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Research paper

Corn oil versus lard: Metabolic effects of omega-3 fatty acids in mice fed obesogenic diets with different fatty acid composition

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abstract

Mixed results have been obtained regarding the level of insulin resistance induced by high-fat diets rich in saturated fatty acids (SFA) when compared to those enriched by polyunsaturated fatty acids (PUFA), and how metabolic effects of marine PUFA of *n*-3 series, i.e. docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), depend on dietary lipid background. Here we compared two high-fat diets, in which the major lipid constituent was based either on SFA in the form of pork lard (LHF diet) or PUFA of *n*-6 series (Omega-6) as corn oil (cHF diet). Both cHF and LHF parental diets were also supplemented with EPAþDHA (~30 g/kg diet) to produce cHFþF and LHFþF diet, respectively. Male C57BL/6N mice were fed the experimental diets for 8 weeks. Insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamps in mice fed LHF and cHF diets, and then metabolic effects of cHFþF and LHFþF diets were assessed focusing on the liver and epididymal white adipose tissue (eWAT). Both LHF and cHF induced comparable weight gain and the level of insulin resistance, however LHF-fed mice showed increased hepatic steatosis associated with elevated activity of stearoyl-CoA desaturase-1 (SCD1), and lower plasma triacylglycerol levels when compared to cHF. Despite lowering hepatic SCD1 activity, which was concomitant with reduced hepatic steatosis reaching the level observed in cHFþF mice, LHFþF did not decrease adiposity and the weight of eWAT, and rather further impaired insulin sensitivity relative to cHFþF, that tended to improve it. In conclusion, high-fat diets containing as much as ~35 weight% as lipids induce similar weight gain and impairment of insulin sensitivity irrespective whether they are based on SFA or Omega-6. Although the SFA-rich diet containing EPAþDHA efficiently reduced hepatic steatosis, it did so without a corresponding improvement in insulin sensitivity and in the absence of effect on adiposity.

Abbreviations: AA, arachidonic acid; *Acaca*, acetyl-CoA carboxylase isoform 1 gene; *Acly*, ATP citrate lyase gene; *Acot1*, acyl-CoA thioesterase 1 gene; *Acox1*, peroxisomal acyl-CoA oxidase 1 gene; *Adgre1*, adhesion G protein-coupled receptor E1 gene; *Apob*, apolipoprotein B gene; *Arg1*, arginase 1 gene; ATM, adipose tissue macrophages; AUC, area under the glucose curve; *Ccl2*, chemokine (C-C motif) ligand 2 gene; cHF, corn oil-based high-fat diet; cHFþF, cHF diet supplemented with EPA/DHA in the form of a triacylglycerol concentrate; CLS, crown-like structures; *Dgat1*, diacylglycerol O-acyltransferase 1 gene; DHA, docosahexaenoic acid; *Ehhadh*, enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase gene; *Elovl5*, fatty acid elongase 5 gene; EPA, eicosapentaenoic acid; eWAT, epididymal white adipose tissue; *Fasn*, fatty acid synthase gene; GIR, glucose infusion rate; GTO, glucose turnover; *Hadha*, hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase gene; HGP, hepatic glucose production; LHF, pork lard-based high-fat diet; LHFþF, LHF diet supplemented with EPA/DHA in the form of a triacylglycerol concentrate; HOMA-IR, homeostatic model assessment of insulin resistance; LA, linoleic acid; *Mttp*, microsomal triglyceride transfer protein gene; NEFA, non-esterified fatty acids; *Nos2*, inducible nitric oxide synthase 2 gene; Omega-3, marine polyunsaturated fatty acids of *n*-3 series; Omega-6, polyunsaturated fatty acids of *n*-6 series; PUFA, polyunsaturated fatty acids; SCD1, stearoyl-CoA desaturase-1; *Scd1*, stearoyl-CoA desaturase-1 gene; SFA, saturated fatty acids; *Srebf1*, sterol regulatory element-binding transcription factor 1 gene; VLDL, very low-density lipoproteins; WAT, white adipose tissue.

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1. Introduction

Obesity with excessive accumulation of white adipose tissue (WAT) is associated with a cluster of metabolic abnormalities (i.e. metabolic syndrome), which predispose to the development of cardiovascular disease. Obesity is also accompanied by insulin resistance that is driven by the activation of an inflammatory response in WAT, and later also at the systemic level (reviewed in Ref. [1]). While pharmacological interventions for the treatment of metabolic disturbances in obesity require the use of multiple agents, and are often associated with side-effects, lifestyle modifications including increased physical activity and dietary manipulations remain an essential component of any prevention and/or treatment strategy (see e.g. Ref. [2]).

With regard to nutrition and its role in the development of obesity, not only the quantity but also the type of dietary lipids is an important determinant. For instance, the chain lengths of dietary fatty acids, degree of their unsaturation, and double bond configuration all could have a significant impact on their metabolism [3,4]. Of note, the typical Western diet of today is dramatically different to that of Paleolithic people, especially with regard to dietary fat; modern people have switched from a diet of animal protein consisting of wild game, which was rich in polyunsaturated fatty acids (PUFA) and contained more PUFA of n-3 series (Omega-3), to one containing more fat per unit of weight and much less Omega-3 in favor of increased content of PUFA of n-6 series (Omega-6), namely linoleic acid (LA; 18:2n-6) and arachidonic acid (AA; 20:4n-6; reviewed in Refs. [5,6]). A recent study by Alvheim et al. [7] reported that in the United States dietary content of LA increased from 1 to 8 energy% during the 20th century; furthermore, a significant increase in LA, likely derived from foods containing corn oil, in the diet of Americans is largely due to recommendations to minimize saturated fatty acids (SFA) intake to help preventing cardiovascular disease [5]. Regarding the presumed detrimental role of SFA, it has been shown that the diet containing a higher proportion of monounsaturated fatty acids as compared to SFA improves insulin sensitivity in healthy subjects receiving controlled, isoenergetic diets for 3 months, however only when a total fat intake was below 37 energy% (the KANWU study; [8]). Furthermore, feeding mice a high-fat diet enriched with monounsaturated fatty acids resulted in improved insulin sensitivity coincident with attenuated adipose interleukin-1 b secretion, reduced interleukin-1 b precursor priming, and sustained adipose AMP-activated protein kinase activation as compared with mice fed SFA-rich high-fat diet [9]. In healthy humans, overeating SFA promoted hepatic and visceral fat storage, whereas excess energy from PUFAs instead promoted lean tissue [10,11]. Similar data showing more detrimental metabolic effects of SFA-when compared to PUFA-based high-fat diets have been obtained also in rodent models of obesity [12e16].

Increased dietary intake of Omega-3, either plant-derived (a-linolenic acid; 18:3n-3) or especially fish-derived [i.e. eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3)], has been associated with a lower risk of cardiovascular disease [17]. Several studies in obese humans also demonstrated a reduction of adiposity after supplementation with fish-derived Omega-3 [18,19]. However, different results have been obtained regarding the effect of Omega-3 supplementation on glucose homeostasis in animal models and human patients with obesity and insulin resistance. While in obese adults with impaired glucose tolerance Omega-3 administration did not improve glycemic control ([20] and reviewed in Ref. [21]), admixing EPAþDHA in the form of fish oils (i.e. Omega-3 as triacylglycerols) to various high-fat diets rich in Omega-6 partially prevented obesity, dyslipidemia, insulin resistance, and WAT inflammation in various rodent models [22e27]. The reason for reduced efficacy of fish oil in states of obesity and insulin resistance especially in humans is not clear, however background lipids and/or other macronutrients in the diet could also play a role in influencing metabolic efficacy of dietary EPAþDHA supplementation, as suggested by some experimental studies in rodents ([13,14,28] and reviewed in Ref. [29]).

In the present study, using our established model of obesity-prone C57BL/6 mice fed a corn oil-based high-fat diet (see e.g. Refs. [25e27]), as well as mice fed a commercial high-fat diet based on pork lard, we aimed (i) to evaluate the potency of either SFA- or Omega-6-based high-fat diets to induce obesity and insulin resistance, the latter assessed by hyperinsulinemic-euglycemic clamps, and (ii) to explore the ability of dietary supplementation with EPAþDHA in the form of triacylglycerols to induce beneficial effects on glucose and lipid metabolism depending primarily on the lipid background of the diet.

2. Materials and methods

2.1. Animals and dietary interventions

Before the experiments, male C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) were maintained for 2 weeks on a 12-h light/dark cycle (light from 6:00 a.m.) at 22 °C, with *ad libitum* access to water and a low-fat standard diet (Chow; extruded Ssniff R/M-H diet; Ssniff Spezialdieten GmbH, Soest, Germany). As a general approach, single-caged mice at the age of 12 weeks were randomly divided into two experimental groups in order to get accustomed for 2 weeks to one of the following obesogenic diets: corn oil-based high-fat diet (cHF; lipids ~32% wt/wt; corn oil contains ~50% of LA), prepared at the Department of Adipose Tissue Biology of the Institute of Physiology in Prague (see e.g. Ref. [25]), and lard-based high-fat diet (LHF; lipids ~35% wt/wt; Ssniff EF acc. D12492 (I) mod., product #E15742-34; Ssniff Spezialdieten GmbH, Soest, Germany; http://www.ssniff.com/documents/gereinigte_diaeten_experimentaldiaeten.pdf). Following a 2-week adaptation period, each of the above mentioned groups was randomly divided into two subgroups, one of which continued on a respective parental high-fat diet (cHF or LHF) while the other subgroup received the same parental diet supplemented with Omega-3 in the form of a triacylglycerol concentrate of EPAþDHA (Epax 1050 TG; DHA, ~47% wt/wt, EPA, ~11% wt/wt; Epax, Sandvika, Norway). Thus, 52.5 g of the Epax 1050 TG was used to replace an equivalent part of the major lipid source in each diet (corn oil or lard), resulting in the concentration of ~30 g of EPAþDHA per 1 kg of diet. The supplemented diets were designated either as cHFþF (see also ref. [25]) and LHFþF, depending on whether the background diet was cHF or LHF, respectively. For details regarding the macronutrient composition of experimental diets, see Supplementary Table 1; for fatty acid composition in dietary lipids, see Supplementary Table 2. Chow-fed mice served always as lean controls.

Two independent experiments were performed: (i) the first experiment lasting 8 weeks compared the capacity of the LHF and cHF diet (i.e. without EPAþDHA supplementation) to induce insulin resistance and associated impairments of glucose homeostasis, assessed by hyperinsulinemic-euglycemic clamps (for details on clamp procedure, see the Section 2.2), and (ii) the next experiment lasting 6 weeks (plus the 2-week adaptation period e see above), which investigated metabolic effects of dietary Omega-3 with respect to dietary background; thus, it involved five experimental groups including cHF, LHF, cHFþF, LHFþF, and the Chow.

Body weight of single-caged mice was monitored weekly, while a fresh ration was given every 2 days. The calculation of average energy intake was based on food consumption measurements assessed in each mouse during a 24-h period once a week. Mice

were killed following a 6-h fast (food removed at 6:00 a.m.) by cervical dislocation under diethylether anesthesia between 12:00 p.m. and 14:00 p.m. The weights of tissues including white adipose tissue (WAT) from the epididymal depot (eWAT), mesenteric WAT, subcutaneous WAT from dorsolumbar depot, as well as the liver were recorded; aliquots of eWAT and liver were stored in 4% formaldehyde for the histological evaluation, and also snap-frozen in liquid nitrogen and stored at #70 °C for subsequent biochemical analyses. Adiposity index (in %) was defined as a sum of the weights of eWAT, mesenteric WAT, and subcutaneous WAT relative to body weight of the respective animal. All animal experiments were approved by the Animal Care and Use Committee of the Institute of Physiology, Czech Academy of Sciences (Approval Number: 127/2013) and followed the guidelines.

2.2. Hyperinsulinemic-euglycemic clamp

The clamp procedure performed in conscious mice was a modification of our previously published approach [25,26]. Briefly, approximately 1 week before the end of experiment, i.e. after 7 weeks on experimental diets, mice were equipped with a permanent catheter inserted into *v. jugularis*. Following a post-operative period that lasted a minimum of 2 days, mice were fasted for 6 h (6:00 a.m.e12:00 p.m.) and then infused with insulin HumulinR (Lilly USA, Indianapolis, IN) at a constant rate of 4.8 mU/min per kg body weight, while D-[3- ^{3}H]glucose (Perkin Elmer, Boston, MA) was infused at a rate of 0.26 mCi/min. Throughout the infusion, glucose concentration in tail blood was determined by the use of glucometers Contour™PLUS (Bayer, Leverkusen, Germany). Euglycemia (~5.5 mmol/l) was maintained by periodically adjusting a variable infusion of glucose solution (30% for lean animals, 15% for obese animals). Every 10 min during the last hour of a 3-h infusion period, blood samples were collected for the analysis of D-[3- ^{3}H]-glucose specific activity and other parameters as described before [26]. At the end of the clamp, mice were killed by cervical dislocation in diethylether anesthesia. Liver and EDTA-plasma were collected for biochemical analyses.

2.3. Glucose tolerance

Intraperitoneal glucose tolerance tests were performed similarly as before [30]. Briefly, an injection of D-glucose was applied at a dose of 1 g per kg body wt. to overnight fasted mice, and glucose levels in tail blood were monitored by glucometers (see above) before and 15, 30, 60, 120, and 180 min after the glucose injection. The level of glucose intolerance was then quantified as an area under the glucose curve (AUC), either as incremental or total (i.e. including blood glucose at the basal state) AUC.

2.4. Plasma metabolites and hormones

The levels of total cholesterol and total triacylglycerols in EDTA-plasma were assessed colorimetrically using commercial kits Bio-La-test TG L250S and Bio-La-Test CHOL L250S, respectively (Erba Lachema, Brno, Czech Republic); non-esterified fatty acids (NEFA) were measured by the kit Wako NEFA-HR (Wako Chemicals USA, Inc., Richmond, VA). Plasma levels of insulin and total adiponectin were determined by the Sensitive rat insulin RIA kit and Mouse Adiponectin ELISA kit, respectively (Merck Millipore Co., Billerica, MA). The homeostasis model assessment (HOMA) was applied to quantify insulin resistance (HOMA-IR index) using the following formula: fasting plasma insulin (mU/l) x fasting plasma glucose (mmol/l)/22.5.

2.5. Tissue lipids and fatty acid composition

Liver samples (~50 mg) were digested with 150 ml of 3 M KOH in 65% EtOH at 70 °C for 2 h. Resulting homogenates were cleared from debris by a brief centrifugation and the concentration of total glycerolipids was assessed by the Bio-La-Test TG L250S (see Section 2.4). In the second experiment, the total lipid content in the liver was assessed by gravimetry following the Folch extraction procedure [31]. Fatty acid composition of the triacylglycerol and phospholipid fraction in the liver was analyzed by gas chromatography as before [32].

2.6. Gene expression analysis

Total RNA was isolated either from samples of liver stored in RNAlater (Ambion, Austin, TX, USA) or from the frozen eWAT samples using TRI Reagent (SigmaeAldrich, St. Louis, MO). The levels of mRNA in the liver and eWAT were analyzed by real-time quantitative PCR (qPCR) as described earlier [33]. The expression of lipogenic genes including fatty acid synthase (*Fasn*), acetyl-CoA carboxylase isoform 1 (*Acaca*), ATP citrate lyase (*Acly*), stearoyl-CoA desaturase-1 (*Scd1*), fatty acid elongase 5 (*Elovl5*), sterol regulatory element-binding transcription factor 1 (*Srebf1*), fatty acid oxidation genes such as enoyl-CoA, hydratase/3-hydroxyacyl-CoA dehydrogenase (*Ehhadh*), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (*Hadha*), acyl-CoA thioesterase 1 (*Acot1*), and acyl-CoA oxidase 1 (Acox1), genes encoding enzymes involved in a formation of very-low density lipoproteins (VLDL) including apolipoprotein B (*Apob*), diacylglycerol O-acyltransferase 1 (*Dgat1*), and microsomal triglyceride transfer protein (*Mttp*), as well as macrophage markers such as adhesion G protein-coupled receptor E1 gene (*Adgre1*), chemokine (C-C motif) ligand 2 (*Ccl2*), inducible nitric oxide synthase 2 (*Nos2*), arginase 1 (*Arg1*) were analyzed. Results were normalized to a geometric mean of expressions of several housekeeping genes including eukaryotic translation elongation factor 2, eukaryotic translation elongation factor 1 a 1, and peptidylprolyl isomerase B. Primer sequences are shown in Supplementary Table 3.

2.7. Light microscopy and immunohistochemical analysis

Samples of eWAT and liver fixed in 4% formaldehyde were embedded in paraffin, and 5-mm sections were stained using hematoxylin-eosine as before [30]. In eWAT, a macrophage marker MAC-2/galectin-3 was detected by using specific antibodies in order to calculate a relative density of crown-like structures (CLS), i.e. the aggregates of adipose tissue macrophages (ATM) in inflamed tissue [34]. Digital images were captured using Olympus AX70 light microscope and a DP 70 camera (Olympus, Tokyo, Japan). Adipocyte morphometry was performed using a Lucia IMAGE version 4.81 (Laboratory Imaging, Prague, Czech Republic).

2.8. Statistics

The data are presented as means ± SEM. The comparisons were judged to be significant at p $ 0.05. The effects of obesogenic high-fat diets (cHF or LHF) as compared to the Chow, as well as the effects of EPAþDHA supplemented diets (cHFþF and LHFþF) as compared to their respective parental diets (cHF and LHF), were determined using the Student's t-test or ManneWhitney test for the data with and without normal distribution, respectively. For the overall comparisons among all parental high-fat diets and their EPAþDHA supplemented forms, Two Way ANOVA followed by the Holm-Sidak method for pairwise multiple comparisons was used. All analyses were performed using SigmaStat 3.5 software.

3. Results

3.1. Comparable effects of SFA- and Omega-6-enriched high-fat diets on obesity-associated insulin resistance, but distinct effects on the level of hepatic steatosis

In the first experiment, before the dietary intervention with EPAþDHA, obesity- and insulin resistance-inducing capacity of the two parental high-fat diets differing in the major lipid constituent was analyzed. Body weight gain after 8 weeks of dietary intervention with high-fat diets enriched either in Omega-6 (cHF group) or SFA (LHF group) showed a ~3-fold increase when compared to lean Chow-fed controls, however no significant differences in body weight gain were observed between the cHF and LHF group (Fig. 1a), despite a marginal increase in the average energy intake in case of the cHF group (Fig. 1b). At the end of experiment, hyperinsulinemic-euglycemic clamps were performed in both groups of mice to evaluate whole-body insulin sensitivity (Fig. 1c). Thus, under insulin-stimulated conditions, the amount of exogenous glucose required to maintain euglycemia during the clamp, i.e., the glucose infusion rate (GIR), was ~2.4-fold lower in both groups of mice fed high-fat diets than in Chow-fed mice, suggesting a profound diet-induced insulin resistance (Fig. 1c; *GIR*). Insulin resistance in mice fed either the cHF or LHF diet was also characterized by a ~1.6-fold reduction in the glucose turnover rate (GTO) despite a ~4.1-fold increase in hepatic glucose production (HGP), which suggests an impaired suppression of HGP by insulin in mice fed high-fat diets (Fig. 1c). As might be expected in the states of obesity-induced insulin resistance, the whole-body glycogen synthesis was markedly reduced in both the cHF and LHF group as compared with lean, insulin-sensitive Chow-fed mice (Fig. 1c; *Glycogen*). No differences in the whole-body glycolysis were found among the groups (not shown). Thus, high-fat feeding in general produced a full spectrum of known defects in glucose metabolism; however there were no significant differences between the cHF and LHF group with regard to GTO, HGP,or whole-body glycogen synthesis (Fig. 1c). In contrast, the content of glycerolipids in the livers of clamped mice was significantly increased only in the LHF group when compared to lean Chow-fed mice (Fig. 1d).

3.2. Differential effects of EPAþDHA supplementation on adiposity and glucose homeostasis depending on dietary background of their supplementation

Although the obesogenic cHF and LHF diets showed a comparable potency to impair glucose homeostasis (see Section 3.1), the ability of dietary EPAþDHA to improve various aspects of metabolism under obesogenic conditions could differ depending on whether they are supplemented onto a lard- or corn oil-enriched high-fat background. Therefore, metabolic effects of a 6-week dietary intervention with EPAþDHA in the form of triacylglycerols using the LHF and cHF background (i.e. the LHFþF and cHFþF supplemented diets, respectively) were analyzed in mice following a 2-week adaptation period (see Section 2.1). As shown in Table 1, both the cHF and LHF diet induced a significant but comparable weight gain of ~18e19 g, which was similar to that in the " clamp" experiment (Section 3.1). The average energy intake of mice fed the cHF and LHF diet was comparable; however, when these diets were supplemented with EPAþDHA, the average energy intake in the LHFþF group was ~12% and ~17% higher than in the LHF and cHFþF group, respectively, corresponding to a larger weight gain in LHFþF mice (Table 1). Furthermore, a tendency for decreased weight gain in the cHFþF group was associated with a significant reduction in the adiposity index, which was in turn largely attributable to a preferential reduction in the weight of eWAT as compared to other fat depots (Table 1). No such effects were observed in LHFþF mice. The cHF and LHF diets impaired glucose homeostasis to a similar level; however, while EPAþDHA in the form of LHFþF diet increased fasting blood glucose (FBG) and tended to further deteriorate glucose homeostasis, the cHFþF diet reduced fasting plasma insulin and in general tended to improve insulin sensitivity, as also suggested by a significant reduction of HOMA-IR by ~35% when compared to the LHFþF diet (Table 1). The levels of total adiponectin, i.e. the adipokine with insulin-sensitizing properties, were higher in mice fed LHF diet as compared to cHF-fed mice, however they increased similarly in both the LHFþF and cHFþF groups with EPAþDHA supplementation (Table 1). Plasma levels of triacylglycerols, NEFA and total cholesterol, analyzed after a 6-h fast, were reduced to a similar extent in both the cHFþF and LHFþF group as compared to their respective parental dietary groups (Table 1). Interestingly, plasma triacylglycerol levels were significantly lower in mice fed the LHF diet as compared to cHF group (Table 1).

3.3. Pronounced hepatic steatosis in SFA-enriched lard-based LHF diet is associated with up-regulation of SCD1, which is reduced by EPAþDHA supplementation

Histological analysis of liver sections stained with hematoxylin-eosin (Fig. 2) indicated a higher level of hepatic steatosis in the LHF than in the cHF group, while EPAþDHA supplementation reduced it. The liver weight was increased only in the LHF and LHFþF group but not in the cHF-based groups (Fig. 3a). Confirming the results of histological analysis in the liver (see above), the amount of tissue lipids measured either by the estimation of released glycerol (not shown) or by gravimetry (Fig. 3b) was much higher in the LHF than in cHF group, showing a ~2.6-fold and ~1.6-fold elevation in tissue lipid accumulation, respectively, as compared to lean Chow-fed mice (Fig. 3b). Regarding the effects of EPAþDHA supplementation, there was a robust reduction of tissue lipids by ~40% in response to EPAþDHA administered as LHFþF, while there was a ~20% decrease in the cHFþF group (Fig. 3b). Although dietary content of SFA (e.g. 16:0 and 18:0) was markedly increased in both the LHF and LHFþF diet (Fig. 4a), the SFA levels in hepatic triacylglycerol (Fig. 4b) and total phospholipid (Fig. 4c) fractions were already quite similar between the LHF and cHF groups, which is compatible with elevated stearoyl-CoA desaturase-1 (SCD1) enzyme activity in the livers of LHF mice. Indeed, marked hepatic steatosis in the parental LHF group was associated with a large elevation in the activity of SCD1, as documented by increased desaturation indexes 16:1(n-7)/16:0 and 18:1(n-9)/18:0 in hepatic triacylglycerol fraction (Fig. 3c and d); see also the data on fatty acid composition in hepatic triacylglycerol fraction in Supplemental Table 4. Similar differences in the indexes of hepatic SCD1 activity between the cHF and LHF group were also observed at the level of tissue phospholipids (Supplemental Table 5). Of note, administration of EPAþDHA reduced the levels of AA in the hepatic phospholipid fraction by ~50% in mice fed the cHFþF diet as well as in mice fed the LHFþF diet (Supplemental Table 5), which is consistent with the observed metabolic effects of EPAþDHA administration on the liver, irrespective of the dietary background.

Furthermore, the magnitude of changes in the lipid content induced by Omega-3 either in the form of the cHFþF or LHFþF diet corresponded with the suppressive effect of these diets on hepatic SCD1 activity (Fig. 3c and d). Interestingly, the cHF and LHF diets induced similar increases in the expression of lipogenic genes such as *Fasn*, *Acaca*, *Acly*, as well as their transcriptional regulator *Srebf1*, however hepatic *Scd1* expression was more than 4-fold higher in the LHF than in cHF group (Fig. 3e). Similarly, the expression of *Elovl5*, which is implicated in the elongation of long-chain fatty

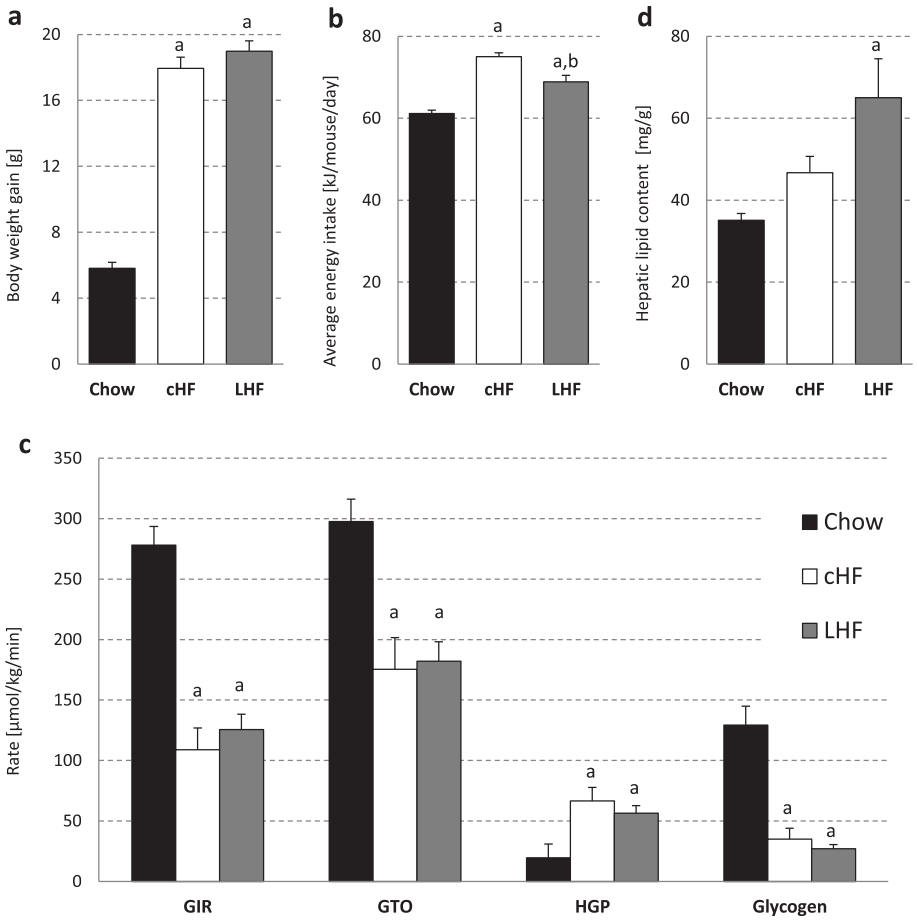

Fig. 1. Glucose homeostasis assessed by the hyperinsulinemic-euglycemic clamps. Mice were fed for 8 weeks high-fat diets based on either corn oil (cHF diet) or lard (LHF diet), and then subjected to hyperinsulinemic-euglycemic clamps to assess whole-body insulin sensitivity and related parameters. Standard diet-fed mice (Chow) served as lean controls. Body weight gain of mice at the end of study (a) and average energy intake (b). Various parameters of glucose homeostasis (c) assessed by hyperinsulinemic-euglycemic clamps using D-[3- ^{3}H]glucose: glucose infusion rate (*GIR*), glucose turnover (*GTO*), hepatic glucose production (*HGP*), and whole-body glycogen synthesis (*Glycogen*). Lipid content in the liver (d) was assessed in tissue samples removed immediately after the clamp. Data are means ± SEM (n ¼ 11e12 except Chow, n ¼ 8). ^{a}p $ 0.05 cHF or LHF vs. Chow; ^{b}p $ 0.05 LHF vs. cHF (t-test).

acids, showed a ~2-fold increase selectively in the LHF group (Fig. 3e). As would be expected in case of Omega-3 supplementation, hepatic expression of lipogenic genes was generally depressed and the magnitude of this decrease was very similar in the cHFþF and LHFþF groups (Fig. 3e); however, the effect of LHFþF on *Scd1* and *Elovl5* expression was much stronger when compared to cHFþF, thus closely reflecting differential effects of the Omega-3-supplemented diets on the SCD1 activity (Fig. 3c) and hepatic lipid content (Fig. 3b). The expression of genes involved in peroxisomal (*Acox1*, *Ehhadh*) but not mitochondrial (*Acot1*, *Hadha* e not shown) fatty acid oxidation was higher in the LHF than in cHF group, while it was generally increased in response to Omega-3 supplementation (Fig. 3e). The expression of genes encoding enzymes/proteins involved in the formation of VLDL such as *Mttp*, *Apob* or *Dgat1* remained unchanged among all experimental diets (not shown).

3.4. *Dietary background determines the effect of EPAþDHA supplementation on the size of adipocytes and inflammatory profile in WAT*

We next performed a morphological and immunohistochemical assessment of inflammation-related parameters in eWAT (Fig. 5). Both cHF and LHF high-fat diets led to a ~2.7-fold increase in average adipocyte area when compared to the lean controls (Fig. 6a). In agreement with a significant loss of eWAT in the cHFþF group (see Section 3.2), the average size of adipocytes in eWAT of these mice was reduced by ~13% when compared to mice fed the parental cHF diet, and reduced by ~21% with respect to the other Omega-3 supplemented group (i.e. LHFþF), in which the size of adipocytes remained unchanged as compared to its parental LHF diet (Fig. 6a). As expected, feeding mice for 8 weeks either the cHF or LHF diet induced significant although similar increases in the number of CLS, i.e. a marker of ATM accumulation (see Section 2.7 for details) as compared to lean Chow-fed mice (Fig. 6b). However, supplementing the parental diets with Omega-3 for 6 weeks resulted in either no change (in cHFþF mice) or even signi ficant deterioration of eWAT inflammation in case of mice fed the LHFþF diet (Figs. 5 and 6b). The level of ATM accumulation in eWAT based on the CLS counting was generally reflected in the level of gene expression of selected inflammation-related molecules (Fig. 6c). Thus, *Adgre1* (also known as F4/80), a marker of pro-inflammatory M1 macrophages, was increased ~6-fold in both groups of mice fed the parental diets (cHF and LHF), while in Omega-3-supplemented groups *Adgre1* expression was unchanged in the cHFþF mice and further increased ~2-fold in LHFþF mice as compared to their counterparts fed the parental diets (Fig. 6c). A similar pattern was also observed in the expression of *Ccl2*, a chemoattractant molecule

Table 1
Body mass, adiposity, glucose homeostasis, and plasma lipid parameters of mice fed two different high-fat diets with or without EPAþDHA supplementation.

	cHF	cHFþF	LHF	LHFþF	Chow
Energy balance					
Body weight e *initial* (g)	23.3 ± 0.2	23.4 ± 0.3	23.2 ± 0.2	23.4 ± 0.2	23.4 ± 0.3
Body weight e f*inal* (g)	43.2 ± 1.2 [a]	42.0 ± 1.0	44.4 ± 1.5 [a]	45.5 ± 0.7 [d]	30.7 ± 0.5
Weight gain (g)	18.2 ± 1.2 [a]	16.8 ± 0.9	19.5 ± 1.4 [a]	20.4 ± 0.7 [d]	5.9 ± 0.4
Energy intake (kJ/mouse)	77.2 ± 1.9 [a]	73.2 ± 1.6	76.7 ± 2.7	85.9 ± 1.6 [b,d]	68.1 ± 0.7
WAT					
Epididymal (g)	2.56 ± 0.11 [a]	2.02 ± 0.11 [b]	2.53 ± 0.13 [a]	2.32 ± 0.07	0.54 ± 0.04
Subcutaneous (g)	0.83 ± 0.04 [a]	0.77 ± 0.05	0.94 ± 0.06 [a]	1.00 ± 0.05 [d]	0.24 ± 0.01
Mesenteric (g)	1.18 ± 0.15 [a]	1.11 ± 0.12	1.20 ± 0.18 [a]	1.28 ± 0.07	0.28 ± 0.01
Adiposity index (%)	10.5 ± 0.4 [a]	9.3 ± 0.5 [b]	10.4 ± 0.4 [a]	10.1 ± 0.3	3.4 ± 0.1
Glucose homeostasis					
FBG (mmol/l)	8.7 ± 0.4 [a]	8.5 ± 0.4	9.0 ± 0.6 [a]	10.3 ± 0.4 [b,d]	5.8 ± 0.7
Incr. AUC (mol & 180 min)	1.90 ± 0.21 [a]	1.73 ± 0.19	1.79 ± 0.25 [a]	1.69 ± 0.22	0.91 ± 0.06
Total AUC (mol & 180 min)	3.49 ± 0.24 [a]	3.28 ± 0.22	3.42 ± 0.32 [a]	3.55 ± 0.26	1.94 ± 0.15
HOMA-IR	15.7 ± 1.6 [a]	11.6 ± 1.6	15.4 ± 2.5 [a]	17.9 ± 1.5 [d]	2.8 ± 1.0
Metabolites and hormones in plasma					
Triacylglycerols (mmol/l)	0.75 ± 0.05 [a]	0.61 ± 0.03 [b]	0.60 ± 0.03 [c]	0.46 ± 0.02 [b,d]	0.58 ± 0.05
NEFA (mmol/l)	0.72 ± 0.03	0.63 ± 0.04 [b]	0.67 ± 0.04 [a]	0.59 ± 0.01	0.83 ± 0.05
Cholesterol (mmol/l)	4.04 ± 0.13 [a]	3.16 ± 0.18 [b]	4.48 ± 0.22 [a]	3.75 ± 0.14 [b,d]	2.11 ± 0.05
Insulin (pmol/l)	318 ± 24 [a]	241 ± 29 [b]	297 ± 35 [a]	313 ± 19	79 ± 15
Adiponectin (total; mg/ml)	8.87 ± 0.54	11.88 ± 0.77 [b]	11.03 ± 0.77 [a]	14.7 ± 1.49 [b,d]	8.80 ± 0.25

Data are means ± SEM (n ¼ 9e10 except Chow, n ¼ 6). Average energy intake was based on 24-h food consumption measurements performed weekly during a 5-week period, from Week 3 (i.e. the start of EPAþDHA supplementation) till Week 7 of dietary intervention. Tissues and blood (for the isolation of EDTA-plasma) were collected from 6-h (6:00 a.m.e12:00 p.m.) food-deprived mice. Blood glucose and plasma insulin were measured in overnight fasted animals before the start of glucose tolerance test. A.U., arbitrary units; AUC, area under the glucose curve; FBG, fasting blood glucose; NEFA, non-esterified fatty acids; WAT, white adipose tissue. [a]p $ 0.05 for difference from Chow (Student's t-test); [b,c,d]p $ 0.05 for difference from parental diets (either cHF or LHF), cHF diet, and cHFþF diet, respectively (Two Way ANOVA).

also known as MCP-1, which was even reduced in the cHFþF group, while the expression of *Nos2* was similar in all groups except the cHFþF mice, where it was reduced as compared to mice fed the parental cHF diet (Fig. 6c). Similar increases in the expression of *Arg1*, a marker of anti-inflammatory M2 macrophages, were observed in the groups fed different high-fat diets as compared to Chow-fed lean controls (Fig. 6c).

In contrast to the liver, no consistent pattern was observed in the expression of fatty acid oxidation genes such as *Ehhadh*, *Acot1* and *Acox1* in adipose tissue (not shown). The expression of lipogenic enzymes including *Acaca*, *Acly*, *Fasn*, *Scd1* and *Elovl5* was in general more decreased by EPAþDHA supplemented within the cHF as compared to LHF parental diet (Fig. 6d).

4. Discussion

Dietary supplementation using Omega-3 has benefits for human health. While their apparent hypolipidemic and also certain anti-inflammatory properties (for review see Ref. [35]) could explain the beneficial role of EPAþDHA (i.e. Omega-3 of marine origin) in the primary prevention of cardiovascular disease, their anti-obesity effects and especially their metabolic efficacy in the secondary prevention of cardiovascular disease or in diabetic patients are much less obvious (reviewed in Ref. [21]). In this regard, besides a plethora of confounding factors such as heterogeneity of population, the use of other medication, and variability in Omega-3 dose, the type of background lipids and potentially other macronutrients in the diet could also contribute to the magnitude and type of metabolic effects induced by dietary interventions with EPAþDHA, as suggested in rodent models of obesity ([13,14,28] and reviewed in Ref. [29]). Here, using a complex panel of physiological, cell biology, molecular biology, and lipidomics analyses, we demonstrate in obesity-prone C57BL/6N mice that (i) two high-fat diets with very similar energy densities (20e21 kJ/g diet) and total lipid contents (32e35 weight%) differing in their major lipid source [corn oil (cHF diet) vs. pork lard (LHF diet)] induce similar levels of weight gain and insulin resistance following 8 weeks of dietary intervention, and (ii) dietary supplementation with EPAþDHA leads to lowering of plasma as well as hepatic lipid levels irrespective of the type of lipids in the background diet, however beneficial effects on adiposity and glucose metabolism might be reduced when EPAþDHA are supplemented onto the LHF dietary background.

In general, previous studies comparing obesogenic and metabolic effects of different types of dietary lipids suggested that SFA-rich diets cause larger increases in hepatic and/or visceral fat than PUFA-enriched diets, both in animal models [13e16] as well as in human subjects [10,11]. Based on the results of hyperinsulinemic-euglycemic clamps [12] or on surrogate measures of insulin sensitivity such as serum insulin or HOMA-IR [11,13,14], the studies also suggested that SFA-rich diets impair insulin sensitivity to a greater extent than PUFA-based diets in both rodents [12e14] and humans [11]. In our study, we compared metabolic effects of two high-fat diets; the cHF diet was based on corn oil (~50% of LA) and was previously shown to induce obesity and insulin resistance in mice (e.g. Refs. [25e27,30,33,36]), while the LHF diet was a commercial diet from Ssniff Spezialdieten GmbH (see Section 2.1 for details), which is based on pork lard (enriched with SFA) and is very similar to D12492 diet from Research Diets, widely used for its obesogenic and diabetogenic effects (e.g. Ref. [37]). We first compared the potential of LHF and cHF diets for induction of insulin resistance assessed by hyperinsulinemic-euglycemic clamps, i.e. the state-of-the-art technique in the field (see Section 2.2). However, in disagreement with some previous studies (e.g. Refs. [11e14]), mice fed either the LHF or cHF diet showed the same level of insulin resistance when compared with lean Chow-fed controls, and this whole-body insulin resistance was reflected in the same level of glycogen synthesis reduction and the impairment of hepatic insulin sensitivity (as suggested by elevated HGP in hyperinsulinemic conditions) in both groups of mice. Given the known deleterious effects of dietary SFA on the composition of membrane phospholipids [12] or in the induction of endoplasmic reticulum stress in the liver [38], the same level of insulin resistance induced by the LHF and cHF diet was an unexpected finding. However, high amounts of lipids in both types of diets might play a greater role in the development of insulin resistance than the presence of SFA in

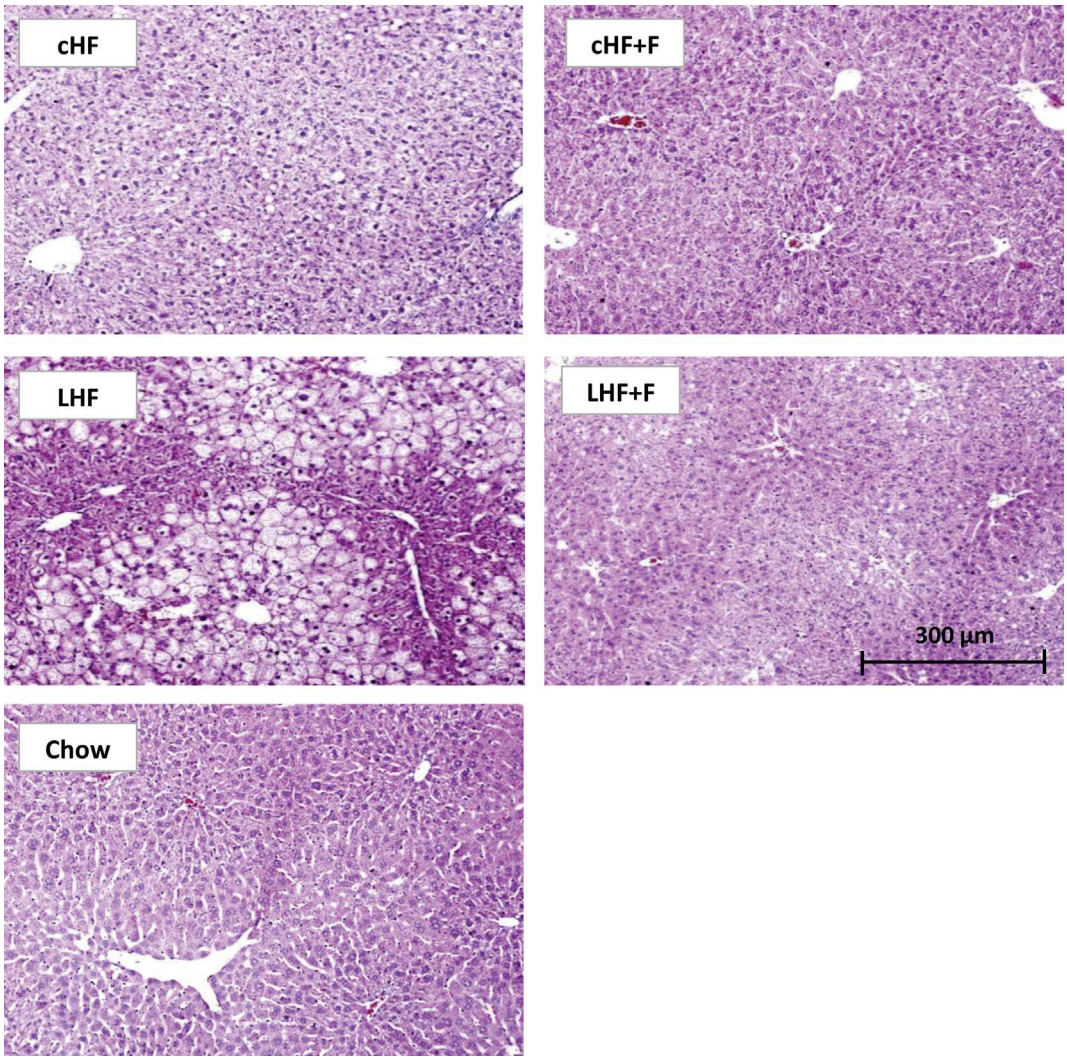

Fig. 2. Histological analysis of the liver. Representative histological sections of liver tissue obtained from mice fed two different high-fat diets based on either corn oil (cHF diet) or lard (LHF diet); the cHF and LHF parental diets were also supplemented by the triacylglycerol concentrate of EPAþDHA to generate either the cHFþF or LHFþF diet, respectively. Standard diet-fed mice (Chow) served as lean controls. Liver sections were stained using hematoxylin-eosine. Scale bars ¼ 300 mm.

the LHF diet. This is also supported by the results of the KANWU study, showing that the isoenergetic diet with a higher proportion of monounsaturated fatty acids as compared to SFA could improve insulin sensitivity in healthy subjects only when a total fat intake was below 37 energy% [8].

Our study also confirms previous findings [10,11,14], by showing a more pronounced hepatic steatosis in response to the SFA-rich diet (LHF) when compared to a diet containing Omega-6 (cHF). We also observed an inverse relationship between the levels of triacylglycerols in plasma and the liver; thus, lower plasma triacylglycerol levels in the LHF group might be at the expense of increased hepatic steatosis, also suggesting possible contribution of a reduced secretion of VLDL to hepatic steatosis in LHF mice. However, based on gene expression profiling in the liver, higher levels of hepatic steatosis in LHF- vs. cHF-fed mice cannot be explained by differences in hepatic *de novo* lipogenesis, fatty acid oxidation, and formation/secretion of VLDL. Furthermore, NEFA levels in plasma, which could drive the synthesis of hepatic VLDL [39], were similar in both groups. On the other hand, hepatic steatosis in LHF-fed mice was associated with increased activity of SCD1 in the tissue. It is known that palmitate toxicity can be prevented by promoting triacylglycerol accumulation through up-regulation of SCD1 [40,41]. Thus, elevated SCD1 activity in the liver of LHF-fed mice likely represents an adaptive response in order to transform SFA into less toxic monounsaturated fatty acids that are subsequently re-esterified to neutral triacylglycerols. This could be analogous to the situation, when low doses of rosiglitazone, i.e. antidiabetic drug from the thiazolidinedione family, induce beneficial metabolic effects despite increasing hepatic steatosis [25,36,42], while up-regulating *Scd1* expression especially in the liver [43].

One of the main findings in this study is that the effects of EPAþDHA on adiposity and glucose homeostasis might depend on the types of lipids in the diet. Thus, as compared to the parental LHF diet, feeding LHFþF diet containing EPAþDHA on the SFA-rich lipid background elevated FBG and tended to worsen insulin resistance (determined as HOMA-IR), the latter being significantly increased when compared to mice fed the cHFþF diet. However, it is important to stress that LHFþF mice ate by an average of ~15% more calories than mice fed either the parental LHF diet or EPAþDHA-supplemented cHFþF diet. EPAþDHA on the cHF background (cHFþF diet) tended to lower HOMA-IR while reducing plasma insulin, which was already observed before [25,26]. Given the fact that EPAþDHA supplementation reduced hepatic steatosis to a similar extent on both dietary backgrounds, the effects of EPAþDHA

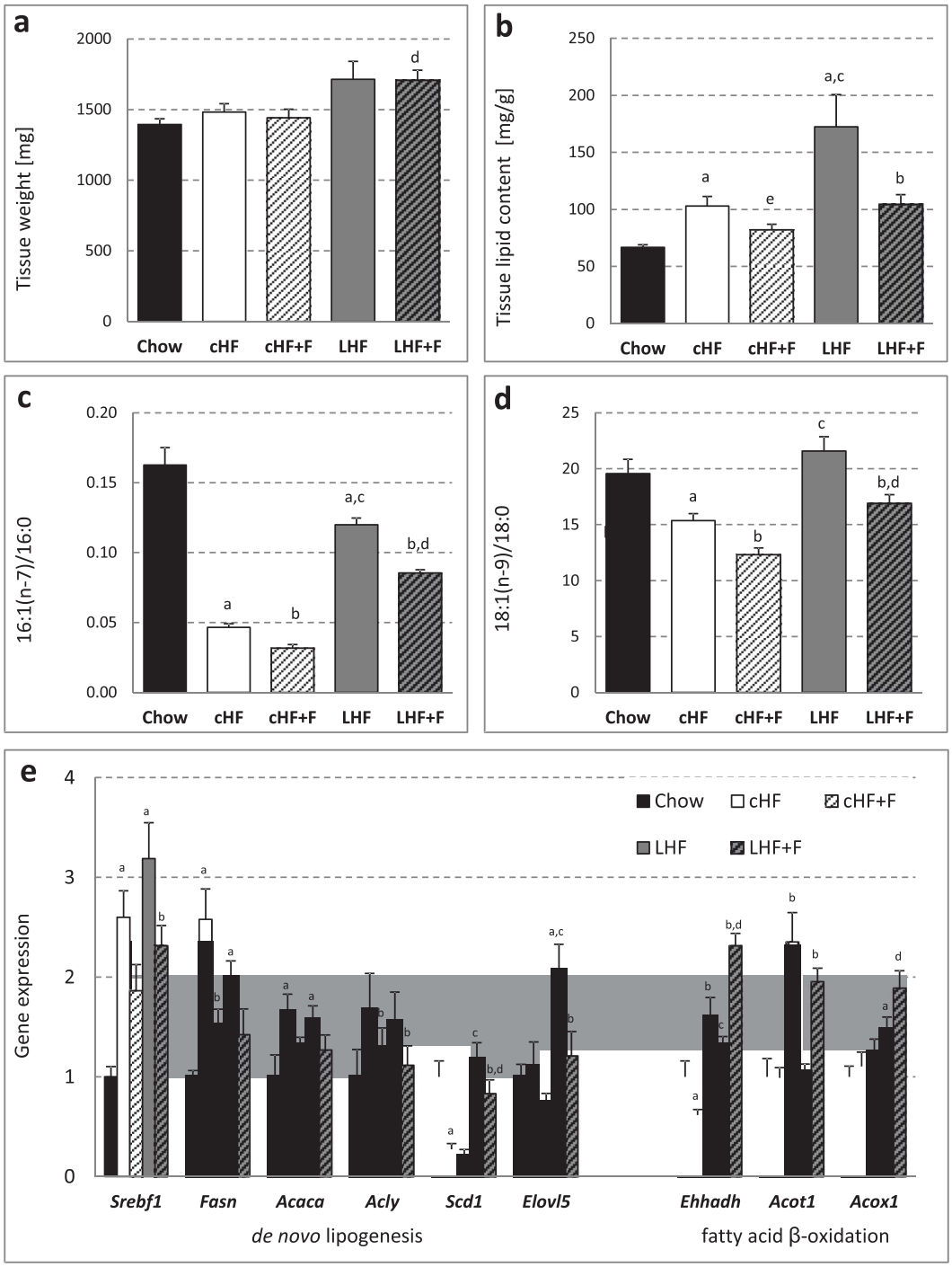

Fig. 3. Selected parameters of lipid homeostasis in the liver. Mice were fed either a corn oil-based (cHF) or lard-based (LHF) high-fat diet; the cHF and LHF parental diets were also supplemented by the triacylglycerol concentrate of EPAþDHA to generate either the cHFþF or LHFþF diet, respectively. Standard diet-fed mice (Chow) served as lean controls. At the end of study, the weight of liver (a), total lipid content (steatosis) in the tissue (b), as well as the activity of SCD1 based on the calculation of desaturation indexes 16:1(n-7)/16:0 (c) and 18:1(n-9)/18:0 (d) in the triacylglycerol fraction were assessed. Hepatic gene expression of key enzymes involved in *de novo* lipogenesis and fatty acid oxidation expressed as a fold-change relative to the Chow-fed mice (e). Data are means ± SEM (n ¼ 9e10 except Chow, n ¼ 6). ^{a}p $ 0.05 cHF or LHF vs. Chow; ^{b}p $ 0.05 cHF or LHF vs. cHFþF or LHFþF, respectively; ^{c}p $ 0.05 cHF vs. LHF; ^{d}p $ 0.05 cHFþF vs. LHFþF (Two Way ANOVA); ^{e}p $ 0.05 cHF vs. cHFþF (t-test).

on glucose homeostasis could be rather explained by a differential modulation of adiposity and the weight of eWAT, both of which were significantly reduced by EPAþDHA only on the cHF background. In this context, a potential involvement of the endocannabinoid system cannot be excluded. This system regulates food intake and feeding efficiency, and is overactive in obesity ([44,45] and reviewed in Ref. [46]). Alvheim et al. showed in mice that dietary intake of LA increased the levels of AA in tissue phospholipids, and, consequently, the levels of AA-derived endocannabinoids such as 2-arachidonoylglycerol and anandamide, which was associated with increased food intake, feeding efficiency, and the development of obesity [7]; in turn, the supplementation of obesogenic LA-

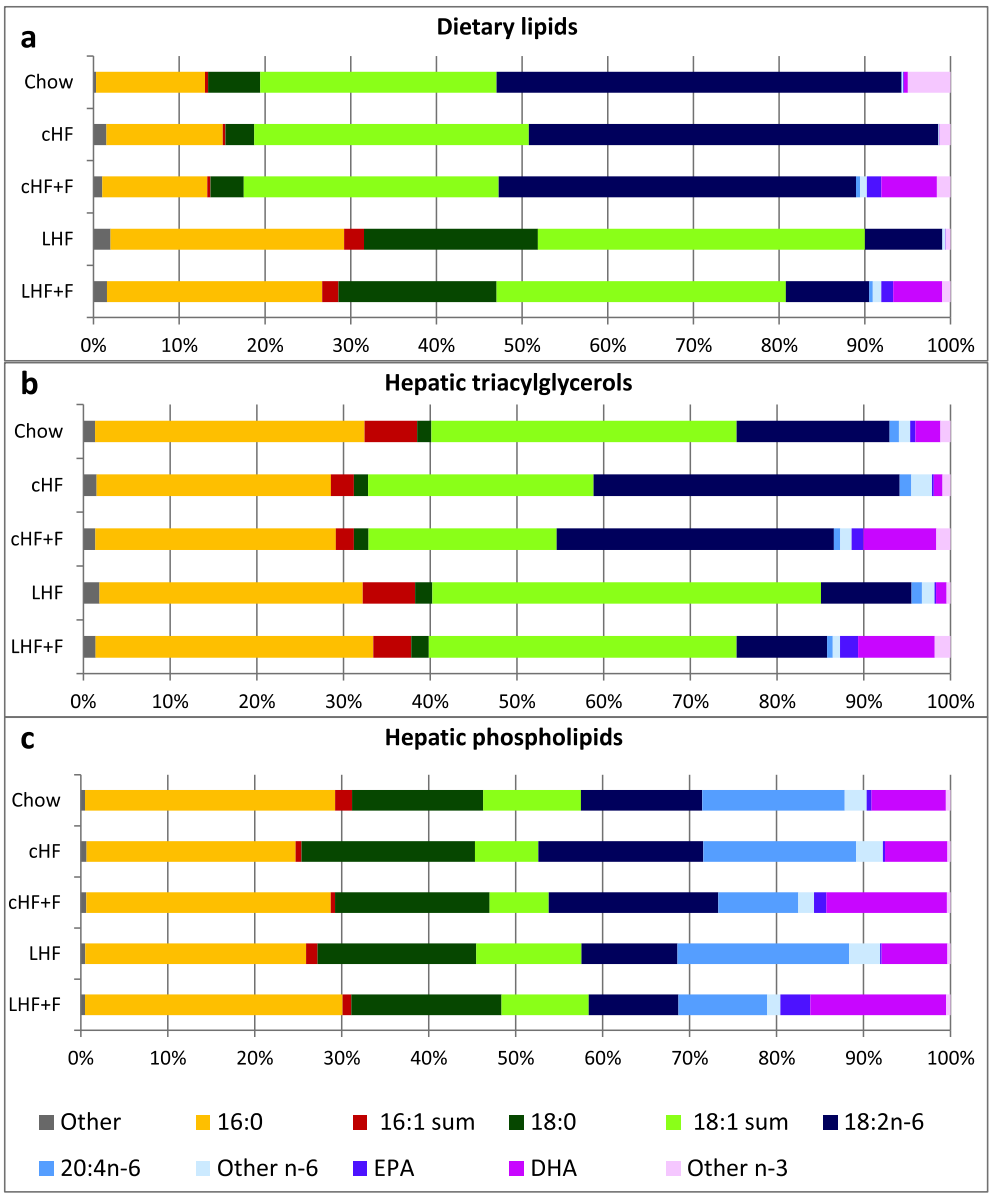

Fig. 4. Graphical representation of fatty acid composition in the experimental diets and in major lipid fractions from the liver. The relative content of individual fatty acids was analyzed by gas chromatography in the standard diet (Chow) and in the experimental diets (a), i.e. in the parental high-fat diets based on corn oil (cHF) and lard (LHF), and in the respective EPAþDHA supplemented diets cHFþF and LHFþF, as well as in the triacylglycerol (b) and phospholipid (c) fraction in the liver. Hepatic profiles of saturated and monounsaturated fatty acids, especially in the triacylglycerol fraction, correspond to the changes in hepatic *Scd1* gene expression (see Fig. 3). The values are expressed as mol%. "Other", sum of fatty acids containing less than 16 carbons; "16:1 sum" and "18:1 sum", sum of n-7 and n-9 isomers, respectively. For details, see Supplementary Tables 4 and 5.

enriched high-fat diets by EPAþDHA is sufficient to reduce the levels of AA in tissue phospholipids and consequently the levels of AA-derived endocannabinoid molecules in various tissues including WAT [7,27]. Therefore, the ability of EPAþDHA to reduce eWAT size and to improve some aspects of glucose homeostasis preferentially on the cHF background might depend on the normalization of the levels of endocannabinoids in adipose tissue, as observed before in case of eWAT [27]. Furthermore, elevated activity of the endocannabinoid system in WAT in response to high-fat feeding is associated with increased lipogenesis (reviewed in Ref. [47]). In agreement with this, a more consistent down-regulation of lipogenic genes was observed in eWAT of mice fed the cHFþF diet when compared to LHFþF group. Moreover, development of insulin resistance in obesity is associated with ATM accumulation and a chronic inflammation of WAT in both mice and men [34,37,48]; thus, by counting the number of CLS to assess the level of ATM infiltration ([34]; see Section 2.7 for details), we found a 2-fold increase in CLS in eWAT of the LHFþF mice as compared to LHF mice, which was associated with increased expression of *Adgre1* (F4/80) and *Ccl2* (MCP-1), i.e. markers of pro-inflammatory M1 macrophages, in the former group. However, in this context, surprisingly low CLS numbers in mice fed the parental LHF diet could be due to a relatively short period of LHF feeding, since other studies suggested that it is not earlier than after 12 weeks of high-fat feeding, when significant levels of expression of pro-inflammatory genes and macrophage infiltration are observed in eWAT of C57BL/6J mice [37,49]. In contrast to the LHFþF diet, the expression of *Ccl2* and *Nos2* was reduced by EPAþDHA administered as cHFþF diet. However, since the expression of *Arg1*, a marker of anti-inflammatory M2 macrophages, was increased in

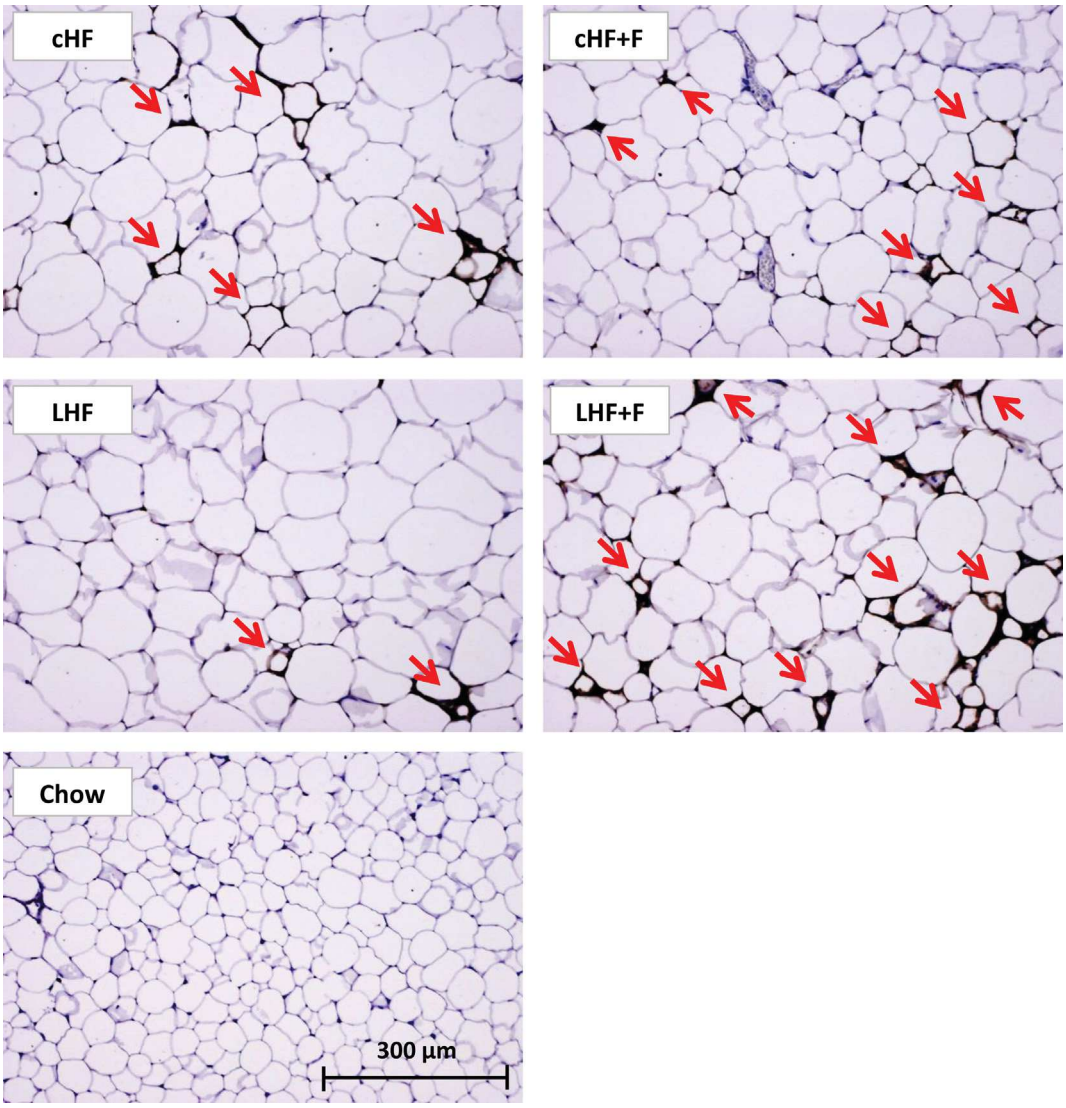

Fig. 5. The morphology of adipocytes and ATM accumulation in adipose tissue. Representative histological sections of eWAT from mice fed either the corn oil-based (cHF) or lard-based (LHF) high-fat diet; the cHF and LHF parental diets were also supplemented by the triacylglycerol concentrate of EPAþDHA to generate either the cHFþF or LHFþF diet, respectively. Standard diet-fed mice (Chow) served as lean controls. The sections were immunostained with specific antibodies against a macrophage marker MAC-2/galectin-3 in order to visualize the CLS (red arrows; see Section 2.7 for details).

eWAT of all groups fed high-fat diets relative to lean Chow-fed mice, it is difficult to conclude what is the real contribution of M1 and M2 macrophages to the CLS counts observed in each group.

As expected from previous studies [28,50e52], also in our study EPAþDHA on both dietary backgrounds down-regulated lipogenic genes and up-regulated fatty acid oxidation (mainly peroxisomal) genes in the liver, which is the likely mechanism that is responsible for reduction of hepatic steatosis. In addition, with regard to the results of previous studies [53,54], demonstrating the involvement of endocannabinoid signaling in the induction of hepatic steatosis, we observed a pronounced decrease in the content of AA in the hepatic phospholipid fraction caused by EPAþDHA administered either as cHFþF or LHFþF diet, which was in agreement with reduced accumulation of tissue lipids on both dietary backgrounds. Paradoxically, administration of EPAþDHA in the form of LHFþF diet decreased *Scd1* expression in both the liver and adipose tissue, which can be counterproductive given the expected beneficial metabolic effects of up-regulated SCD1 activity in mice fed the LHF diet (see above). This could explain rather negative metabolic effects in terms of glucose homeostasis and inflammation of WAT, when EPAþDHA are supplemented onto SFA-rich high-fat background. Although mice fed either the cHFþF or LHFþF diet displayed similar levels of hepatic steatosis, yet there could be differences in hepatic insulin sensitivity between these two EPAþDHA-supplemented groups. For instance, by using hyperinsulinemic-euglycemic clamps, we and others have previously shown [25,26,55] that dietary EPAþDHA improved insulin sensitivity of mice fed a high-fat diet rich in Omega-6 primarily through amelioration of insulin resistance in the liver, which is dependent on a transcription factor peroxisome proliferator-activated receptor- a [55] and AMP-activated protein kinase [26]. Given the fact that the levels of diacylglycerols are major determinants of tissue insulin resistance [56], the lowering of hepatic diacylglycerol levels seems to be one of the main mechanisms how EPAþDHA on this particular lipid background alleviate insulin resistance in the liver. It is not clear whether the same mechanism applies in cases when EPAþDHA are supplied on the SFA-rich background.

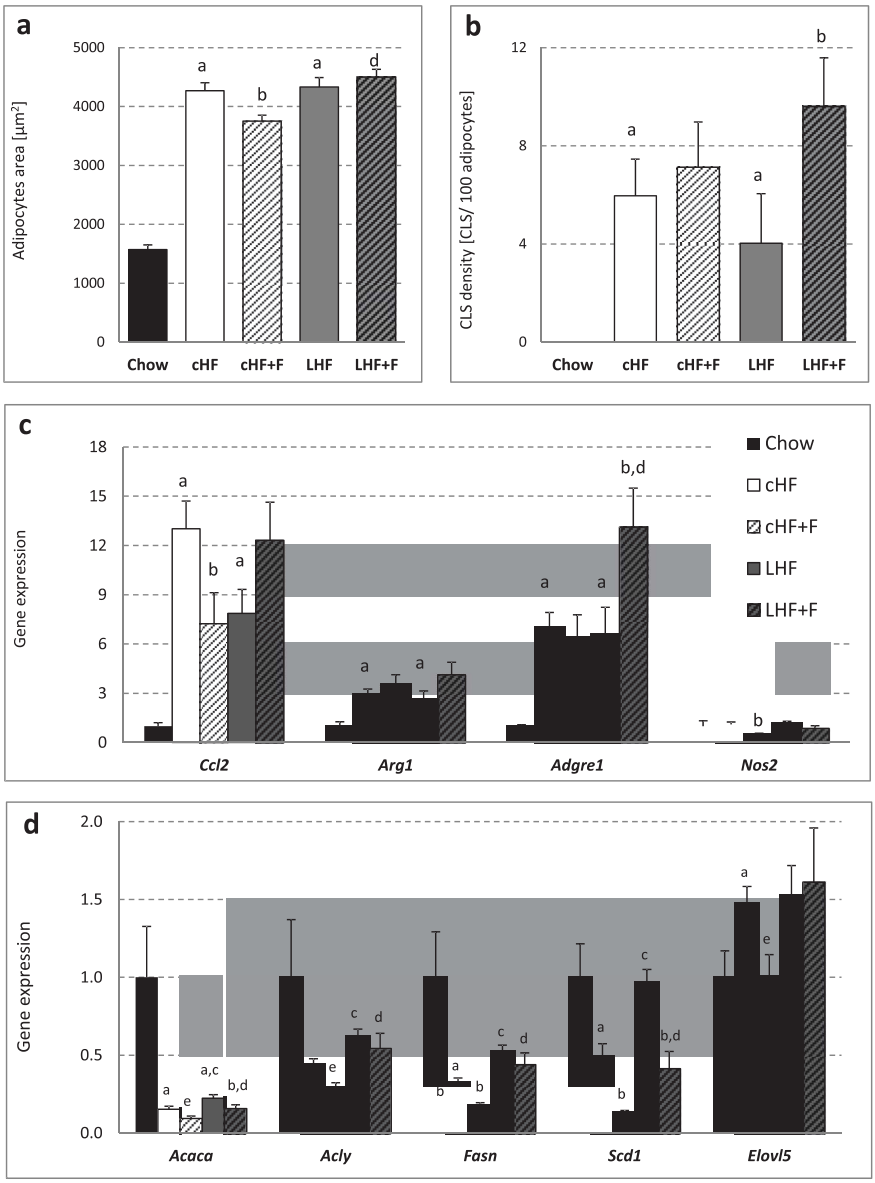

Fig. 6. Major characteristics of adipose tissue based on immunohistochemical and gene expression analyses. Mice were fed either a corn oil-based (cHF) or lard-based (LHF) high-fat diet; the cHF and LHF parental diets were also supplemented by the triacylglycerol concentrate of EPAþDHA to generate either the cHFþF or LHFþF diet, respectively. Standard diet-fed mice (Chow) served as lean controls. Morphological analysis of histological sections of eWAT was used to evaluate the average diameter of adipocytes in the tissue (a), while the overall level of ATM accumulation was assessed by calculating the relative density of CLS in the tissue (b; see Section 2.7 for details). Gene expression in eWAT: (c) the markers of pro-inflammatory M1 (*Ccl2*, *Adgre1*, *Nos2*) and anti-inflammatory M2 (*Arg1*) macrophages, and (d) the key enzymes of *de novo* lipogenesis, expressed as a fold change relative to the Chow-fed mice. Data are means ± SEM (n ¼ 9e10 except Chow, n ¼ 6). [a]p $ 0.05 cHF or LHF vs. Chow; [b]p $ 0.05 cHF or LHF vs. cHFþF or LHFþF, respectively; [c]p $ 0.05 cHF vs. LHF; [d]p $ 0.05 cHFþF vs. LHFþF (Two Way ANOVA); [e]p $ 0.05 cHF vs. cHFþF (t-test).

It is also possible that other macronutrients and components found in the cHF and LHF diet may contribute to the above described differences in metabolic effects induced by EPAþDHA supplementation either in the form of the cHFþF or LHFþF diet. In contrast to cHF, the LHF diet contains cholesterol (290 mg/kg), sucrose (~9.5%), and has a higher content of protein (~22% vs. ~12%), to name a few examples. Despite the fact that the LHF diet contained cholesterol, plasma cholesterol levels in LHF-fed mice were not significantly different from those in the cHF-fed mice; however, the presence of cholesterol in the LHFþF diet could partially explain higher plasma levels of cholesterol in mice fed this diet as compared to cHFþF-fed mice, although the efficiency of EPAþDHA supplementation to reduce plasma cholesterol was comparable in both the LHFþF and cHFþF group (#16% vs. #22%). With regard to sucrose, its content in the LHF diet is in fact comparable to that in the AIN-93M standard rodent chow (i.e. 10%; [57]), thus it is not likely that this amount of sucrose would play an important role in the observed effects. On the other hand, Ma et al. [58] previously showed that the enrichment of a high-fat diet with sucrose (43%; at the expense of dietary protein) led to the development of obesity while counteracting the anti-inflammatory effects of Omega-3 (fish oil) in WAT but not its anti-steatotic effects in the liver. These findings might support our current results showing a higher number of CLS in eWAT of LHFþF mice. Apparently, the influence of the background diet with respect to the metabolic effects of dietary EPAþDHA supplementation is worth further study.

Collectively, our data support the previous findings, showing that instead of their triacylglycerol form (i.e. fish oil), EPAþDHA

should ideally be administered as marine phospholipids ([27,36] and reviewed in Ref. [59]) and/or in combination with other pharmacological and lifestyle interventions (e.g. antidiabetic drugs [25] or calorie restriction [18,33,60]) to ensure their highest possible efficacy, especially with regard to their insulin-sensitizing effects in both the prevention and reversal (i.e. treatment) of obesity and associated metabolic disturbances.

In conclusion, this study demonstrates that a long-term dietary intervention with high-fat diets differing primarily in their major lipid constituent (Omega-6 vs. SFA) and containing as much as ~35 weight% as lipids leads to a similar weight gain and impairment of glucose homeostasis. Supplementing these high-fat diets with marine Omega-3 (i.e. EPAþDHA, ~30 g/kg diet) in the form of triacylglycerols results in comparable reductions of lipid metabolites in plasma and relatively mild effects on glucose homeostasis. Administration of EPAþDHA on a dietary background containing mainly Omega-6 and starch beneficially affected at least some parameters of glucose homeostasis and reduced accumulation of fat. On the other hand, the anti-steatotic effects of EPAþDHA in the liver are even stronger when these fatty acids are supplemented on the SFA-rich dietary background.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2015.07.001 .

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Omega-3 phospholipids from fish suppress hepatic steatosis by integrated inhibition of biosynthetic pathways in dietary obese mice

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abstract

Non-alcoholic fatty liver disease (NAFLD) accompanies obesity and insulin resistance. Recent meta-analysis suggested omega-3 polyunsaturated fatty acids DHA and EPA to decrease liver fat in NAFLD patients. Anti-inflammatory, hypolipidemic, and insulin-sensitizing effects of DHA/EPA depend on their lipid form, with marine phospholipids showing better efficacy than fish oils. We characterized the mechanisms underlying beneficial effects of DHA/EPA phospholipids, alone or combined with an antidiabetic drug, on hepatosteatosis. C57BL/6N mice were fed for 7 weeks an obesogenic high-fat diet (cHF) or cHF-based interventions: (i) cHF supplemented with phosphatidylcholine-rich concentrate from herring (replacing 10% of dietary lipids; PC), (ii) cHF containing rosiglitazone (10 mg/kg diet; R), or (iii) PC + R. Metabolic analyses, hepatic gene expression and lipidome profiling were performed. Results showed that PC and PC + R prevented cHF-induced weight gain and glucose intolerance, while all interventions reduced abdominal fat and plasma triacylglycerols. PC and PC + R also lowered hepatic and plasma cholesterol and reduced hepatosteatosis. Microarray analysis revealed integrated down-regulation of hepatic lipogenic and cholesterol biosynthesis pathways by PC, while R-induced lipogenesis was fully counteracted in PC + R. Gene expression changes in PC and PC + R were associated with preferential enrichment of hepatic phosphatidylcholine and phosphatidylethanolamine fractions by DHA/EPA. The complex down-regulation of hepatic lipogenic and cholesterol biosynthesis genes and the antisteatotic effects were unique to DHA/EPA-containing phospholipids, since they were absent in mice fed soy-derived phosphatidylcholine. Thus, inhibition of lipid and cholesterol biosynthesis associated with potent antisteatotic effects in the liver in response to DHA/EPA-containing phospholipids support their use in NAFLD prevention and treatment.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), a premorbid condition that can lead to fibrosis and cirrhosis, is frequently driven by obesity and insulin resistance [1]. Pharmacological interventions to treat obesity-associated diseases require multiple agents and are often associated with adverse side effects, as in the case of thiazolidinedione (TZD) antidiabetic drugs, thus lifestyle interventions remain an essential component of any treatment strategy. Marine fish oils, namely long-chain polyunsaturated fatty acids (FA) of *n*-3 series (omega-3), such as docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3), were shown to reduce the incidence and mortality of cardiovascular disease [2,3]. This beneficial effect was attributed to a reduction in plasma triacylglycerol (TG) levels as well as to the anti-inflammatory action of omega-3, both in rodents [4–6] and in humans [7,8]. Omega-3 supplementation potentiates health benefits of reduced calorie intake in humans [9] and obese mice [10]. While omega-3 improved insulin sensitivity in rodent models of metabolic syndrome [11], they could not revert insulin resistance in diabetic patients

Abbreviations: cHF, corn oil-based high-fat diet; Chow, standard low-fat diet; CLS, crown-like structures; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; FDR, false discovery rate; GEO, Gene Expression Omnibus; GSEA, Gene Set Enrichment Analysis; HDL-C, high-density lipoprotein cholesterol; HMW, high molecular weight; HOMA, homeostasis model assessment; LA, linoleic acid; NAFLD, non-alcoholic fatty liver disease; NEFA, non-esterified fatty acids; omega-3, long-chain *n*-3 polyunsaturated fatty acids of marine origin; omega-3 PL, omega-3 as marine phospholipids; omega-3 TG, omega-3 as triacylglycerols in fish oil; oPLS-DA, orthogonal partial least squares-discriminant analysis; PC, cHF supplemented with marine phospholipids; PCA, principal component analysis; PPAR, peroxisome proliferator-activated receptor; PC + R, cHF supplemented with marine phospholipids and rosiglitazone; qPCR, real-time quantitative RT-PCR; R, cHF supplemented with an antidiabetic drug rosiglitazone; SHP, small heterodimer partner; TC, total cholesterol; TG, triacylglycerol; TZD, thiazolidinedione; VIP, variable important to projection; VLDL-TG, very low-density lipoprotein triacylglycerol; WAT, white adipose tissue

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[12]. However, even these patients benefited from omega-3 supplementation through prevention of cardiovascular disease and improvement of dyslipidemia [13]. Moreover, omega-3 limited hepatosteatosis in rodents [5,6,10,11,14–16] and humans, as documented by a number of studies (e.g. [17,18]), as well as by a recent meta-analysis [1].

Metabolic effects of omega-3 are determined by: (i) modulation of expression by master transcriptional regulators, mainly by peroxisome proliferator-activated receptor α (PPARα; [19]) and sterol regulatory element-binding protein-1c [20], (ii) tissue production of eicosanoids and other lipid mediators [21], (iii) induction of adiponectin [22], and (iv) changes in the levels of endocannabinoids in metabolically relevant tissues [5,15,23]. White adipose tissue (WAT) and the liver represent the primary targets for omega-3 in dietary obese mice, as evidenced by induction of mitochondrial biogenesis and FA oxidation specifically in epididymal WAT [24] and by AMP-activated protein kinase dependent improvement of hepatic insulin sensitivity [11]. Hypolipidemic effects of omega-3 depend on complex metabolic and gene expression changes (see above), resulting in suppression of hepatic lipogenesis and increased FA oxidation [6].

Recent studies[5,6,15,16] document amelioration of hepatic steatosis in obese rodents in response to omega-3 administered as marine phospholipids (omega-3 PL), mostly as krill oil [6,15,16]. When compared with omega-3 contained in TG (omega-3 TG) in fish oils, omega-3 PL were more efficient in the reduction of steatosis [5,15] and improvement of metabolic profile [5]. Low-fat diet supplementation with krill oil resulted in decreased activities of gluconeogenic, lipogenic and cholesterol biosynthetic pathways, with enhanced mitochondrial oxidative activity, which was not observed after omega-3 TG supplementation [25]. However, a detailed analysis of pathways that are affected in response to omega-3 PL using a more clinically relevant setting, i.e. in the steatotic livers of mice with diet-induced obesity, is lacking. Moreover, although sea fish may contain up to one third of omega-3 as PL [26], and thus could serve as an abundant source of omega-3 PL, studies on the effects of fish-derived omega-3 PL are scarce[5].

We used our well-established mouse model of dietary obesity [4,5,10,11,22] to identify mechanisms involved in the antisteatotic action of omega-3 PL in the liver. By means of physiological assessment, gene expression profiling and lipidomic analysis, we examined the metabolic effects of phosphatidylcholine-rich omega-3 PL isolated from herring, administered either alone or in combination with a low dose of TZD rosiglitazone. Furthermore, in an attempt to distinguish the importance of FA composition and that of the PL moiety with regard to their contribution to the overall metabolic effect of omega-3 PL, we also compared herring-derived omega-3 PL with a phosphatidylcholine-rich concentrate isolated from soy, i.e. the type of PL molecule characterized by a completely different FA profile. We show here that besides preventing weight gain and dyslipidemia and preserving glucose homeostasis, omega-3 PL markedly reduced hepatic steatosis, which was associated with a profound suppression of hepatic biosynthetic processes, above all characterized by integrated inhibition of de novo FA synthesis and cholesterol biosynthesis. Omega-3 PL were even able to override the permissive effect (see refs. [4,27]) of TZD rosiglitazone on hepatic lipogenesis. Lastly, the strong down-regulation of the lipogenic and cholesterol biosynthesis pathways was specific for the livers of mice subjected to dietary intervention with omega-3-containing PL, since it was not observed in mice fed a high-fat diet supplemented with the phosphatidylcholine-rich PL concentrate isolated from soy. Furthermore, in mice fed soy-derived PL, the lack of effect on hepatic expression of enzymes involved in lipid metabolism was also associated with unchanged plasma lipid levels and hepatosteatosis.

2. Material and methods

2.1. Animals and dietary interventions

Male C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) were fed a standard diet (Chow; extruded Ssniff R/M-H diet; Ssniff Spezialdieten GmbH, Soest, Germany) and were maintained on a 12-h light-dark cycle (light from 6:00 a.m.) at 22 °C. Three independent experiments labeled A, B and C were performed:

(A) Three-month-old mice were randomly assigned for 7 weeks to a corn oil-based high-fat diet (cHF; lipids ~35% wt/wt; see [4]) or to various cHF-based interventions (n = 8): (i) cHF with ~10% lipids replaced by herring-derived omega-3 PL concentrate rich in phosphatidylcholine (PC diet; produced by EPAX AS, Aalesund, Norway; 5 g DHA/EPA per kg diet); (ii) cHF supplemented with rosiglitazone (R diet; 10 mg/kg diet); and (iii) cHF with both omega-3 PL and rosiglitazone (PC + R diet). Chow-fed mice served as lean controls. See Supplemental Tables 1 and 3 for details regarding the macronutrient and FA composition of the diets, and Supplemental Table 2 regarding the PL composition of the phosphatidylcholine-rich omega-3 PL concentrate used to prepare the PC diet.

(B) Three-month-old male C57BL/6N mice were randomly assigned either the cHF diet or various cHF-based interventions (n = 6–8): (i) cHF with ~ 10% lipids replaced by herring-derived omega-3 PL of a similar composition as in the experiment A above (PC-M; produced by EPAX AS, Aalesund, Norway; 3.4 g DHA/EPA per kg diet – i.e. ~30% lower when compared with the phosphatidylcholine-rich PL concentrate used in the experiment A); and ii) cHF with ~10% lipids replaced by phosphatidylcholine-rich PL concentrate from soy (PC-S; L-α-Phosphatidylcholine, Cat. no. P3644, Sigma-Aldrich). Both supplemented diets were matched for the content of phosphatidylcholine (~ 14 g/kg diet), which represents the major PL fraction contained in both concentrates (see Supplemental Table 2 for details on the PL composition of the PL concentrates used in this study). Dietary interventions lasted for a period of 7 weeks.

(C) Three-month-old male C57BL/6N mice were randomly assigned either the cHF diet (see the experiment A above for details) or cHF-based intervention (PC; n = 7), in which ~ 10% of lipids in the cHF was replaced by herring-derived omega-3 PL of a similar composition as in the experiment A (5 g DHA/EPA per kg diet; see also Supplemental Table 2 for details on the PL composition of the omega-3 PL concentrate used in this study). Dietary intervention lasted for a period of 7 weeks. During the week 6 of the experiment, feces were collected over a 24-h period to estimate the loss of lipids in feces. At the end, mice were used for the measurement of hepatic secretion of TG in the form of VLDL (VLDL-TG).

Body weight of single-caged mice was monitored weekly, while a fresh ration was given every 2 days. Food intake of each mouse was assessed weekly during a 24-h period and recalculated per week. In the experiments A and B, mice were killed by cervical dislocation under diethylether anesthesia between 9:00 a.m. and 11:00 a.m. Dorsolumbar (subcutaneous) and epididymal (abdominal) WAT and the liver were snap-frozen in liquid N_2. Tissues and EDTA-plasma were stored at − 70 °C. The experiments followed the guidelines for the use and care of laboratory animals of the Institute of Physiology.

2.2. Plasma metabolites, hormones and enzymes

Plasma lipids were assessed by using a clinical analyzer Hitachi 902 (Roche Diagnostics) and enzymatic kits [28]. Plasma HDL cholesterol (HDL-C) levels were assessed by the kit Biolatest HDL Cholesterol Direct Liquid from Erba Lachema (Brno, Czech Republic). Bile acids were assessed using a Mouse Total Bile Acids Kit (Crystal Chem, IL, USA). Plasma levels of insulin were determined using the Sensitive rat insulin RIA kit from Linco Research (St. Charles, MO, USA). The distribution of adiponectin multimeric complexes in plasma including its high molecular weight (HMW) form was determined by Western blotting [4]. The homeostasis model assessment (HOMA) was applied to quantify insulin

resistance (HOMA-IR index) using the following formula: fasting plasma insulin (mIU/l) × fasting glucose (mmol/l)/22.5.

2.3. Glucose tolerance

Intraperitoneal glucose tolerance tests were performed on overnight fasted mice as described [28].

2.4. Light microscopy and immunohistochemistry

Epididymal WAT samples were fixed in 4% formaldehyde, embedded in paraffin, and 5 μm-sections were stained using hematoxylin–eosine; in epididymal WAT, macrophage marker MAC-2/galectin-3 was used to detect crown-like structures (CLS) density as a measure of inflammation as before [4,5,28].

2.5. Lipid content in the liver and feces

Liver glycerolipid content was estimated in ethanolic KOH tissue solubilisates as before [28]. For estimation of hepatic bile acids [29], livers (100 mg) were homogenized in 1 ml of 75% ethanol, incubated for 2 h at 50 °C, followed by centrifugation for 10 min at 4 °C and 6000 *g*. Hepatic cholesterol content was assessed by a modified Folch's protocol [30]; samples were homogenized in H_2O (1:4), lipid phase was extracted using a mixture (2:1:1.2) of chloroform, methanol, and 3% KH_2PO_4. Chloroform phase was dried under N_2, and resuspended in absolute isopropanol. Cholesterol was assayed by Cholesterol Liquid kit (Erba Lachema, Brno, Czech Republic). Total lipids in feces were isolated by the Folch's protocol and assessed gravimetrically, while fecal cholesterol content was assayed by the kit from Erba Lachema as above.

2.6. FA composition of hepatic lipids

The FA composition of TG, phosphatidylcholine, phosphatidylethanolamine, and cholesteryl ester fractions was analyzed by using gas chromatography as before [5]. For the lipidomics analysis, the detection limit of 0.1 mol% was used.

2.7. Gene expression

Total RNA was isolated from liver samples stored in RNAlater (Ambion, Austin, TX) using TRI Reagent (Sigma-Aldrich, Prague, Czech Republic), and purified by using RNeasy columns (Qiagen, Venlo, The Netherlands). Two-dye labeling, hybridization to whole mouse genome (4 × 44 k) Agilent microarrays (G4122F, Agilent Technologies, Inc., Santa Clara, CA), and scanning were performed as described ([31] and Supplementary data). Real-time quantitative RT-PCR (qPCR) was performed as before [10]. For the gene names, their acronyms, and sequences of oligonucleotide primers, see Supplemental Table 5. All microarray data are in Gene Expression Omnibus (GEO) under accession GSE45235.

2.8. In vivo hepatic VLDL-TG secretion

In a separate experiment ("C"), the rate of liver TG synthesis was evaluated by using Tyloxapol, an inhibitor of plasma VLDL clearance. After fasting for 6 h (6:00 a.m. to 12:00 p.m.), mice were first anesthetized with Pentobarbital (Sanofi-Aventis; 90 mg/kg of body wt.) and then injected into the jugular vein with Tyloxapol (Sigma-Aldrich, Cat. no. T8761) at a dose 500 mg/kg of body wt. as before [32,33]. Plasma TG levels were determined before and 1, 2 and 3 h after injection using a kit Triacylglyceroly Liquid from Erba Lachema (Brno, Czech Republic).

2.9. Statistical analysis

All values are means ± SEM. Logarithmic transformation was used to stabilize variance when necessary. Data were analyzed by paired *t*-test or ANOVA with Holm–Sidak post-hoc tests using SigmaStat 3.5 software. Comparisons were judged to be significant at p b0.05. Principal component analysis (PCA) of microarray data as well as orthogonal partial least squares-discriminant analysis (oPLS-DA) of lipidomics data was performed by using SIMCA-P + 12 software (Umetrics AB, Umea, Sweden). Microarray data (see also Supplementary data; FDR p b 0.05, absolute fold change ≥1.20) were analyzed using MetaCore (GeneGO, Carlsbad, CA, USA) and Gene Set Enrichment Analysis (GSEA [34]).

3. Results

3.1. Prevention of obesity, dyslipidemia, glucose intolerance and WAT inflammation

Body weight gain of the cHF mice was ~4-fold higher as compared with the Chow mice (Table 1). Supplementation of cHF using either omega-3 PL (PC) or both omega-3 PL and rosiglitazone (PC + R) completely prevented the obesogenic effect, while this was not affected in the R mice (Table 1). Effects on weight gain correlated with changes in adiposity (Table 1). Energy intake of mice on cHF-based diets was higher than Chow, and was further increased in response to the omega-3 PL (PC and PC + R;Table 1). Daily lipid losses in feces were estimated in the cHF and PC group using data from an independent experiment C. When compared to the cHF mice, total lipids in feces were significantly decreased in the PC group (cHF, 71 ± 10 vs. PC, 51 ± 2 mg per 24 h; p b 0.05), which could be largely explained by differences in the fecal content of cholesterol (cHF, 19.2 ± 1.3 vs. PC, 8.4 ± 0.8 mg per 24 h; p b 0.001). Furthermore, plasma levels of TG and non-esterified FA (NEFA) were completely normalized while total cholesterol (TC) levels were reduced in the PC and PC + R (Table 1). Since HDL-C was not significantly affected, lower plasma TC levels in the PC group could be likely explained by marked reductions of cholesterol in non HDL-C fraction (Table 1). In the PC + R group, a modest lowering of cholesterol content in both HDL-C and non HDL-C fraction likely contributes to reduced plasma TC levels in these animals (Table 1). Fasting blood glucose (Table 1), glucose tolerance (Fig. 1A, B), fasting plasma insulin (Fig. 1C) and HOMA-IR (Fig. 1D) were improved by all dietary interventions. Plasma levels of HMW adiponectin were increased by all interventions, with the PC + R being highest (Table 1). Accordingly, adipocyte hypertrophy and inflammation (i.e. CLS density) were both reduced by all interventions, especially by PC + R (Table 1).

3.2. Reduced tissue levels of TG and cholesterol associate with specific changes of FA in hepatic PL fraction

Despite unchanged liver weight in cHF (Fig. 2A), hepatic TG (steatosis; Fig. 2B) and cholesterol (Fig. 2C) concentrations were ~ 2.0-fold and ~ 2.7-fold increased, respectively, relative to Chow. Steatosis was completely prevented (Fig. 2B) and cholesterol content almost normalized (Fig. 2C) in both PC and PC + R, while R alone had no effect (Fig. 2B, C). Hepatic bile acid content was reduced in cHF relative to Chow, while omega-3 PL supplementation tended to normalize it (Fig. 2D). Lipidomic analysis of the TG, phosphatidylcholine, phosphatidylethanolamine and cholesteryl esters fractions revealed distinct changes in the FA profiles in response to all the interventions (Supplemental Fig. 1). The analysis of lipidomics data by oPLS-DA (Fig. 3A) separated dietary interventions into four distinct groups according the presence of omega-3 (first principal component) and rosiglitazone (second component). Variable important to projection (VIP) plot identified phosphatidylcholine and phosphatidylethanolamine species containing arachidonic acid, DHA or EPA as the most discriminating analytes (Fig. 3B), while lipids containing the most abundant FA were little

Table 1
Body mass, adiposity and plasma parameters of mice given high-fat diets with or without omega-3 PL and given antidiabetic drug rosiglitazone.

	cHF	PC	R	PC + R	Chow
Energy balance					
Body weight – *initial* (g)	28.5 ± 0.5	28.7 ± 0.5	28.6 ± 0.5	28.9 ± 0.6	30.3 ± 1.0
Body weight – f*inal* (g)	44.4 ± 1.3	33.6 ± 0.9 [ab]	40.7 ± 1.8	30.9 ± 0.8 [ab]	34.3 ± 1.3 [d]
Weight gain (g)	15.9 ± 1.2	4.9 ± 1.1 [ab]	12.1 ± 1.9	2.0 ± 0.9 [ab]	4.0 ± 0.5 [d]
Energy intake (MJ/mouse)	4.13 ± 0.13	5.14 ± 0.18 [ab]	4.10 ± 0.26	4.95 ± 0.22 [ab]	3.38 ± 0.14 [d]
WAT					
Epididymal fat (g)	2.70 ± 0.14	1.2 ± 0.17 [ab]	1.86 ± 0.25 [a]	0.71 ± 0.12 [ab]	0.79 ± 0.08 [d]
Adipocyte size (μm^2)	4066 ± 146	2772 ± 281 [a]	3120 ± 285 [a]	1851 ± 168 [abc]	1984 ± 207 [d]
Inflammation (CLS)	2.55 ± 0.61	0.29 ± 0.12 [a]	0.73 ± 0.30	0.07 ± 0.03 [a]	0.04 ± 0.02 [d]
Subcutaneous fat (g)	0.91 ± 0.09	0.45 ± 0.05 [a]	0.71 ± 0.13	0.35 ± 0.04 [ab]	0.30 ± 0.03 [d]
Plasma					
TG (mmol/l)	2.16 ± 0.14	1.05 ± 0.22 [a]	1.25 ± 0.12 [a]	1.02 ± 0.22 [a]	1.33 ± 0.05 [d]
NEFA (mmol/l)	1.00 ± 0.06	0.44 ± 0.04 [ab]	0.67 ± 0.04 [a]	0.38 ± 0.04 [ab]	0.92 ± 0.06
TC (mmol/l)	4.31 ± 0.07	3.35 ± 0.27 [a]	4.02 ± 0.26	3.27 ± 0.23 [a]	2.60 ± 0.14 [d]
HDL-C (mmol/l)	2.51 ± 0.20	2.32 ± 0.28	2.25 ± 0.31	1.80 ± 0.16 [d]	1.91 ± 0.26
Non HDL-C (mmol/l)	1.96 ± 0.11	1.13 ± 0.23 [a]	1.88 ± 0.29	1.56 ± 0.09 [d]	0.87 ± 0.26 [d]
Glucose (mmol/l)	6.66 ± 0.36	5.32 ± 0.38 [d]	5.22 ± 0.23 [d]	5.54 ± 0.50	4.97 ± 0.21 [d]
Insulin (ng/ml)	4.18 ± 0.48	1.73 ± 0.38 [a]	1.58 ± 0.23 [a]	1.68 ± 0.16 [a]	0.94 ± 0.19 [d]
Adiponectin - HMW (A.U.)	0.34 ± 0.03	0.56 ± 0.09 [a]	0.82 ± 0.11 [a]	1.13 ± 0.18 [ac]	0.33 ± 0.03
HMW: total	0.39 ± 0.02	0.47 ± 0.03	0.50 ± 0.03 [a]	0.59 ± 0.03 [ac]	0.42 ± 0.02

Data are means ± SEM (n = 8 except Chow, n = 5). Cumulative energy intake was assessed during a 7-week period of dietary intervention. Inflammation was assessed as the number of crown-like structures (CLS) per 100 adipocytes. Blood glucose was measured after an overnight fast right before glucose tolerance testing. Non HDL-C was calculated as a difference between TC and HDL-C. A.U., arbitrary units; HDL-C, High-density lipoprotein cholesterol; TC, total cholesterol; TG, triacylglycerols; WAT, white adipose tissue.
[a]p≤0.05 for difference from cHF (ANOVA); [b]p≤0.05 for difference from R (ANOVA); [c]p≤0.05 for difference from PC (ANOVA); [d]p≤0.05 for difference from cHF (t-test).

affected. As expected, arachidonic acid (20:4n-6) decreased and EPA and DHA increased in the PL fractions upon omega-3 intervention (Fig. 3C), while R had relatively little effect.

3.3. Complex down-regulation of hepatic biosynthetic pathways by omega-3 PL

Hepatic gene expression profiles of individual mice (n = 6–8) revealed significantly differentially expressed genes by One-way ANOVA [false discovery rate (FDR)-adjusted p-value ≤ 0.05; Supplemental Table 4] within the PC, R, PC + R, and the control cHF group, as well as with sub-analyses between each intervention group and cHF by Students' t-test (FDR p b 0.05). We continued using the latter approach (Table 2, heatmaps in Supplemental Fig. 2A). The highest number of regulated genes was observed in PC + R, with twice as many down-regulated than up-regulated genes (Table 2A). PCA of gene expression profiles (Supplemental Fig. 2B) revealed a distinct separation of individual mice into the respective dietary groups; the first principal component separated mice according to the presence of omega-3 PL and second component to the presence of rosiglitazone (Supplemental Fig. 2B and

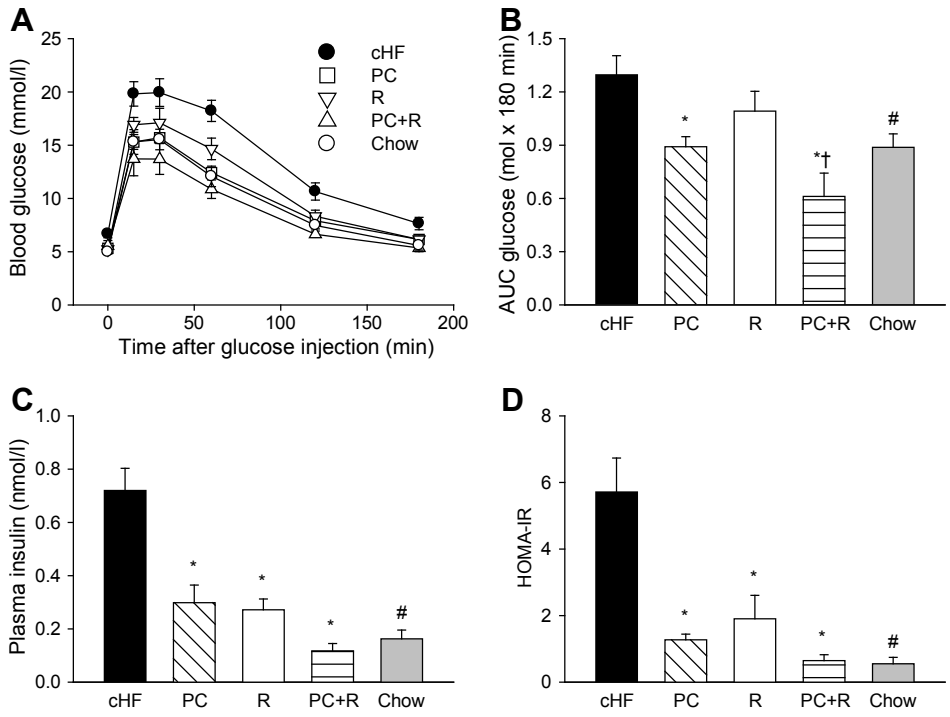

Fig. 1. Prevention of glucose intolerance and insulin resistance. Glucose tolerance test was performed in overnight (15-h) fasted mice fed various diets (A) and area under the glucose curves (AUC) was calculated (B). Plasma levels of insulin in the fasted state (C) as well as HOMA index of insulin resistance (D) are shown. Data are means ± SEM (n = 8, except Chow, n = 5). *p≤0.05 vs. cHF; †p≤0.05 vs. R (ANOVA). #p≤0.05 vs. cHF (t test).

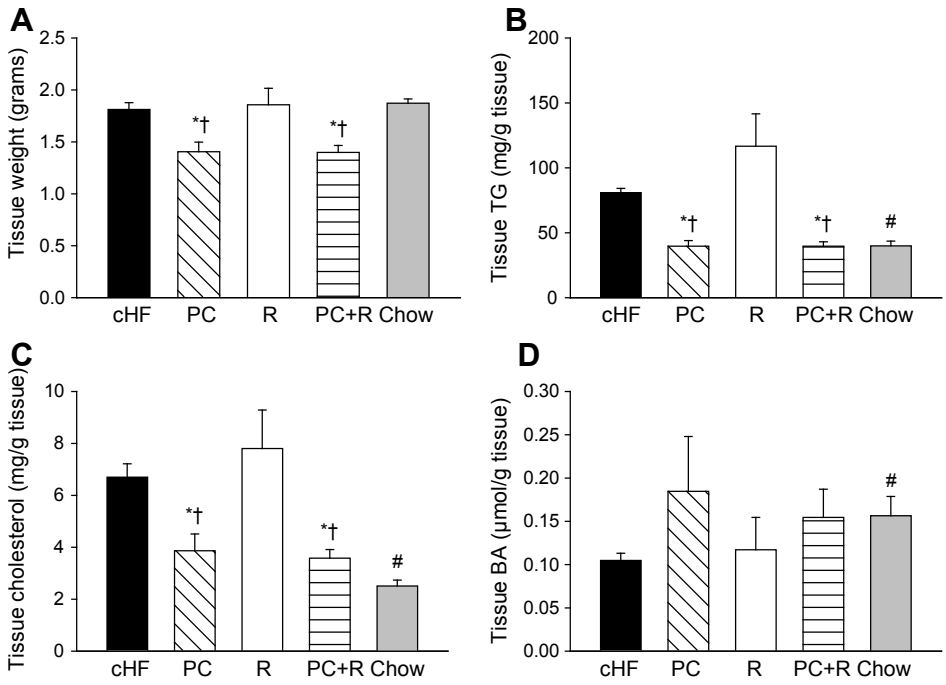

Fig. 2. Reduced hepatic lipid content in response to omega-3 PL. Liver weight (A) and hepatic content of triacylglycerols (B), cholesterol (C), and bile acids (D) was assessed in the ad libitum fed state. Data are means ± SEM (n = 8, except Chow n = 5). $^{*}p \leq 0.05$ vs. cHF; $^{\dagger}p \leq 0.05$ vs. R (ANOVA). $^{\#}p \leq 0.05$ vs. cHF (*t* test).

Supplemental Table 4). Pathway analysis identified FA metabolic processes (all interventions), as well as changes in biosynthesis and metabolism of steroids, sterol metabolism including cholesterol biosynthesis, and FA oxidation (PC and partially also PC + R) as the most affected metabolic processes (Table 2B). Indeed, inhibition of biosynthetic processes in PC was remarkably strong, evidenced by down-regulation of a majority of genes within the de novo FA synthesis pathway (Fig. 4A) and even stronger and complex down-regulation of expression of cholesterol biosynthesis pathway genes (Fig. 4B). GSEA (Supplemental Table 6) confirmed FA and lipid metabolism as most regulated processes in response to omega-3 PL. In contrast, the number of pathways regulated in R was relatively small (Table 2 and Supplemental Table 6).

3.4. Verification of the reduced expression of genes encoding for lipogenic and cholesterol biosynthetic enzymes

In agreement with microarray data showing profound inhibition of biosynthetic pathways in PC (Table 2, and Fig. 4), qPCR analysis revealed up to a 3-fold down-regulation of expression of key genes encoding lipogenic enzymes (*Acly*, *Fasn*, *Scd1* and *Elovl5*; for gene names see Supplemental Table 5) or enzymes involved in the regulation of lipogenesis (*Acacb*) in response to omega-3 PL (both in PC and PC + R), while these genes were up-regulated in the R group (Fig. 5A). In PC + R, the effect of omega-3 PL always outweighed that of rosiglitazone (Fig. 5A). The expression of genes encoding peroxisomal FA oxidation enzymes (*Ehhadh* and *Acox1*), and to a lesser extent also genes encoding mitochondrial FA oxidation enzymes (*Acadm*, *Acadl* and *Acot1*), were up-regulated in response to all dietary interventions (Fig. 5A and Supplemental Table 4). Expression of key cholesterol biosynthesis genes, including *Hmgcs1*, *Fdps* and *Sqle*, was down-regulated, while the expression of *Scarb1* and *Abcg5*, which are involved in cholesterol excretion, was up-regulated by omega-3 PL (Fig. 5B). Moreover, the expression of *Cyp7a1* encoding cholesterol 7α-hydroxylase, the rate-limiting enzyme in the synthesis of bile acids from cholesterol via the classic pathway, was reduced by omega-3 PL (Fig. 5B). Accordingly, the expression of *Nr0b2* encoding small heterodimer partner (SHP), a negative regulator of *Cyp7a1* expression [35], was increased and that of *Nr1h4* encoding farnesoid X receptor was unchanged (Fig. 5B). Genes within the cholesterol and bile acid synthesis pathways were not affected in R.

3.5. Differential metabolic effects and modulation of hepatic gene expression by phosphatidylcholine-rich PL concentrates differing in their FA profiles

To decipher the contribution of a particular FA content for the metabolic effect of various phosphatidylcholine-rich PL concentrates, an independent experiment ("B") was designed, in which we compared herring-derived omega-3 PL (i.e. the PC-M diet) of a similar composition as in case of the PC diet in the experiment A with a phosphatidylcholine-rich PL preparation from soy (PC-S diet; Fig. 6). Each of these two PL concentrates was admixed to the cHF diet at the same concentration (10% of dietary lipids) and both of the subsequent PL-supplemented diets were matched for the content of phosphatidylcholine, i.e. the major type of PL contained in these concentrates. As shown in Fig. 6A, PC-S is relatively enriched in stearate (18:0), α-linolenic acid (18:3n-3), and especially linoleic acid (LA; 18:2n-6), while PC-M is enriched in palmitate (16:0) and, as expected, in EPA and DHA. While feeding control mice obesogenic cHF diet resulted in marked obesity, mice consuming cHF diet supplemented with PC-M partially resisted the development of obesity (Fig. 6B), which could be attributed to a significant reduction in adiposity (Fig. 6C). In contrast, mice fed PC-S gained the same weight and adiposity as obese cHF-fed controls during the 7-week period (Fig. 6B, C). Furthermore, hepatic steatosis was markedly reduced (Fig. 6D) and insulin resistance was ameliorated (Fig. 6E) by PC-M, while PC-S was completely ineffective. Plasma TG and TC levels were reduced by ~25% and 15% in response to PC-M but not in response to PC-S (Fig. 6F). In agreement with the reductions in hepatic and plasma lipids, the expression of selected lipogenic (*Acly*, *Fasn*, *Elovl5*) as well as cholesterol biosynthesis (*Hmgcs1*, *Fdps*, *Sqle*) genes was down-regulated in the livers of mice fed PC-M, while no such effect was observed in mice fed PC-S (Fig. 6G).

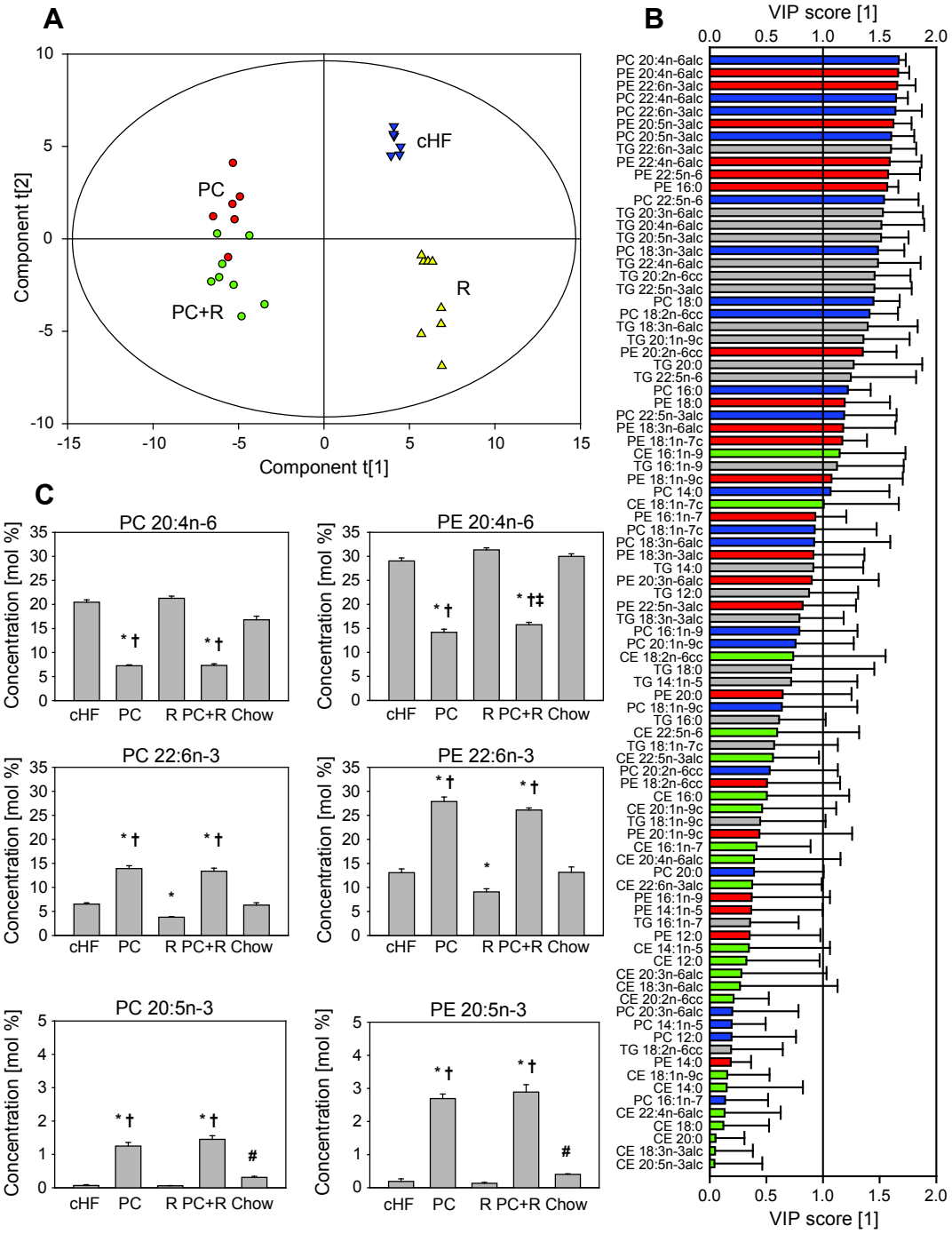

Fig. 3. Hepatic PL fraction as the primary target for omega-3 PL-induced changes in lipid spectra. FA composition of hepatic lipid fractions in mice killed in ad libitum fed state was analyzed (see also Supplemental Fig. 1). (A) Score plot based on the oPLS-DA of the lipidomics data. (B) Corresponding variable important to projection (VIP) plot showing variables important for the separation of dietary interventions along the first major oPLS-DA component. Blue columns, phosphatidylcholines (PC); red columns, phosphatidylethanolamines (PE); gray color, triacylglycerols (TG); green columns, cholesteryl esters (CE). (C) The effect of dietary interventions on FA concentration in the lipid species, representing the highest-scoring variables in the VIP plot. Data are means ± SEM (n = 6–8 except Chow, where n = 5). $^{*}p \leq 0.05$ vs. cHF; $^{\dagger}p \leq 0.05$ vs. R; and $^{\ddagger}p \leq 0.05$ vs. PC (ANOVA). $^{\#}p \leq 0.05$ vs. cHF (*t* test).

4. Discussion

We have demonstrated in mice fed a high-fat diet that dietary supplementation with herring-derived omega-3 PL: (i) prevented weight gain, dyslipidemia, glucose intolerance, hyperinsulinemia and WAT inflammation; (ii) markedly ameliorated hepatic steatosis and reduced accumulation of tissue cholesterol, which was supported by a complex down-regulation of de novo FA synthesis and cholesterol biosynthesis pathways in the liver; and (iii) augmented insulin-sensitizing effect of a low dose rosiglitazone while counteracting its lipogenic effect in the liver. Furthermore, the anti-obesity and antisteatotic effects of omega-3 PL could not be recapitulated when a soy-derived phosphatidylcholine-rich concentrate devoid of DHA/EPA was used.

The effects of omega-3 may depend on the ratio of omega-6 to omega-3 in the diet, which is relatively high in affluent societies, reflecting an increased intake of LA; mimicking this situation in mice [36] resulted in obesity and increased tissue levels of arachidonic acid, the precursor for endocannabinoid biosynthesis [37], which could be prevented by dietary supplementation with DHA and EPA. Also here and in our previous study using LA-rich cHF diet supplemented

Table 2
Differentially expressed genes and major affected pathways in the liver of mice subjected to dietary interventions with omega-3 PL alone or in combination with antidiabetic drug rosiglitazone.

A: *Regulated genes*

Dietary treatment	Number of regulated genes			Fold-change range
	Downregulated	Upregulated	Total	
PC	448	372	820	5.46 to 14.50
R	464	182	646	2.98 to 24.45
PC + R	1377	641	2018	4.69 to 10.40

B: *Major biological processes affected by dietary interventions*

Dietary treatment	Biological process	p-value
PC	Biosynthetic processes	9.6×10^{-11}
	Lipid biosynthetic process	1.2×10^{-10}
	Cholesterol metabolic process	1.6×10^{-10}
	Steroid metabolic process	1.9×10^{-10}
	Steroid biosynthetic process	2.4×10^{-10}
	Cholesterol biosynthetic process	3.2×10^{-10}
	Acetyl-CoA metabolic process	4.8×10^{-10}
	FA metabolic process	9.6×10^{-10}
	Carboxylic acid biosynthesis	8.7×10^{-08}
	FA oxidation	1.1×10^{-07}
	Sterol metabolic process	1.4×10^{-07}
R	Lipid metabolic process	7.9×10^{-10}
	Monocarboxylic acid metabolism	4.0×10^{-07}
PC + R	Biosynthetic process	1.1×10^{-07}
	Coenzyme metabolic process	1.2×10^{-07}
	Lipid biosynthetic process	1.4×10^{-07}
	Innate immune response	1.6×10^{-07}
	Sterol metabolic process	1.8×10^{-07}
	Cholesterol metabolic process	2.2×10^{-07}
	Steroid metabolic process	2.8×10^{-07}
	Lipid transport	3.7×10^{-07}
	Steroid biosynthetic process	5.5×10^{-07}
	FA metabolic process	1.1×10^{-06}

A: Microrarray identified significantly regulated hepatic genes in response to various dietary interventions compared with cHF-fed mice (Students' *t*-test for each intervention versus control cHF group using FDR-adjusted p b 0.05 and absolute fold change ≥ 1.20).
B: Major affected processes (pathways) in the liver identified by MetaCore, using subsets of probes as shown in Table 2A.

with two different doses of omega-3 PL [5], weight gain was reduced and hepatic steatosis prevented in response to herring-derived omega-3 PL. Surprisingly, both effects were stronger in this study, despite a 2- and 6-fold lower dietary content of DHA/EPA (with respect to a high- and low-dose of DHA/EPA in the previous study [5]) and 1.4- and 4.3-fold lower content of PL (see also Tables S1–S3 in ref. [5]). However, the difference may be due to 2- and 6-fold higher levels of lysophosphatidylcholine used here, since lysophosphatidylcholine is well absorbed in the intestine [38] and could augment EPA and DHA bioavailability. Neither here nor in our previous study [5] the anti-obesity effect of omega-3 PL could be explained by reduced food intake or by increased thermogenic activity in brown adipose tissue (based on the expression of uncoupling protein 1; see Supplemental Fig. 3). It is also unlikely that fecal losses of lipids would contribute to the anti-obesity effect of omega-3 PL, since the amount of total lipids in feces of the PC mice was actually reduced. On the other hand, energy-dissipating oxidation of FA in liver peroxisomes could be involved, similarly as documented in mice fed a fish oil-supplemented high-fat diet[39]. Accordingly, the expression of mitochondrial and especially peroxisomal FA oxidation genes was markedly up-regulated in the livers of the PC mice in this study.

Coinciding with a markedly reduced hepatic expression of cholesterol biosynthesis genes, plasma TC levels were decreased by about 20% in response to the PC diet, also consistent with the effect of omega-3 PL in our previous study [5]. The fact that fecal cholesterol content was dramatically decreased (2.3-fold) in the PC mice might suggest the existence of some compensatory mechanisms at the level of enterocytes of the small intestine, analogous to FA-induced down-regulation of intestinal cholesterol transporters observed in mice fed various high-fat Western-type diets [41,42]. In contrast with relatively small changes in plasma TC levels, TG as well as NEFA levels in plasma were strongly reduced in the PC mice as compared with the cHF mice. Moreover, similarly as in most of the human studies on the effects of omega-3, including an intervention trial in NAFLD patients [40], the ratio between HDL-C and non HDL-C increased in response to omega-3 PL supplementation. That omega-3 PL could decrease elevated plasma lipid levels in dietary obese mice [5], but not in lean low-fat diet-fed mice [25] was previously observed. Since the TG-lowering effect of the PC diet could not be explained either by reduced VLDL-TG secretion from the liver (see Supplemental Fig. 4 and below) or by a massive loss of lipids via feces, it probably reflects enhanced lipid catabolism in the liver and possibly in other tissues, as documented in our previous studies showing the induction of FA oxidation both in the small intestine [4] and WAT [24] in response to omega-3 TG.

In accordance with previous studies in obese rodents (see Introduction), omega-3 PL ameliorated hepatic steatosis. Conversely, supplementation of cHF diet with omega-3 TG did not significantly prevent hepatic lipid accumulation in our previous studies [4,5], despite the fact that dietary DHA/EPA content was 6-fold higher when compared with this current study. Antisteatotic action of omega-3 PL could involve strong down-regulation of hepatic genes of de novo FA synthesis, as suggested by the effects of herring-derived omega-3 PL in mice [5] or by krill oil in rats [6] fed high-fat diet. The role of de novo FA synthesis in the development of hepatic steatosis is well recognized [43], as also demonstrated in NAFLD patients [44]. However, reduced supply of NEFA to the liver could also contribute to the amelioration of hepatic steatosis by omega-3 PL. In contrast, there were no significant changes between the cHF-fed mice and mice subjected to dietary omega-3 PL (PC diet) with regard to hepatic secretion of VLDL-TG (see Supplemental Fig. 4), thus ruling out possible changes in the activity of this pathway as a major cause of reduced lipid accumulation in the livers of PC-fed animals. Of note, we have previously shown [4] that dietary intervention with DHA/EPA admixed to the cHF diet as omega-3 TG at a dose ~30 g/kg diet, i.e. 6-fold higher than the dose administered as omega-3 PL in the current study, resulted in a reduced VLDL-TG secretion. Thus, the lack of effect of omega-3 PL on VLDL-TG secretion in the current study might be explained by relatively low DHA/EPA doses rather than by the type of lipid carrier (i.e. PL) used to deliver DHA/EPA. Of note, the liver weight was similar in the cHF-fed and Chow-fed mice despite a marked hepatic steatosis in the former mice. The origin of this phenomena remains to be further explored.

A recent study showed lipid and cholesterol synthesis among the most affected pathways in lean mice fed krill oil-supplemented Chow [25]. Here, in mice fed the obesogenic cHF diet, sterol metabolism was also among the most regulated processes in response to omega-3 PL supplementation. Both hepatic and plasma cholesterol levels were decreased by omega-3 PL, and this effect was associated with an integrated inhibition of genes engaged in hepatic cholesterol synthesis with elevated gene expression of sterol transporters ABCG5 and ABCG8 (Supplemental Table 4) and scavenger receptor SR-BI. Although in lean mice fed krill oil-containing Chow, down-regulated expression of *Srebf-2* encoding the major transcriptional regulator of cholesterol biosynthesis genes was observed [25], the expression of *Srebf-2* remained unchanged in response to omega-3 PL in our study. The complex inhibition of both lipogenic and cholesterol biosynthesis pathways by herring-derived omega-3 PL could be linked to the inhibition of the activity of mitochondrial citrate carrier (encoded by *Slc25a1*), as previously observed in krill oil-supplemented rats fed high-fat diet [6]. In our study, microarray analysis revealed that hepatic *Slc25a1* expression was reduced by 25% in the PC mice. Furthermore, hepatic bile acid content tended to increase despite reduced gene expression of *Cyp7a1*, the rate-limiting enzyme in bile acid synthesis from cholesterol via the classic pathway, and despite increased gene expression of SHP, that is involved in the

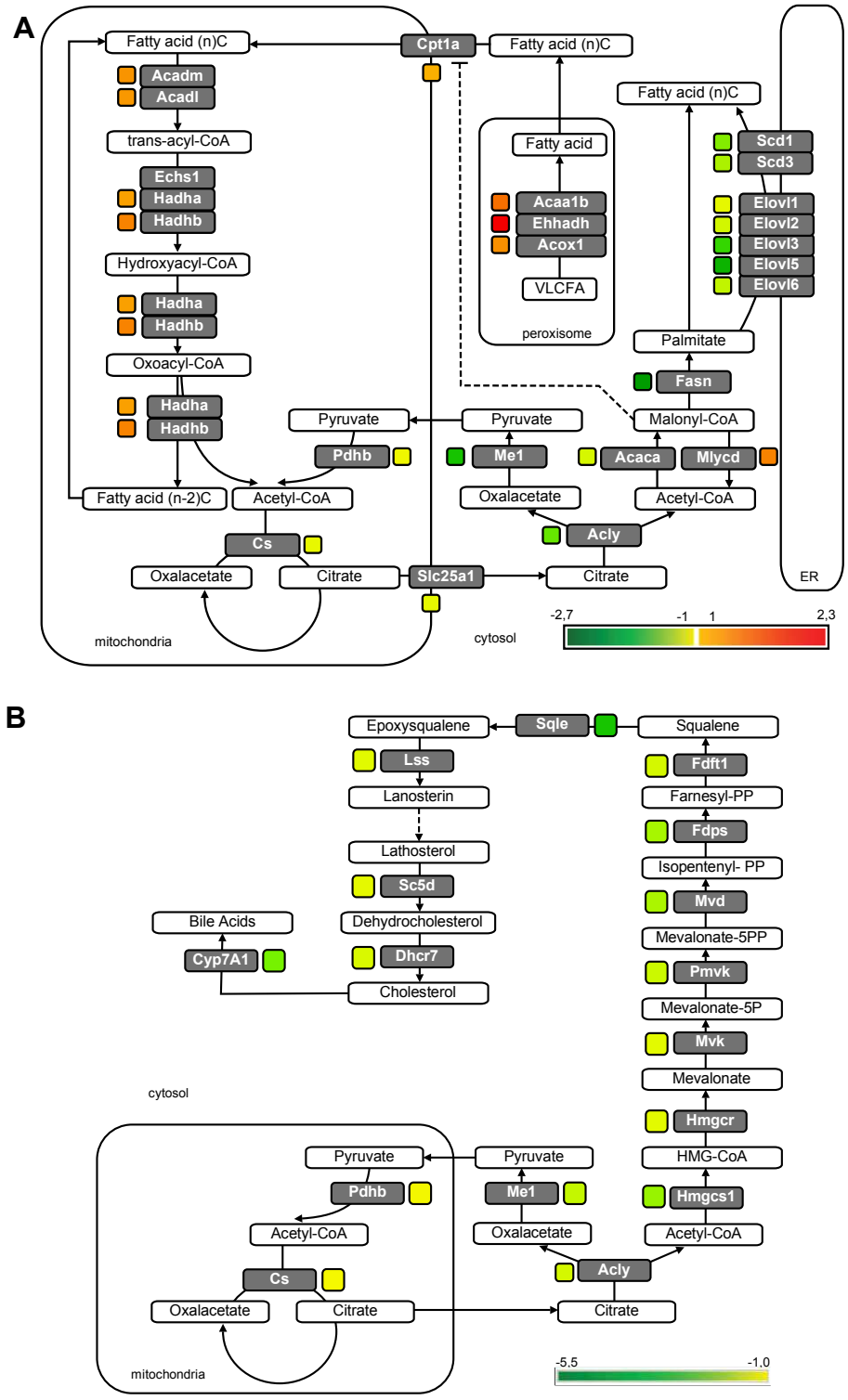

Fig. 4. Modulation of hepatic FA metabolism and cholesterol biosynthesis pathways by omega-3 PL. Microarray-analyzed gene expression changes in major pathways affected in response to dietary omega-3 PL, including FA synthesis and oxidation (A) and cholesterol biosynthesis (B), are schematically shown (see also Fig. 5). Genes up- and down-regulated in response to dietary omega-3 PL (PC intervention) are depicted in red and green, respectively (color bars indicate fold change). Only genes showing significant regulation (FDR, p b 0.05) were used. See Supplemental Table 4 for a complete list of significantly regulated genes and corresponding fold-changes induced by dietary interventions.

repression of *Cyp7a1* and bile acid synthesis [35]. Thus, the precise regulation of bile acids pool in response to dietary omega-3 PL remains to be clarified. Our results support the concept that reduced lipid synthesis together with increased cholesterol excretion represents the major mechanisms by which dietary omega-3 PL could affect lipid and cholesterol metabolism in the liver [26]. At the same time, omega-3 PL induced the expression of genes encoding mitochondrial and especially peroxisomal FA oxidation enzymes, known targets of PPARα [19]. Accordingly, increase in the hepatic CPT-1 activity and carnitine levels in response to krill oil-supplemented high-fat diet in rats was recently demonstrated [6].

It should be noted, however, that some of the effects of omega-3 PL could originate from the specific PL constituent itself, independently of

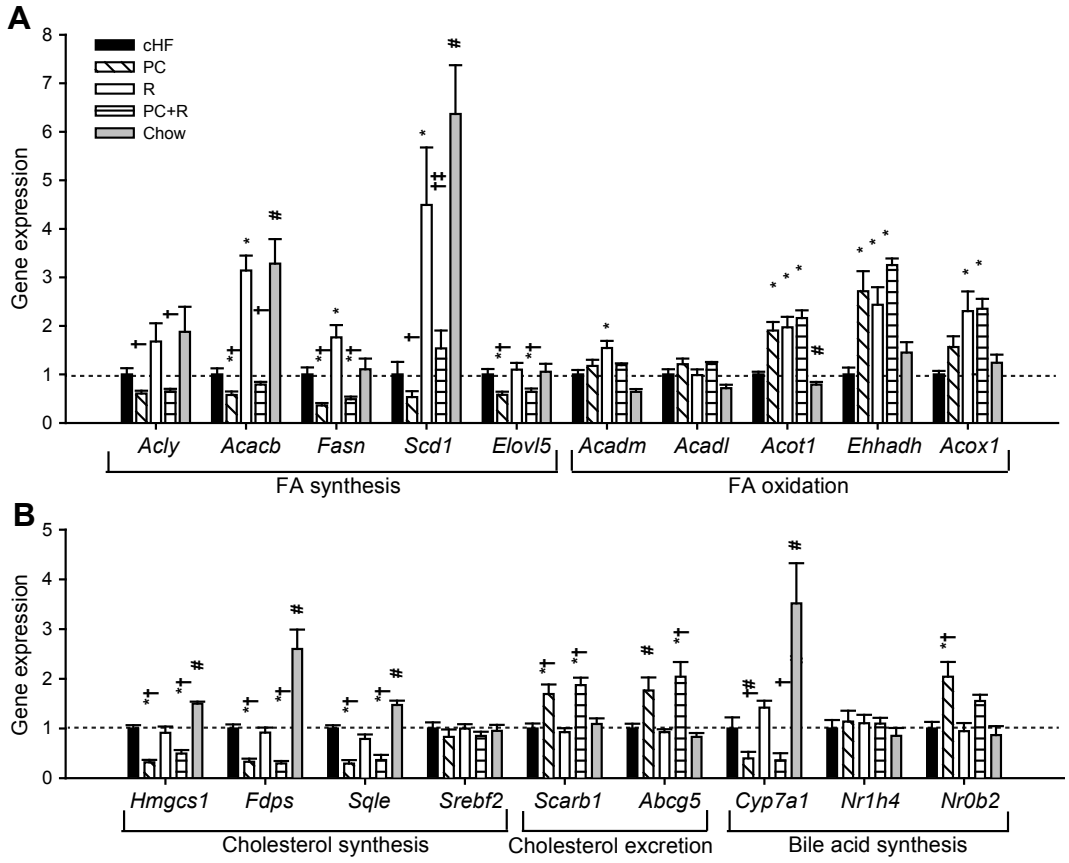

Fig. 5. Dietary interventions regulate hepatic expression of genes involved in FA and cholesterol metabolism. Hepatic expression of selected genes involved in FA (A) and cholesterol (B) metabolism was assessed using qPCR. Data are means ± SEM (n = 8, except Chow n = 5). $^{*}p \le 0.05$ vs. cHF; $^{\dagger}p \le 0.05$ vs. R (ANOVA). $^{\#}p \le 0.05$ vs. cHF (*t*-test).

omega-3. For instance, it has been shown in rabbits and rats that dietary soy-derived phosphatidylcholine was able to alleviate hepatic steatosis [45], reduce FA synthesis in the liver [46], and stimulate bile acid pool size and cholesterol output in the bile [45,47,48], as well as lower cholesterol absorption in the intestine [49]. Since phosphatidylcholine also represented the major PL fraction in herring-derived omega-3 PL concentrates used in our current studies (see above and Supplemental Table 2), we could directly compare metabolic effects of omega-3 PL with soy-derived PL, i.e. the PL concentrates with completely different FA profiles (see Fig. 6A), under obesogenic conditions induced by high-fat feeding. Surprisingly, in contrast to omega-3 PL, soy-derived PL containing mostly LA, as well as minor palmitic, stearic, oleic and α-linolenic acids, completely failed to prevent weight gain, body fat accretion, insulin resistance, dyslipidemia and hepatic steatosis, as well as failed to down-regulate hepatic expression of genes encoding FA synthesis and cholesterol biosynthesis enzymes. This demonstrates that DHA/EPA is a necessary component in the PL molecule in order to carry out its anti-obesity and antisteatotic action under these conditions. Moreover, in our previous study [5], in which either omega-3 PL or omega-3 TG were supplemented to the cHF diet at a much higher dose of ~30 g/kg diet, omega-3 TG failed to significantly down-regulate hepatic genes involved in FA synthesis and cholesterol biosynthesis in association with a much weaker effect on hepatic and plasma lipids when compared to omega-3 PL (our unpublished data). The above data suggest that it is both the FA profile enriched with DHA/EPA and the proper lipid carrier (likely PL in the form of phosphatidylcholine) that ensure the highest efficiency regarding the prevention of various metabolic abnormalities associated with dietary obesity.

Multiple mechanisms may be responsible for the relatively strong biological effects of omega-3 PL of marine origin, including: (i) the effect of this molecular form on absorption, transport and organ distribution of omega-3 FA [50,51], namely the superior bioavailability of DHA and especially EPA (refs. [5,52] and recently reviewed in [53]); (ii) relatively strong depression of endocannabinoid system activity in the tissues by omega-3 PL[5,15,23,37,54]; and (iii) regulation of cellular metabolism by yet unidentified omega-3 PL species functioning as ligands to specific nuclear receptors similarly as in case of 16:0/18:1-phosphatidylcholine being a ligand to PPARα [55] or 12:0/12:0-phosphatidylcholine acting as an agonist to liver receptor homolog-1 [56].

It has previously been shown that omega-3 TG could improve hepatic insulin sensitivity [4,11], and that the combined intervention using omega-3 TG and TZD rosiglitazone in an additive manner reduced weight gain, adiposity, WAT inflammation and dyslipidemia associated with cHF feeding, while inducing adiponectin and improving whole-body and muscle insulin sensitivity [4]. Here, using herring-derived omega-3 PL, we could recapitulate all the above additive effects. Rosiglitazone was administered in a low-dose, which by itself could not affect hepatic insulin sensitivity [4], but instead support in situ lipogenesis [27]. Except for the FA oxidation pathway, the rosiglitazone effects on the major omega-3 PL-regulated pathways such as lipogenesis differed quite substantially, and were eventually fully counteracted when rosiglitazone was combined with omega-3 PL. Future studies regarding hepatic effects of omega-3 PL might reveal novel targets for treatment of insulin resistance.

In conclusion, our results demonstrate that omega-3 PL supplemented to a high-fat diet in mice efficiently prevent weight gain, insulin resistance and dyslipidemia, while reducing hepatic steatosis, which is associated with integrated inhibition of FA synthesis and cholesterol biosynthesis in the liver. These complex effects are unique to omega-3 PL, since they were absent in response to soy-derived PL that do not

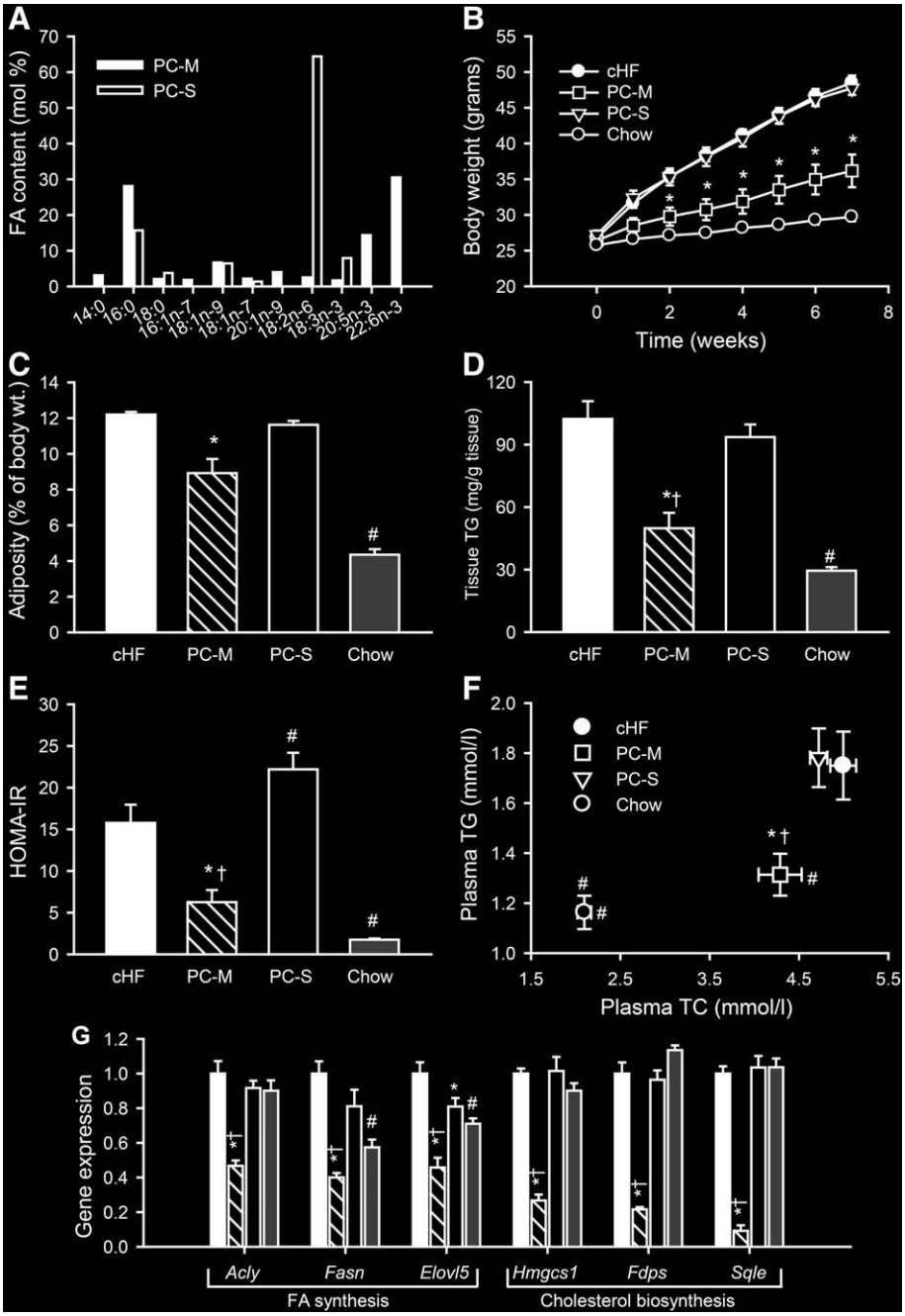

Fig. 6. Differential effects of phosphatidylcholine-rich PL concentrates varying in their FA profiles. Mice were fed for 7 weeks the cHF diet (obese controls) or cHF-based diets supplemented with PL concentrates isolated either from herring (PC-M) or soy (PC-S). (A) The profiles of major FAs contained in the PL concentrates used in this study; see also Supplemental Table 2 and its legend. (B) Body weight gain during a 7-week-long period of differential dietary intervention. (C) Adiposity index calculated as a sum of weights of all three major fat depots in the body (epididymal, dorsolumbar subcutaneous, and mesenteric) and expressed as a percentage of body weight. (D) Hepatic content of triacylglycerols. (E) The level of insulin resistance expressed as HOMA-IR index. (F) Plasma levels of lipid metabolites (x-axis: TC, total cholesterol; y-axis: TG, triacylglycerols). (G) mRNA levels of selected genes within the lipogenic (FA synthesis) and cholesterol biosynthesis pathways. All data are means ± SEM (n = 6–8). $^{*}p \leq 0.05$ vs. cHF; $^{\dagger}p \leq 0.05$ vs. PC-S (ANOVA). $^{\#}p \leq 0.05$ vs. cHF (t-test).

contain DHA or EPA. Thus, omega-3 PL, either alone or in combination with antidiabetic drugs, could be of therapeutic value in obese subjects with NAFLD.

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