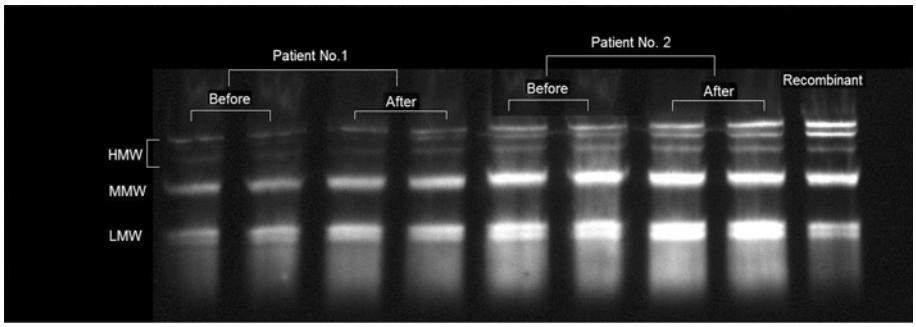

Figure 1 A representative example of the chemiluminescent detection of adiponectin multimeric complexes

Each sample was run in duplicate. Samples were drawn before and after the intervention and were run on the same gel. Recombinant human adiponectin served as a standard for the normalization of the individual band intensities of the patients' samples. The chemiluminescent signal was detected using the FujiFilm LAS 1000 detection system.

immediately in a refrigerated centrifuge. The plasma was stored at −80 °C until analysis.

Dietary intervention

The diet was designed to provide 600 kcal/day (where 1 kcal ≈ 4.184 kJ) less than individually calculated energy requirements, based on the subject's measured pre-treatment resting metabolic rate multiplied by 1.3 (a coefficient of correction for physical activity level, assuming a sedentary lifestyle). Subjects were requested to abstain from alcohol consumption during the study. The diet was designed to provide 25–30 % energy derived from fat, 55–60 % energy from carbohydrates and 10–15 % energy from proteins. Dietary instructions were reinforced and monitored weekly by dieticians throughout the intervention period. Subjects were instructed to follow their habitual patterns of physical activity during the study. A 3-day food record (two weekdays and one weekend day) was obtained from each participant and checked before the study and each week during the study. The dietary records were analysed using a country-specific food-nutrient database [NutriDan 1.2.; Mullerova D., Tychtl Z., Muller L. and Brazdova Z. (2002), produced in cooperation with the Danone Institute and distributed by DADI, Plzen, Czech Republic]. All of the subjects finished the study and, based on follow-up interviews with the study dietician, their compliance with the diet was very high.

Analytical methods

Plasma glucose was determined using the glucose-oxidase technique (Beckman Instruments). Plasma insulin concentration was measured using an Insulin RIA kit (Immunotech; CV, 2.8–4 %). Total plasma adiponectin concentration was measured using an Adiponectin Human ELISA kit (Biovendor Laboratory Medicine; CV, 4.1 %).

The HOMA (homoeostasis model assessment) index was calculated using the following equation: [fasting glucose (mmol/l) × fasting insulin (milli-international units/ml)]/22.5. Plasma LDL-cholesterol was calculated using the Friedewald equation: LDL-cholesterol = total cholesterol − HDL-cholesterol − (triacylglycerols/5).

Quantification of the adiponectin multimeric complexes

Samples (10 μl) of plasma, diluted (1:2) with Laemmli sample buffer (without 2-mercaptoethanol and SDS), were resolved using PAGE under non-reducing and non-denaturing conditions, as described previously [18,26]. Proteins were then transferred on to a nitrocellulose membrane, blocked for 1 h with 5 % (w/v) low-fat milk in PBS-T (PBS containing 0.5 % Tween 20) and incubated overnight with primary rabbit polyclonal anti-(human adiponectin) sera (Biovendor Laboratory Medicine) diluted 1:1000 in 1 % (w/v) low-fat milk in PBS-T. A second incubation (45 min) was carried out with secondary antibody [goat anti-(rabbit IgG) conjugated with horseradish peroxidase; Jackson ImmunoResearch] diluted 1:10 000 in 1 % (w/v) low-fat milk in PBS-T. Antibody binding was detected using a chemiluminescent substrate (Luminol; Sigma–Aldrich) [27] and was visualized using a FujiFilm LAS 1000 detection system. Band intensities were analysed using AIDA software. Plasma samples taken before and after the intervention were run on the same gel in duplicate (Figure 1). Signal intensities from the duplicate samples were averaged and used for statistical analysis. Native molecular-mass standards (Protein Markers for Native PAGE; Serva) and recombinant adiponectin (Adiponectin Human-HEK; Biovendor Laboratory Medicine) were also run on each gel. The individual signal intensity of each band was normalized using the intensity of the MMW form of the recombinant adiponectin protein. The standard was run using an identical concentration on all of the gels. These data were analysed using non-parametric tests and are shown in the Tables. CV of the Western blot analysis was 7.5 %.

Table 1 Anthropometric and biochemical characteristics of subjects before and after a 12-week LCD

Values are means ± S.D., $n = 20$. mIU, milli-international units.

Characteristic	Before LCD	After LCD	*P* value
Body weight (kg)	89.8 ± 16.4	83.1 ± 15.6	< 0.001
BMI (kg/m^2)	32.2 ± 6.4	29.8 ± 6.2	< 0.001
Fat mass (kg)	37.8 ± 11.4	33.3 ± 11.7	< 0.001
Waist circumference (cm)	96.4 ± 13.3	88.8 ± 12.0	< 0.001
Hip circumference (cm)	118.6 ± 11.8	112.7 ± 12.0	< 0.001
WHR	0.81 ± 0.07	0.79 ± 1.0	0.004
Fasting glucose (mmol/l)	5.1 ± 0.4	5.0 ± 0.4	0.120
Fasting insulin (mIU/l)	8.2 ± 3.2	6.6 ± 2.9	0.014
Total cholesterol (mmol/l)	5.8 ± 1.0	5.3 ± 0.8	0.003
HDL-cholesterol (mmol/l)	1.7 ± 0.3	1.5 ± 0.2	< 0.001
LDL-cholesterol (mmol/l)	3.8 ± 0.8	3.6 ± 0.6	0.062
Triacylglycerols (mmol/l)	1.3 + 0.4	1.0 ± 0.3	0.002
HOMA index	1.9 ± 0.8	1.5 ± 0.7	0.013

Table 2 Adiponectin multimeric complexes and total plasma adiponectin before and after a 12-week LCD

Values are means ± S.D., $n = 20$. QL, quantity of light units normalized by reference to the QL of recombinant adiponectin MMW.

	Before LCD	After LCD	*P* value
Total adiponectin			
Determined by ELISA (μg/ml)	3.2 ± 1.7	4.4 ± 3.9	0.080
Determined by Western blotting (QL)	575.0 ± 215.1	655.5 ± 219.1	0.007
HMW (QL)	84.9 ± 37.1	89.6 ± 37.9	0.008
MMW (QL)	133.9 ± 57.3	145.1 ± 55.2	0.045
LMW (QL)	356.2 ± 138.6	420.8 ± 145.2	0.003
HMW/total ratio	0.14 ± 0.04	0.13 ± 0.04	0.009
HMW/LMW ratio	0.55 ± 0.25	0.52 ± 0.25	0.026
HMW/MMW ratio	0.65 ± 0.25	0.63 ± 0.24	0.461

Although Western blot analyses are semi-quantitative in nature, comparisons of samples were possible when plasma samples derived before and after the intervention were run on the same gel. Therefore Western blots provide a useful tool in analysing all of the adiponectin multimeric complexes in human plasma, as has been demonstrated in other studies [25].

Statistical analysis

Statistical analysis was performed using SPSS 12.0 for Windows. The effect of weight loss was tested using the Wilcoxon sign rank test for paired observations for all of the variables studied. Univariate correlations were analysed using Spearman's correlation test. Values are means ± S.D. A level of $P \leqslant 0.05$ was considered statistically significant in all of the tests. The analyses of the differences of the means of all variables reached a power exceeding 80 %, except for total adiponectin (ELISA), MMW and LMW forms and insulin, where the power was 44, 66, 58 and 41 % respectively.

RESULTS

Anthropometric and biochemical parameters

Anthropometric and biochemical characteristics of the subjects before and after the diet intervention are summarized in Table 1. An LCD for 12 weeks resulted in a reduction in body weight, BMI and waist circumference by 7.4, 7.3 and 7.9 % respectively. Fat mass diminished by 11.8 % and was also accompanied by a 4 % decrease in fat-free mass. The diet intervention improved the plasma lipid profile of the subjects; total cholesterol decreased by 8.5 % and triacylglycerol decreased by 21 % during the study. An 11 % decrease in HDL-cholesterol was also observed.

Fasting plasma insulin levels and HOMA index of insulin resistance were lowered by 19.3 and 21.9 % respectively, reflecting an improvement in whole-body insulin sensitivity. Fasting plasma glucose concentrations remained unchanged following the intervention.

Total plasma adiponectin and adiponectin multimeric complexes

Total plasma adiponectin levels, measured using ELISA, increased by 36 % as a result of the LCD intervention; however, this difference was of borderline significance ($P = 0.08$). When the total adiponectin concentration was determined using Western blotting with chemiluminescence detection, a 13.5 % increase ($P = 0.007$) was observed. The LCD induced increases in HMW, MMW and LMW plasma levels by 5.5, 18.1 and 8.5 % respectively. Reductions in HMW/total adiponectin and HMW/LMW ratios were detected, whereas the HMW/MMW ratio remained unchanged. These results are summarized in Table 2.

Relationship between total plasma adiponectin and anthropometric/metabolic variables

Total plasma adiponectin levels were negatively associated with fasting insulin, HOMA index and HDL-cholesterol; however, these correlations were not significant before or after the study. No other associations between total plasma adiponectin and the biochemical, anthropometrical and insulin-sensitivity parameters analysed were observed before or after the

Table 3 Spearman's correlation coefficients between the variables before a 12-week LCD

*Significant difference. TPA-ELISA, total plasma adiponectin measured using ELISA.

	HMW		MMW		LMW		TPA-ELISA	
Variable	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
Weight	0.206	0.384	0.347	0.133	0.373	0.105	0.172	0.468
BMI	0.132	0.578	0.141	0.552	0.319	0.170	−0.310	0.180
Fat mass	0.094	0.693	0.162	0.496	0.228	0.334	0.028	0.907
Fat-free mass	−0.094	0.693	−0.162	0.496	−0.228	0.334	−0.028	0.907
Waist circumference	−0.043	0.857	−0.072	0.762	0.177	0.455	0.101	0.672
Hip circumference	0.269	0.251	0.378	0.100	0.337	0.146	0.204	0.388
WHR	−0.292	0.211	0.378	0.100	−0.157	0.507	0.006	0.981
Triacylglycerols	−0.125	0.600	−0.335	0.149	−0.221	0.349	−0.139	0.560
HDL-cholesterol	0.269	0.265	0.527*	0.021	0.101	0.681	0.290	0.229
LDL-cholesterol	0.261	0.266	0.107	0.663	0.093	0.696	0.221	0.349
Fasting glucose	−0.564*	0.010	−0.447*	0.055	−0.262	0.265	−0.013	0.957
Fasting insulin	−0.196	0.409	−0.305	0.192	0.091	0.703	−0.276	0.239
HOMA index	−0.262	0.265	−0.328	0.158	0.032	0.895	−0.257	0.274

Table 4 Spearman's correlation coefficients between the variables after a 12-week LCD

*Significant difference. TPA-ELISA, total plasma adiponectin measured using ELISA.

	HMW		MMW		LMW		TPA-ELISA	
Variable	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
Weight	0.168	0.478	0.358	0.121	0.268	0.254	0.186	0.431
BMI	0.033	0.890	0.192	0.416	0.168	0.478	0.102	0.668
Fat mass	0.131	0.582	0.212	0.369	0.179	0.450	0.021	0.930
Fat-free mass	−0.131	0.582	−0.047	0.845	−0.161	0.498	−0.182	0.442
Waist circumference	−0.153	0.519	0.042	0.860	0.017	0.945	0.222	0.346
Hip circumference	0.260	0.269	0.275	0.241	0.069	0.771	−0.042	0.859
WHR	−0.491*	0.028	−0.371	0.107	−0.147	0.535	0.198	0.403
Triacylglycerols	0.005	0.985	−0.201	0.396	−0.300	0.199	0.160	0.500
HDL-cholesterol	0.256	0.154	0.313	0.179	0.106	0.656	0.032	0.892
LDL-cholesterol	0.235	0.319	−0.102	0.668	0.195	0.409	−0.256	0.277
Fasting glucose	−0.299	0.214	−0.220	0.366	0.006	0.981	−0.047	0.843
Fasting insulin	0.116	0.627	−0.131	0.582	0.060	0.801	−0.129	0.589
HOMA index	0.051	0.830	−0.108	0.650	0.054	0.821	−0.110	0.645

intervention period. These results are summarized in Tables 3 and 4.

Relationship between adiponectin multimeric complexes and anthropometric/metabolic variables

Among the multimeric complexes analysed, the HMW form was closely associated with fasting glucose level ($r = -0.564$, $P = 0.010$) and the MMW form was closely associated with HDL-cholesterol ($r = 0.527$, $P = 0.021$) at the beginning of the study. An association between the MMW form and fasting glucose was of borderline significance ($r = -0.447$, $P = 0.055$). The HMW form was negatively associated with WHR (waist/hip ratio; $r = -0.491$, $P = 0.028$) at the end of the study. No other correlations between adiponectin multimeric complexes and biochemical and anthropometrical indices (lipid profile, BMI, waist circumference, fat mass and body weight) were found to be significant. No association between the HOMA index and any of the adiponectin multimeric complexes was detectable. These results are summarized in Tables 3 and 4.

Diet-induced changes in the HMW form were negatively associated with changes in the percentage of fat mass ($r = -0.474$, $P = 0.035$). Changes in the MMW and LMW forms were not significantly associated with changes in the HOMA index or plasma insulin. No other correlations were observed between diet-induced changes in the total adiponectin, HMW, MMW and LMW

Table 5 Spearman's correlation coefficients between the diet-induced changes in the variables

*Significant difference. TPA-ELISA, total plasma adiponectin measured using ELISA.

	HMW		MMW		LMW		TPA-ELISA	
Variable	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
	0.076	0.750	− 0.226	0.337	0.202	0.392	0.181	0.444
Weight	0.005	0.982	− 0.270	0.249	0.160	0.502	0.177	0.456
BMI	− 0.474*	0.035	− 0.037	0.877	0.256	0.276	0.003	0.990
Fat mass	0.409	0.073	0.117	0.624	− 0.022	0.927	0.130	0.584
Fat-free mass	− 0.129	0.587	− 0.235	0.319	− 0.089	0.710	− 0.110	0.643
Waist circumference	0.215	0.362	− 0.054	0.822	0.334	0.150	0.165	0.486
Hip circumference	− 0.244	0.301	− 0.328	0.158	− 0.400	0.081	− 0.432	0.057
WHR	− 0.107	0.654	− 0.079	0.740	− 0.203	0.390	0.166	0.485
Triacylglycerols	− 0.284	0.226	0.023	0.922	− 0.260	0.269	0.049	0.838
HDL-cholesterol	− 0.151	0.525	0.113	0.636	− 0.106	0.656	0.080	0.736
LDL-cholesterol	− 0.313	0.179	− 0.074	0.758	− 0.275	0.240	− 0.126	0.596
Fasting glucose	0.120	0.613	− 0.105	0.659	− 0.027	0.910	− 0.005	0.985
Fasting insulin	0.017	0.942	− 0.325	0.162	− 0.184	0.437	− 0.013	0.957
HOMA index	− 0.050	0.835	− 0.343	0.139	− 0.254	0.280	− 0.008	0.975

forms and changes in anthropometrical or biochemical parameters. These results are summarized in Table 5.

DISCUSSION

In the present study, we have demonstrated for the first time that weight loss induced by an LCD in obese and overweight women is accompanied by an increase in plasma levels of all of the adiponectin multimeric complexes (HMW, MMW and LMW) studied. The most responsive form was shown to be the LMW form, with an 18.1 % increase following dietary intervention, followed by the MMW and HMW complexes (with increases of 8.5 and 5.5 % respectively). Whole-body insulin sensitivity, estimated using the HOMA index, improved following the diet. Reduction in anthropometrical parameters and improvement in lipid profile were also achieved.

To the best of our knowledge, three reports on weight-loss-induced changes in plasma distribution of adiponectin multimeric complexes have been published so far, showing either no changes in multimeric complexes distribution [25] or an increase in the HMW and MMW forms [8,28]. The major advantage of our present study is in the number and homogeneity of the subjects studied. Our sample consisted of 20 pre-menopausal obese and overweight women compared with 17 subjects (15 women and two men) in the study by Bobbert et al. [8], 12 subjects (eight women and four men) in the study by Abbasi et al. [25] and six subjects (three women and three men) in the study by Kobayashi et al. [28]. As gender differences in total adiponectin levels, as well as in the distribution of multimeric complexes, have been found, the results of the above-mentioned studies may be biased [5,18,29].

Our present finding of increased HMW and MMW forms after weight loss is in agreement with earlier studies [6,28]. However, we have demonstrated for the first time that the LMW form also increased after dietary intervention. In fact, the LMW form was the isoform with the highest increase. The degree of obesity and achieved weight loss in the subjects in our present study are comparable with those reported by Abbasi et al. [25] [(BMI, 32.7 ± 1.7 kg/m^2; average weight reduction, 7.4 kg) and Bobbert et al. [28] (BMI, 35.1 ± 1.2 kg/m^2; average weight reduction, 6.2 kg). The specific biological role and function of the LMW form relative to the other adiponectin multimeric complexes has not yet been established, so the interpretation of the increased LMW adiponectin remains open for discussion.

The HMW form has been suggested to be the most physiologically potent form of adiponectin and might be the form responsible for its beneficial insulin-sensitizing and anti-atherosclerotic effects [12,28]. It has been shown that the HMW/total adiponectin and HMW/LMW ratios are plausible indicators of TZD (thiazolidinedione)-induced changes in insulin sensitivity [12].

In the present study, we found an association between the HMW form and fasting glucose levels before the weight loss, which was compatible with the hypothesis of an important role of the HMW form in the regulation of insulin sensitivity under basal steady-state conditions. The HMW form increased by 5.5 % after weight loss, but no association with fasting glucose was observed at the end of the study. This small elevation in HMW adiponectin is probably of limited clinical significance, and other regulatory mechanisms possibly play more important roles in the control of fasting glucose following acute weight loss, i.e. changes in other plasma cytokines (interleukin-6, tumour necrosis factor and leptin) [30,31]

or a reduction in fat cell size and intrahepatic lipid content [32]. It has also been proposed that the caloric restriction can, by itself, improve glycaemic control, regardless of weight loss [33].

We observed a reduction in both HMW/total adiponectin and HMW/LMW ratios, but there was no change in the HMW/MMW ratio, reflecting the relatively large increase in the LMW form compared with HMW adiponectin. An association between the HMW form and whole-body insulin sensitivity has been suggested [12,25]. Nevertheless, no significant association between total adiponectin or its multimeric complexes and insulin sensitivity, as evaluated using a euglycaemic hyperinsulinaemic clamp, was found in the study by Bobbert et al. [8]. No associations between the HOMA index and any of the adiponectin oligomeric complexes, ratios or total plasma adiponectin were observed in our present study either at baseline or with respect to the diet-induced changes. On the basis of these findings and on those by Bobbert et al. [8], it can be hypothesized that the above-mentioned ratios and associations of HMW adiponectin with parameters of insulin sensitivity might be specific to TZD treatment and may play only a minor role in LCD-induced changes in insulin sensitivity. A recent study [34] showing that TZD treatment selectively stimulates the secretion of the HMW form in human adipocytes supports this hypothesis further.

The results concerning changes in total plasma adiponectin concentration after weight loss are inconsistent. No change in total plasma adiponectin during moderate weight loss was found in several studies [8,25,35], whereas an increase in plasma adiponectin following large weight reduction subsequent to bariatric surgery [36–38] or intensive lifestyle counselling [39] have been described by others. No change in plasma adiponectin or insulin sensitivity was demonstrated following liposuction [40]. Thus it might be suggested that adiponectin plays a minor role in the regulation of the changes in insulin sensitivity during the moderate weight loss induced either by diet or physical exercise [41]. Despite the absence of a significant association between changes in plasma adiponectin and insulin sensitivity, clinically beneficial effects of increased plasma adiponectin following weight loss might persist, since it also has marked anti-inflammatory and anti-atherosclerotic effects in humans [7,42], effects which are independent of its insulin-sensitizing action.

It has been shown previously [21,43–45] that total adiponectin levels are associated with plasma HDL-cholesterol. In our present study, a correlation between total adiponectin and HDL-cholesterol had only borderline significance ($r = 0.43$, $P = 0.07$). This might be explained by a substantially lower number of subjects participating in our present study (20 women) compared with larger samples in studies describing such an association (407 and 1174 subjects) [21,45].

BMI and total adiponectin were negatively associated in our present study at baseline ($r = -0.31$, $P = 0.18$); however, according to calculations of sample size, the minimal number of subjects required to obtain a statistically significant correlation would have been 73 women.

We observed a correlation between the MMW form and HDL-cholesterol; however, this association was not observed with other multimeric complexes or with total plasma adiponectin. Our observations are in contrast with recent findings [8,10], which have shown that HMW adiponectin is predominantly responsible for the correlation between total adiponectin and HDL-cholesterol, perhaps through its impact on hepatic metabolism [8]. It should be noted here that both the HMW and MMW forms are able to stimulate AMPK in primary culture hepatocytes [18] and might therefore have similar effects on hepatocytes.

The 11.8 % reduction in plasma HDL-cholesterol seen in our present study is in agreement with observations of several other studies, including a meta-analysis [46], and it might be partially explained in terms of the impaired activity of lipoprotein lipase [47] and changes in the macronutrient composition of the diet [48–50]. HDL-cholesterol decreases during an active weight-loss phase in contrast with a stabilized period, when HDL-cholesterol is increased following the reduction of body weight [46].

The increase in all three types of adiponectin multimeric complexes in the presence of a non-significant change in total plasma adiponectin levels measured using ELISA is due to intrinsic differences between the two methods. ELISA provides a quantitative determination of actual plasma concentration, whereas Western blotting yields semi-quantitative data in the form of arbitrary units (quantity of light). Moreover, the difference could be due to different binding capacities of the respective clones of antibodies used in the ELISA and Western blotting.

In conclusion, diet-induced weight loss associated with insulin-sensitizing effects promotes an increase in the amount of HMW, MMW and LMW adiponectin multimeric complexes in plasma. No direct relationships between the diet-induced changes in individual adiponectin multimeric complexes and those of insulin sensitivity were found. Further studies elucidating the physiological relevance and function of adiponectin multimeric complexes with respect to obesity and insulin resistance are warranted.

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SHORT COMMUNICATION

Negative association between plasma levels of adiponectin and polychlorinated biphenyl 153 in obese women under non-energy-restrictive regime

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The aim of this study was to reveal whether accumulation of the persistent organic pollutants (POPs), especially polychlorinated biphenyl (2,2′,4,4′,5,5′-hexachlorobiphenyl, PCB 153), affects plasma levels of adiponectin in obese patients. The study was designed as a longitudinal intervention trial with a control group, where 27 obese women (body mass index (BMI) > 30 kg/m^2; age 21–74 years) were studied before (OB) and after (OB-LCD) a 3-month low-calorie-diet intervention (LCD; 5 MJ daily). As the control group, 9 female volunteers without LCD intervention were used (C; BMI = 19–25 kg/m^2; age 21–64 years). Plasma levels of PCB 153 were measured by high-resolution gas chromatography with electron capture detection; total adiponectin and insulin plasma levels were quantified by immunoassays; and adiponectin multimeric complexes were quantified by immunoblotting. Plasma levels of total adiponectin, high and medium molecular weight multimers significantly negatively correlated with plasma levels of PCB 153 in OB, but not in C or in OB-LCD, whereas the LCD intervention lowered BMI by 3.3 ± 3.0 kg/m^2. Our results may suggest suppression of adiponectin by PCB 153 in obese women under non-energy-restrictive regime, which may contribute to the known association of PCB 153 and other POPs with type 2 diabetes.
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Keywords: persistent organic pollutants; PCB 153; adiponectin; insulin

Introduction

Epidemiological studies have shown that some of the persistent organic pollutants (POPs), namely 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153)—one of 209 known polychlorinated biphenyl congeners (PCBs)—and 2,2-bis-(4-chlorophenyl)-1,1-dichloroethene (*p,p*′-DDE), both highly concentrated POPs in humans, determined in plasma, are associated with increased risk of type 2 diabetes.[1,2] PCB 153, used in technical application until the 1970s, is still present in food, as a main source of human exposition with a half life of 27.5 years.[3]

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Adipose tissue is a storage site for POPs, such as PCBs, polychlorinated pesticides, insecticides, dioxins and dibenzofurans. In essence, POPs show resistance to biological degradation and may exert a large variety of adverse health effects. In adipose tissue, a lot of POPs show endocrine-disrupting potency, may interact for instance with peroxisome proliferator-activated receptor-γ or aryl hydrocarbon receptor, and thus modulate metabolism, differentiation and secretory functions of adipose tissue (for review, refer the article by Mullerova and Kopecky[4]). Adipocytokines play a role in the link between obesity and insulin resistance.[5] In fact, tetrachlorodibenzo-p-dioxin (Agent Orange), one of the POPs, could induce low-grade inflammation of adipose tissue in mice[6] and also downregulation of adiponectin, one of the major secretory products of adipose tissue known to augment the effects of insulin on glucose homeostasis. Adiponectin plasma levels have been shown to be reduced, particularly in obese,[7] insulin-resistant patients[8] and in type 2 diabetics.[9] From three distinct multimeric complexes

identified in human plasma, high (HMW), medium (MMW) and low (LMW) molecular weight, only the HMW adiponectin plasma levels are positively associated with insulin sensitivity.[10]

To verify a hypothesis that PCB 153 may modify secretory functions of adipose tissue in obese women, plasma levels of PCB 153, adiponectin and other inflammatory markers were measured in a small cohort of obese patients before and after a weight-reducing regimen, which was based on a low-calorie-diet intervention. Lean patients were used as controls.

Materials and methods

Cohort of 27 obese adult women, aged 21–74 years, body mass index (BMI) $>30\,kg/m^2$, were randomly recruited from patients of Centre for Obesity Treatment (Teaching Hospital at Plzen, Czech Republic) during their first visit, and 9 nonobese healthy control women volunteers (C), aged 21–64 years, BMI = 19–25 kg/m^2, were recruited as a control group. Formal consent was obtained from all patients involved in the study. Anthropometry (body weight, height and BMI were calculated) and plasma analysis (see below) were performed in all the subjects. Obese patients were studied just before (OB) and after 3 months of LCD intervention (5 MJ daily; OB-LCD). Their diaries were checked on a monthly basis using nutritional software NutriDan 1.2 (DADI Ltd, Plzen, Czech Republic, 2002). Venous blood was collected from fasted (12 h) patients between 0700 and 1000 hours. Plasma samples were divided into 50 μl aliquots and stored at −80 °C for subsequent analysis of total immunoreactive adiponectin and its multimeric forms, C-reactive protein, interleukin-6, glucose, insulin, and PCB 153. The HOMA (homeostasis model assessment) index was calculated using the equation: (fasting glucose ($mmol\,l^{-1}$) × fasting insulin (milli-international units ml^{-1}))/22.5.

C-reactive protein was determined immunoturbidimetrically using a commercial kit (Kamiya Biomedical Company, Seattle, WA, USA). Automatic clinical analyser Olympus AU 2700 (Olympus, Mishima, Japan) was used. Interleukin-6 was measured using ELISA Quantikine HS Human interleukin-6 (R&D Systems, Inc., Minneapolis, MN, USA). Total adiponectin concentration in plasma was measured using a kit from ALPCO Diagnostics (Salem, NH, USA) and adiponectin multimeric forms were determined by Western blotting.[11] Plasma glucose was determined using the glucose-oxidase photometric method (Dialab, Vienna, Austria), plasma insulin was measured using the chemiluminescent microparticle immunoassay on Architect $i2000_{SR}$ Analyzer (Abbott Laboratories, Abbott Park, IL, USA).

Plasma levels of PCB 153 were determined by high-resolution gas chromatography with electron capture detection (HRGC/μECD; Agilent Technologies 6890 Series, Palo Alto, CA, USA), Capillary Column DB-5 (0.25 mm × 60 m × 0.25 μm; J&W Scientific, USA[12]).

Due to abnormal distribution for most of the examined variables non-parametric tests were used: Wilcoxon's test for comparison of obese and control groups and Spearman's test for correlation of variables within one group. Regression analyses of PCB 153 and adiponectin, or its multimeric forms, respectively, was performed. Logarithms of the adiponectin data, having normal distribution, were used for the analysis. The regression model has a form $y = \exp(rx + a)$, where x represents the PCB 153 and y the adiponectin. We also tested mathematical model of dependence of adiponectin (y) on insulin (x) and PCB 153 (z); $y = x^{\alpha} \cdot z^{\beta}$.

We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research. Approval with the protocol examination was authorized by the Medical School and Teaching Hospital at Plzen Ethical Committee.

Results

The basic data about subjects (OB, OB-LCD, and C) are depicted in Table 1. From the 27 OB plasma samples, only 22 showed measurable values of PCB 153, while in 5 cases the concentrations were below the quantification limit of the method (that is, <0.02 ng of PCB 153 per g). Spearman's test revealed a negative correlation ($P<0.05$) between plasma levels of total immunoreactive adiponectin for both (i) measurable levels of PCB 153; and (ii) pooled measurable and immeasurable levels of PCB 153, with the latter substituted by the values corresponding the quantification limit of the method (see above). The negative correlation between total adiponectin levels and both (i) measurable; and (ii) pooled (see above) PCB 153 plasma levels was confirmed by using a regression analysis. In the first case, the parameters of the above described regression model equal $r=-2.567$, $a=9.2$ ($P<0.003$, $R^2=0.332$, see also Figure 1), while in the second case the corresponding parameters were $r=-2.590$, $a=9.2$ ($P<0.001$, $R^2=0.335$).

The negative correlation between plasma total adiponectin and PCB 153 was not proved in C and disappeared in OB-LCD, while BMI decreased significantly due to LCD (Table 1, Figure 1). This finding of the negative correlation between total adiponectin and PCB 153 levels in plasma in OB was further supported by a negative correlation between both high (HMW) and medium molecular weight (MMW) form of adiponectin and PCB 153 ($P<0.01$, and $P<0.05$, respectively).

Monofactorial analysis has not revealed any correlation between PCB 153 and either inflammatory markers (C-reactive protein, interleukin-6) in plasma, insulinemia or HOMA (data not shown). However, using mathematical modeling of dependence of adiponectin (y) on insulin

Table 1 Basic characteristics of OB, OB-LCD and C patients

	OB	*OB-LCD*	*C*	P-*value* <
n	27	22	9	
BMI (kg/m^2)	39.4 (35.4; 44.7)	36.1 (33.8; 41.1)	22.7 (20.9; 24.1)	0.001 (OB vs C) 0.001 (OB vs OB-LCD) 0.001 (OB-LCD vs C)
Adiponectin (μg l^{-1})	6110 (4650; 8890)	7560 (5970; 9160)	7370 (4770; 7960)	*N* (between studied groups)
Adiponectin HMW (μg l^{-1})	1060 (760; 1340)	—	840 (600; 1220)	*N* (between examined groups)
Adiponectin MMW (μg l^{-1})	2130 (1470; 3190)	—	2060 (1410; 2770)	*N* (between examined groups)
Adiponectin LMW (μg l^{-1})	2780 (2110; 3360)	—	3040 (2120; 3640)	*N* (between examined groups)
PCB 153 (ng ml^{-1})	0.15 (0.1; 0.24)	0.16 (0.14; 0.21)	0.14 (0.11; 0.17)	*N* (between studied groups)
PCB 153 under QL	5	5	2	
Insulin (mU l^{-1})	11.7 (8.5; 18.6)	10.6 (7; 16.4)	5.7 (3.6; 7.5)	0.001 (OB vs C) 0.05 (OB vs OB-LCD) 0.01 (OB-LCD vs C)
HOMA	2.5 (1.8; 4.2)	2.3 (1.5; 3.6)	1.2 (0.8; 1.4)	0.001 (OB vs C) 0.01 (OB-LCD vs C)
CRP (mg l^{-1})	6 (3; 9.8)	6 (3; 12)	1 (1; 5)	0.01 (OB vs C) 0.05 (OB-LCD vs C)
IL-6 (ng l^{-1})	1.8 (1.4; 3.2)	1.8 (1.3; 2.5)	0.9 (0.8; 2.3)	*N* (between studied groups)

Abbreviations: BMI, body mass index; CRP, C-reactive protein; HMW, high molecular weight; HOMA, homeostasis model assessment; IL-6, interleukin-6; LMW, low molecular weight; MMW, medium molecular weight; OB, before a 3-month low-calorie-diet intervention; OB-LCD, after a 3-month low-calorie-diet intervention; PCB 153 under QL: number of samples with PCB 153 levels under quantification limit of the method. Data are medians (value of first quartile; third quartile). Statistical analysis was performed using a Wilcoxon's test. Adiponectin multimeric forms were quantified using immunoblotting in a subset of cases (OB, $n=14$; C, $n=8$; not all samples were left for the analysis). $N=P$-value above 0.05 threshold limit.

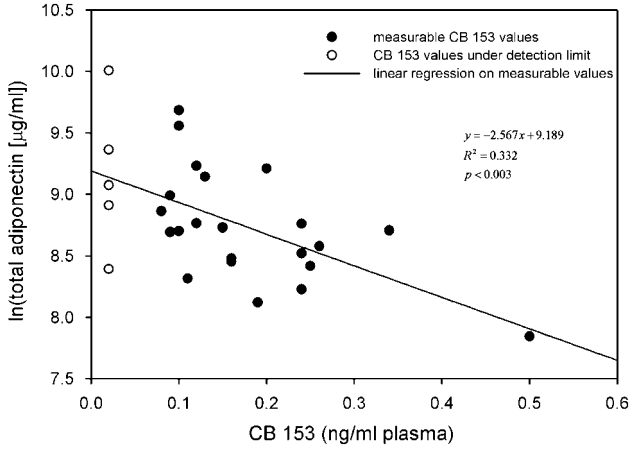

Figure 1 Negative correlation between plasma levels of total immunoreactive adiponectin and PCB 153 in OB. The negative correlation between plasma total adiponectin and PCB 153 was not proved in C (for measurable data only: $P<0.123$, $r=2.28$, $a=3.38$, $R^2=0.2692$; for pooled data: $P<0.337$, $r=-5.989$, $a=3.86$, $R^2=0.0268$) and disappeared in OB-LCD, whereas BMI decreased significantly due to LCD (for measurable data only: $P<0.026$, $r=9.921$, $a=3.56$, $R^2=0.4037$; for pooled data: $P<0.139$, $r=3.803$, $a=3.77$, $R^2=0.0782$).

(x) and CB 153 (z); $y=x^{\alpha}\times z^{\beta}$, where the null hypothesis that α or $\beta \geqslant 1$ was rejected in OB for α and β on $P<0.01$ and $P<0.05$, respectively, suggests that insulin affects the interaction between PCB 153 and adiponectin.

Discussion

For the first time, a negative correlation between adiponectin and PCB 153 levels in human plasma was described. This correlation disappeared as a consequence of a LCD regime with weight loss, and it was not apparent either in lean subjects. Our results might indicate suppression of adiponectin by PCB 153 in obese women under non-energy-restrictive regime, which may contribute to the association of this and other POPs with type 2 diabetes, as revealed by epidemiological studies.[1,2] Indeed, in support of this notion, a negative association between POPs and adiponectin was found with tetrachlorodibenzo-p-dioxin[13] or PCB 77,[14] both POPs having dioxin-like properties while interacting with the aryl hydrocarbon receptor. However, PCB 153 displays little or no binding affinity for aryl hydrocarbon receptor, and PCB 153 is considered to be a partial androgen antagonist.[15] Similar to phenobarbital, PCB 153 interacts with cytochrome P450, the CYP 1A and especially with CYP 2B enzymes. Yoshinari *et al.*[16] also demonstrated that in white adipose tissue of rats, phenobarbital induced CYP 2B enzymes, and hence the same effect could be expected in human adipose tissue. Interestingly, xenobiotic-mediated increases of CYP 2B in rat hepatocytes is augmented by insulin,[17] and expression of CYP 2B is increased in diabetic patients.[18] Furthermore, n-3 polyunsaturated fatty acids downregulate phenobarbital-induced CYP 2B expression,[19] whereas they upregulate adiponectin and improve glucose homeostasis.[20] Therefore, a hypothesis may be tested whether PCB 153 downregulates adiponectin through induction of CYP 2B, or whether adiponectin and CYP 2B represent two independent targets for PCB 153 in adipose tissue. Further studies should be performed to reveal (1) whether it was the negative energy balance or the new achieved lighter body weight in itself that resulted in the disappearance of negative correlation between adiponectin

and PCB 153; and (2) whether PCB 153 was not only a biomarker of body exposition to some other biological active pollutant, which has not been measured.

In conclusion, we found a negative association between plasma levels of PCB 153 and adiponectin, including its HMW and MMW forms, in obese women in nondiet-restrictive regime. The interaction between PCB 153, and possibly also other POPs, and adiponectin may contribute to the relatively high risk of development of type 2 diabetes in humans exposed to environmental pollutants.

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