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Fish oil minimises feed intake and improves insulin sensitivity in Zucker fa/fa rats

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Abstract

Long-chain *n*-3 PUFA (LC *n*-3 PUFA) prevent, in rodents, insulin resistance (IR) induced by a high-fat and/or fructose diet but not IR induced by glucocorticoids. In humans, contrasting effects have also been reported. We investigated their effects on insulin sensitivity, feed intake (FI) and body weight gain in genetically insulin resistant male obese (fa/fa) Zucker (ZO) rats during the development of obesity. ZO rats were fed a diet supplemented with 7 % fish oil (FO) + 1 % corn oil (CO) (wt/wt) (ZO_{FO}), while the control group was fed a diet containing 8 % fat from CO (wt/wt) (ZO_{CO}). Male lean Zucker (ZL) rats fed either FO (ZL_{FO}) or CO (ZL_{CO}) diet were used as controls. FO was a marine-derived TAG oil containing EPA 90 mg/g + DHA 430 mg/g. During an oral glucose tolerance test, glucose tolerance remained unaltered by FO while insulin response was reduced in ZO_{FO} only. Liver insulin sensitivity (euglycaemic–hyperinsulinaemic clamp + 2 deoxyglucose) was improved in ZO_{FO} rats, linked to changes in phosphoenolpyruvate carboxykinase expression, activity and glucose-6-phosphatase activity. FI in response to intra-carotid insulin/glucose infusion was decreased similarly in ZO_{FO} and ZO_{CO}. Hypothalamic ceramides levels were lower in ZO_{FO} than in ZO_{CO}. Our study demonstrates that LC *n*-3 PUFA can minimise weight gain, possibly by alleviating hypothalamic lipotoxicity, and liver IR in genetically obese Zucker rats.

Keywords: **Long-chain *n*-3 fatty acids: Obesity: Insulin resistance: Ceramides: Lipotoxicity**

Long-chain *n*-3 PUFA (LC *n*-3 PUFA) have repeatedly demonstrated to partially or completely prevent insulin resistance (IR) in rodents as well as in humans, but contrasting effects have been observed in both species[1,2]. In rats, they can prevent IR induced by a high-fat, a high-sucrose or a high-fructose diet[3–5]. Similarly, in humans, they also prevent or even decrease IR. Furthermore, when given before and throughout gestation and lactation in dams fed a high-fat diet, they can hamper materno-fetal-transmitted IR in male offspring[6]. However, in rats made insulin resistant by a glucocorticoid (dexamethasone), they amplify the decrease in liver and muscle PI3K activity, resulting in an increase in the dexamethasone-induced alteration of insulin signalling[7]. We introduced dexamethasone as a model of IR here to underline that there was contradictory effects of LC *n*-3 PUFA towards IR, which led to the possibility that they have different effect depending on the model studied (diet, pharmacological, genetic). Similarly, in healthy humans, LC *n*-3 PUFA aggravate dexamethasone-induced IR[8]. In patients with type 2 diabetes, most studies have been unable to show an alleviation of IR, but the recent meta-analysis by Delpino *et al.*[2] suggests that they could ameliorate the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), an index of IR. Thus, the metabolic effects of LC *n*-3 PUFA can differ depending on the cause of IR.

Abbreviations: BW, body weight; CO, corn oil; DAG, diacylglycerol; FI, feed intake; FO, fish oil ; G6Pase, glucose-6-phosphatase; IR, insulin resistance; LC *n*-3 PUFA, long-chain *n*-3 PUFA; OGTT, oral glucose tolerance test; PEPCK, phosphoenolpyruvate carboxykinase; PKC, protein kinase C.

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To further explore this contrasting effect, we aimed to study the effects of LC *n*-3 PUFA on main metabolic parameters (feed intake (FI), body weight (BW), glucose tolerance and whole body and tissue insulin sensitivity) in genetically insulin resistant non-diabetic obese (fa/fa) Zucker rats (ZO)[9–11]. To that end, male ZO rats and their lean Zucker (Fa/Fa) control (ZL) were fed a corn oil (CO) diet (ZO_{CO}) or an equienergetic fish oil (FO) diet containing (ZO_{FO}) for 9 weeks. In the case where FO had an inhibitory effect on FI, we hypothesised that this effect could be examined by a reduction in hypothalamic lipotoxicity. To this end, we determined the hypothalamic concentrations of ceramides and diacylglycerol (DAG).

Experimental methods

Animals' husbandry

The experimental procedure was approved by the Animal Ethical Committee of the University of Brest, and the protocol was carried out in conformity with French laws and regulations and the ARRIVE Guidelines for Reporting Animal Research.

ZO male rats and ZL rats were obtained from Charles River Laboratories. The obese or fatty condition appeared spontaneously in the 13M strain maintained at the Laboratory of Comparative Pathology of Theodore and Lois Zucker in Stow, MA. Rats were individually housed in a temperature-, humidity- and light-controlled room. Single housing was necessary to measure food intake accurately in every rat; the cages were transparent and the rats could see each other. Due to practical reasons and the already large number of single-housed rats, we chose to perform this study in males only, which is a limitation of the study.

The groups of animals used in this study are described below.

On the day of sacrifice, all rats were killed in the morning before 10 hours after being anaesthetised with pentobarbital sodium (50 mg/kg BW). One rat from each four groups was chosen randomly, and these four rats were killed simultaneously and this was repeated until all the rats had been killed. By killing one rat from each group at the same time, this prevented a potential time effect of sacrifice. After decapitation, trunk blood was immediately collected in heparinised tubes, and the plasma was separated by centrifugation and stored at –20°C until subsequent analysis. We used –80°C only for storage of tissues in order to save space in the freezer because several other things were stored or had to been stored from other studies. Plasma samples were analysed within 1 month prior to assays for proteins of interest (insulin, leptin, adiponectin). We assume that this 1-month storage at –20°C had no deleterious effect on protein preservation and integrity[12,13]. Moreover, the leptin value we measured was similar to that of several publications, for example, this one by Holm *et al.*[14], where plasma leptin in ng/ml is around 25 in Zucker rats, close to the value we observe, 26·8 (Table 3). In the other published article, we also have a leptin value of 35 ng/ml[15], again in the same order of magnitude as our present study.

Liver, leg muscles (soleus and tibialis anterior), adipose tissue (epididymal and inguinal) and hypothalamus were immediately frozen, powdered into liquid N_2 and stored at –80°C until subsequent analysis. Epididymal fat pad is part of abdominal fat in rodents both with mesenteric fat[16] whereas inguinal fat is representative of subcutaneous fat pad. Skeletal muscles were chosen as representative of oxidative (soleus) and glycolytic (tibialis anterior) muscles. In addition, these muscles are those usually used when assessing muscle insulin sensitivity in rats because they are considered as representative of insulin sensitivity of whole striated muscles of the animals[17].

Experiment 1: assessment of insulin sensitivity and glucose tolerance (Fig. 1(a))

Twenty-four ZO and 24 ZL 3-week-old male rats were studied. Each group was further divided into two subgroups of twelve rats receiving either a control or a FO diet over 9 weeks. The control diet contained 8 % fat from CO rich in *n*-6 fatty acids; the FO diet contained 7 % of FO rich in LC-*n*-3 PUFA + 1 % CO. FO used was a marine-derived TAG oil containing EPA 90 mg/g + DHA 430 mg/g (Omegavie® 1050 TG Quality Silver) provided by Polaris. Both diets were prepared by the Unité de Préparation des Aliments Expérimentaux (UPAE, INRAE), and their composition is presented in Tables 1 and 2. To study the specific effect of FO replacement, we chose to not include any FO in the control diet because even a low dose of FO inside CO diet could have masked some differences between the two diets. It would have been better to include a low dose of FO in the control diet to be absolutely certain that such would or would not have any masking effect and that not trying this is a limitation of the study.

FI was recorded daily and BW once a week.

Physiological studies of glucose metabolism were performed in these four groups of twelve rats (two groups of twelve ZO and two groups of twelve ZL rats). Each of these subgroups was further divided into two subgroups of six rats (Fig. 1(a)). In one subgroup of six rats, glucose tolerance and insulin sensitivity were assessed by using an oral glucose tolerance test (OGTT) at day 0, week 4 and week 9. In the other subgroup of six rats, glucose metabolism was assessed at week 9 under euglycaemic–hyperinsulinaemic clamp with tritiated glucose infusion for determination of hepatic glucose production and with 2-deoxyglucose infusion for determination of muscle and adipose tissue glucose uptake.

Oral glucose tolerance test. At each time, rats were fasted overnight, and then a local anaesthetisation was performed on the tail with lidocaine (EMLA 5 % cream; AstraZeneca) to take vein iterate blood samples. The first vein blood sample was taken (T0) (50 µl) and immediately after, an oral glucose solution (3 g/kg BW) was administered. Blood samples were collected at times 10, 20, 30, 45, 60, 90 and 120 min after glucose administration (50 µl at each time point). A fraction was immediately used for glucose determination using a glucometer (OneTouch®, Lifescan SAS); another fraction was collected in tubes containing heparin to separate the plasma by centrifugation at 10·000 ***g***, 4°C. Plasma samples were stored at –20°C until further analysis of glucose, insulin, NEFA, leptin and adiponectin plasma concentrations. Plasma adiponectin and leptin concentrations were determined in ZO rats only.

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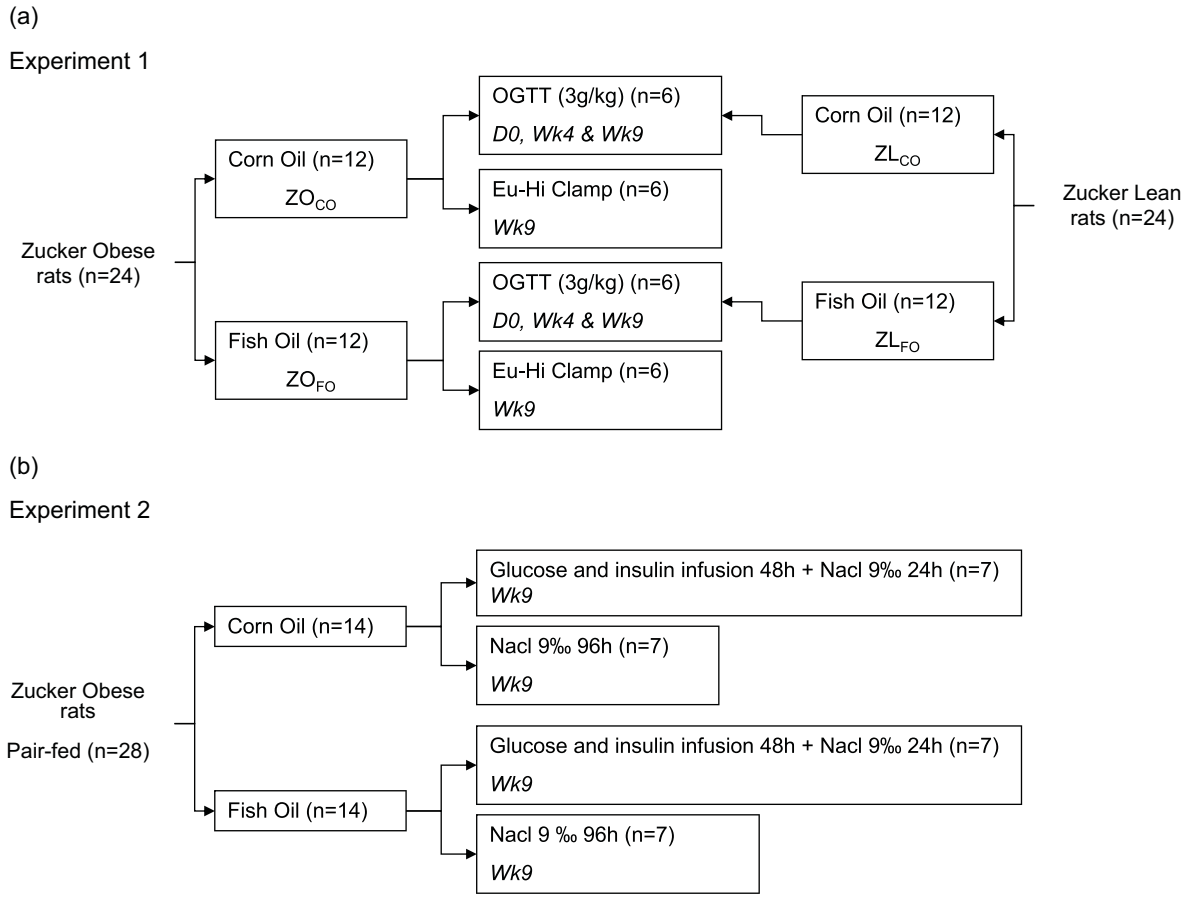

Fig. 1. Schematic overview of the different experimental procedures. (a) Experiment 1: assessment of insulin sensitivity and glucose tolerance in lean and obese Zucker male rats fed corn oil diet (CO) or fish oil diet (FO); (b) Experiment 2: glucose and insulin carotid infusion in male obese Zucker rats fed CO or FO.

Euglycaemic–hyperinsulinaemic clamp studies and 2-deoxyglucose experiments. The clamp was carried out in ZO only. Rats were subjected to clamp studies after 9 weeks of diet as described[18,19]. Briefly, rats were deprived of feed for 5 h (from 09.00 to 14.00) and anaesthetised with pentobarbital sodium (50 mg/kg BW). A catheter was inserted into the right jugular vein for blood sampling. Infusions (insulin, labelled and unlabelled glucose) were carried out using butterfly needles inserted in the saphenous vein. Before insulin infusion, two sets of blood samples were drawn for determination of basal blood glucose and insulin concentrations. A priming dose of [3–^{3}H] glucose (5 μCi) and a priming dose of 20 mUI of insulin (Actrapid®, Novo) dissolved in isotonic saline were injected through the saphenous vein, followed by a continuous infusion of tritiated glucose (0·2 μCi/min) and of insulin (0·4 U/kg/h) at a constant rate of 20 μl/min. During clamp, blood was sampled from caudal vessels every 5 min to determine blood glucose and to adjust the rate of unlabelled glucose infusion to maintain euglycaemia (glucose infusion rate). The euglycaemic clamp was attained within 30–40 min and maintained for 40 min thereafter. Steady-state-specific glucose radioactivity and blood glucose and insulin concentration were determined during the last 20 min of the clamp using three latest blood samples. Then, an injection of a bolus of 2-deoxy-D-[1-^{14}C] glucose (2-DG, 5 μCi, Amersham), a glucose analogue that did not undergo further degradation following the 6-phosphate addition by the hexokinase, was done. Blood samples (50 μl) were collected from the tail at times 0, 5, 10, 20, 30, 40, 50 and 60 min until the end of the experiment where rats were killed by lethal pentobarbital injection, and muscle and adipose tissues were collected.

Calculation of glucose kinetics. Basal and steady-state plasma [3-^{3}H] glucose radioactivity were measured as described[12]. Briefly, for [3-^{3}H] glucose determination, plasma was deproteinised with $Ba(OH)_2$ and $ZnSO_4$. Aliquots of the supernatant of each sample were dried to remove $^3H_20$, and the dry residues were dissolved in 0·5 ml water to which 10 ml scintillation solution was added (Aqualuma Plus® scintillator, Lumac BV). Radioactivity was determined in a Packard Tri-Carb 460C liquid scintillation counter. The rate of glucose disappearance (Rd), reflecting glucose utilisation rate, was calculated according to the formula Rd = Ra (rate of appearance) = [3-^{3}H] glucose infusion rate (dpm/min/kg) divided by blood glucose-specific activity (dpm/mg). Endogenous glucose production (given as mg/kg/min) was determined by subtracting the glucose infusion rate from total Ra (= Rd). *In vivo* glucose uptake (ng/mg of tissue/min) for muscle (tibialis anterior and soleus) and white adipose tissue (inguinal and peri-epididymal) was calculated

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Table 1. Diet composition of corn oil diet (CO) and fish oil diet (FO), g/100 g. The FO used was a marine-derived TAG oil containing EPA 90 mg/g + DHA 430 mg/g (Omegavie® 1050 TG Quality Silver) provided by Polaris

	Corn oil diet (%)	Fish oil diet (%)
Total protein	22·16	22·16
Casein	22	22
DL methionine	0·16	0·16
Total carbohydrates	62·84	62·84
Corn starch	41·89	41·89
Sugar	20·95	20·95
Total lipids	8	8
Corn oil	8	1
Fish oil	0	7
Minerals	4	4
Vitamins	1	1
Fibre	2	2
Energy content (kcal/100 g)	404	404

Table 2. Fatty acid composition of the two diets (g/100 g of lipids)

	Corn oil diet	Fish oil diet
SFA		
C14:0	0·6	8·2
C16:0	25·3	16·1
C18:0	4·9	4
C20:0	0·5	0·2
Σ	31·3	28·5
MUFA		
C16:1 (*n*-7)	0·3	10·4
C18:1 (*n*-9)	11·3	10·2
C18:1 (*n*-7)	3·9	1·1
C20:1 (*n*-9)	0·1	0·9
C20:1 (*n*-7)	0·09	0·1
Σ	15·69	22·7
n-6 PUFA		
C18:2 (*n*-6)	52·9	19·3
C20:3 (*n*-6)	0	0·08
C20:2 (*n*-6)	0	1
C20:4 (*n*-6)	0	0·91
Σ	52·9	20·38
n-3 PUFA		
C20:5 (*n*-3)	0	15·4
C22:5 (*n*-3)	0	2·1
C22:6 (*n*-3)	0	10·9
Σ	0	28·4

based on the accumulation of 2-DG-6-phosphate in the respective tissues and the disappearance rate of 2-DG from plasma, as described[20].

Phosphoenolpyruvate carboxykinase and glucose-6-phosphatase enzymatic activities. Liver phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) enzyme activities were measured in the liver of ZO_{FO} and ZO_{CO} rats only. We previously showed that the best approach to preserve PEPCK and G6Pase activities at their initial level was to quickly freeze-clamp liver and intestine at liquid N_2 temperature and further maintain them at –80°C[21,22]. Frozen liver samples were powdered into liquid N_2 and homogenised by sonication in 20 mM HEPES, 0·25 M sucrose (pH 7·3) (100 µg of wet tissue per millilitre). Homogenates were diluted ten times before determination of G6Pase at maximal velocity (20 mmol/l glucose-6 phosphate) at 30°C by complex formation of the phosphate produced, as previously described[21]. Specific G6Pase activity was cleared of the contribution of non-specific phosphohydrolase activities by subtracting the activity towards β-glycerophosphate (20 mmol/l). PEPCK was determined in 100 000 × ***g*** supernatants of the homogenates, using the decarboxylation assay described by Rajas *et al.*[23] and by Jomain-Baum and Schramm[24].

For PEPCK protein quantification, 30 µg protein was separated by electrophoresis in 9 % polyacrylamide gels in the presence of SDS. Protein concentration was assayed by the Lowry protein assay[25]. The method combines the reactions of copper ions with the peptide bonds under alkaline conditions (the Biuret test) with the oxidation of aromatic protein residues. The Lowry method is based on the reaction of Cu^+, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid). This reagent allows the aromatic amino acids (tyrosine and tryptophan) to be reduced, leading to the formation of a dark blue coloured complex (heteropolymolybdenum blue) whose absorbance will be measured by absorbance at 660 nm. As a result, the total concentration of protein in the sample can be deduced from the concentration of tryptophan and tyrosine residues that reduce the Folin–Ciocalteu reagent.

Total protein extracts were then quantified using a DC protein assay (Biorad), and concentration was determined quantitatively using 96-well micro-plates (Nunc) and a micro-plate reader connected to KC4 v3 software (Bio-Tek Instruments, Inc.).

After electro transfer to polyvinylidene fluoride immobilon membranes (Millipore Corp.), immunodetection was performed using anti-PEPCK antibodies at dilutions of 1:7000[24]. Membranes were re-blotted with anti-*β*-tubulin (use at 1:2000; Santa Cruz Biotechnology, Inc.) for standardisation.

Experiment 2: glucose and insulin intra-carotid infusion (Fig. 1(b))

In a second set of experiments, twenty-eight 3-week-old ZO rats were divided into two groups (n 14 per group) and were pair-fed with CO diet *v.* FO diet for 9 weeks. BW was measured every week. After 9 weeks, these rats were used to measure FI in response to glucose and insulin carotid infusion.

The long-term infusion technique (96 h) under unrestrained conditions was used as previously described[15]. Briefly, 3 days before the experiments, rats were anaesthetised with pentobarbital injection intra-peritoneally (50 mg/0·1 kg of BW) for the placement of a catheter in the carotid artery with the tip being directed towards the brain. The catheters were exteriorised at the vertex of the head and attached to a swivelling infusion device, allowing the rats free access to water and diet. After a week's recovery from surgery, the experiments took place. Briefly, insulin and glucose were infused concomitantly, at a rate of 2·5 pmol/min and 0·50 mg/min, respectively (at a flow rate of 7 µl/min), for 48 h in both ZO_{CO} and ZO_{FO} rats. These rates were shown not to elevate the systemic concentrations of glucose or insulin[15] as we only wanted to induce a supply of glucose and insulin to the central nervous system, without modifying their concentration at the periphery. After 48 h, glucose and insulin were

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Table 3. Blood glucose, plasma insulin, free fatty acid (FFA), leptin and adiponectin levels of obese and lean Zucker rats fed either with corn oil diet (CO) or fish oil diet (FO) at D0, week 4 and week 9 of diet (Mean values with their standard error of the mean)

	Lean CO		Lean FO		Obese CO		Obese FO	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
				Day 0				
Glucose (mmol/l)	6·44	0·23[a]	6·19	0·37[a]	6·32	0·50[a]	5·97	0·30[a]
Insulin (nmol/l)	0·25	0·02[a]	0·24	0·02[a]	0·54	0·06[b]	0·57	0·03[b]
NEFA (mmol/l)	0·84	0·06[a]	0·74	0·17[a]	1·78	0·18[b]	1·73	0·16[b]
Leptin (ng/ml)					26·80	1·74[a]	29·77	3·31[a]
Adiponectin (μg/ml)					19·13	1·83[a]	22·18	1·36[a]
				Week 4				
Glucose (mmol/l)	6·51	0·21[a]	6·08	0·20[a]	7·38	0·31[b]	7·21	0·73[a,b]
Insulin (nmol/l)	0·35	0·03[a]	0·25	0·02[a]	1·81	0·39[c]	2·04	0·25[c]
NEFA (mmol/l)	0·67	0·07	0·50	0·02[a]	1·28	0·17[b]	1·25	0·20[b]
Leptin (ng/ml)					118·72	13·44[b]	111·43	4·54[b]
Adiponectin (μg/ml)					9·56	1·12[b]	9·18	0·97[b]
				Week 9				
Glucose (mmol/l)	7·11	0·18[a]	6·47	0·29[a]	10·66	1·15[c]	8·69	1·01[b,c]
Insulin (nmol/l)	0·52	0·06[a]	0·38	0·05[c]	4·68	0·75[d]	4·16	0·51[c]
NEFA (mmol/l)	0·66	0·10[a]	0·88	0·10[a]	1·58	0·11[b]	1·22	0·10[c]
Leptin (ng/ml)					146·18	5·67[b]	135·65	10·07[c]
Adiponectin (μg/ml)					8·71	0·74[b]	8·16	0·79[b]

Values are presented as mean values with their standard error of the mean (n 6/group). Obese and lean Zucker rats were fed corn oil diet (CO) or fish oil diet (FO) diet for 9 weeks. Rats were evaluated at DO and after 4 and 9 weeks of experimental manipulation. [a,b,c]Parameters with differing superscripts differ from each other at the $P < 0·05$ level by post hoc Bonferroni adjustment after significant intergroup differences were found by the two-way ANOVA.

replaced by NaCl 9‰ over the next 24 h to allow FI return to baseline condition. During the infusion, daily FI was measured manually.

Analytical procedures

Plasma parameters. Blood glucose was determined by reflectometry using a glucose analyser (Accu-Chek®, Roche). The system was calibrated using whole blood with different glucose levels. Reference values were determined using the hexokinase method calibrated against the ID-GCMS method. This reference method was standardised using isotope dilution gas chromatography–mass spectrometry, the best metrological quality assurance method (order), and meets the NIST standard (traceable).

Insulin, leptin and adiponectin plasma concentrations were determined by rat ELISA kits (ALPCO Diagnostics) with a sensitivity of 0·124 ng/ml and dynamic range: 0·15–5·5 ng/ml for insulin, a sensitivity of 0·01 ng/ml and dynamic range: 0·025–1·6 ng/ml for leptin, a sensitivity of 0·08 ng/ml and 0·25–10 ng/ml dynamic range for adiponectin.

NEFA concentrations were measured using an enzymatic assay (NEFA-C test®; Wako Chemicals GmbH) with a sensitivity 0·005 mmol/l. The Wako NEFA C test kit utilises an *in vitro* enzymatic colorimetric method.

Extraction and analysis of fatty acids content in diets. Total lipid extracts from diets were obtained by using the method of Bligh and Dyer[26]. The extract was evaporated, dissolved in $CHCl_3$–MeOH (98:2) and then stored under nitrogen atmosphere at –20°C until analysis. After evaporation to dryness, lipid fractions were esterified with MeOH-H_2SO_4 (98:2) in excess for one night at 50°C. After cooling, 2 ml of hexane and 1 ml of water were added, and the mixture was vortexed. The upper organic phase containing fatty acid methyl esters was collected and separation was carried out on a GC (PerkinElmer Autosystem) equipped with a flame ionisation detector, an on-column injector and a fused silica column (BPX, 60 m × 0·25 mm; 0·25 μm film thickness, SGE) programmed from 55°C (for 2 min) to 150°C at 20°C/min. Identification of fatty acid methyl esters was based on the comparison of their retention times with those of authentic standards, and quantification of peak area was done using margaric acid (17:0) as internal standard added at the time of lipid extraction[27].

Extraction and analysis of ceramides and diacylglycerol content in the hypothalamus. DAG and ceramide levels in tissue extracts were measured by the DAG kinase enzymatic method as previously described[28,29] in ZO rats only. Briefly, aliquots of the chloroform phases from cellular lipid extracts were re-suspended in 7·5 % (w/v) octyl-β-D-glucopyranoside/ 5 mM cardiolipin in 1 mM DETPAC/10 mM imidazole (pH 6·6). The enzymatic reaction was started by the addition of 20 mM DTT, 0·88 U/ml *E. coli* DAG kinase, 5 μCi/10 mM [γ-^{32}P] ATP and the reaction buffer (100 mM imidazole (pH 6·6), 100 mM NaCl, 25 mM $MgCl_2$ and 2 mM EGTA). After incubation for 1 h at room temperature, lipids were extracted with chloroform/methanol/ HCl (100:100:1, v/v) and 1 M KCl. [γ-32P]-phosphatidic acid was resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) and quantified with a Molecular Dynamics Storm PhosphorImager®. Known amounts of DAG and ceramide standards were included with each assay. Ceramide and DAG levels were expressed as picomoles by nanomoles of phospholipid levels.

Measurement of total cellular phospholipids in hypothalamus. In order to normalise the ceramide and DAG content in hypothalamus, total phospholipids present in cellular lipid extracts used for ceramide analysis were quantified as described previously[29] with minor modifications. Briefly, a mixture of

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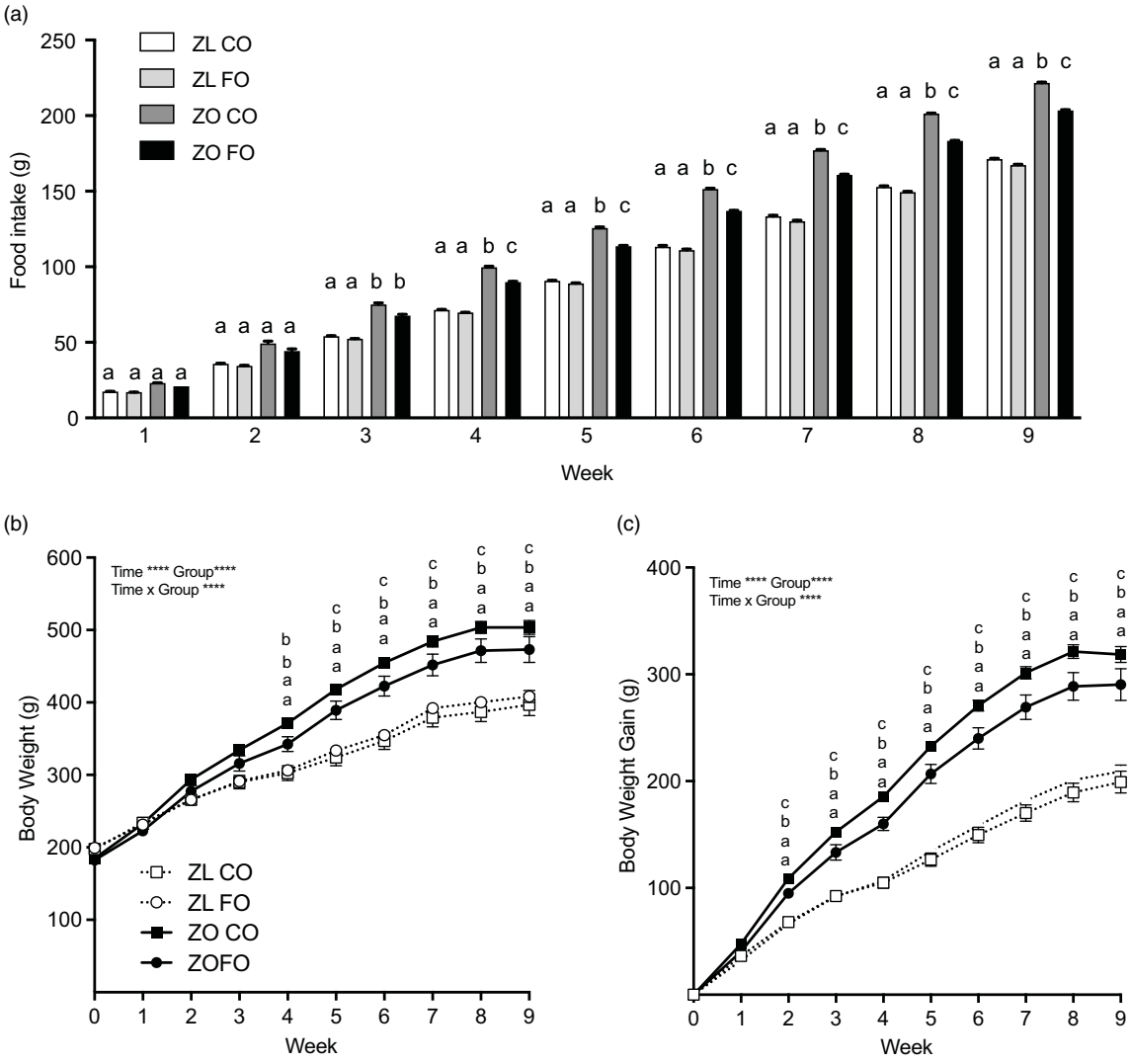

Fig. 2. Feed intake (a) and body weight gain (b) of male obese and lean Zucker rats that were fed either corn oil diet (CO) or fish oil diet (FO) for 9 weeks. Results are expressed as mean values with their standard error of the mean(n 12/group). [a,b,c] Parameters with differing superscripts differ from each $P < 0{\cdot}05$ level by Tukey's post hoc test after significant intergroup differences were found by the two-way ANOVA with repeated measures (factors: time and group).

10N H_2SO_4/70 % perchloric acid (3:1, v/v) was added to lipid extracts, which were incubated for 30 min at 210°C. After cooling, water and 4·2 % ammonium molybdate in 4 N HCl/ 0·045 % malachite green (1:3 v/v) were added. Samples were incubated at 37°C for 30 min, and absorbance was measured at 660 nm. Total phospholipid levels were expressed as nanomoles of phospholipid.

Statistical analysis

Data are reported as mean values with their standard error of the mean. Statistical analyses of physiological data were performed with Prism software (GraphPad Prism). For all statistical tests, a P-value $< 0{\cdot}05$ was considered statistically significant. Information on replicates and significance is reported in the figure legends. We did not perform a power sensitivity analysis, which is a limitation in the interpretation of statistical results.

In the experiment related to Fig. 2 and 3, data were analysed by the two-way ANOVA with repeated measure (time, group). In the experiment related to Fig. 4 and 5, Student's t test was used to compare two groups, for example, obese rats treated or not. When significant differences were found by the two-way ANOVA, further intergroup comparisons were performed by post hoc Bonferroni or Tukey's tests ($P < 0{\cdot}05$). Information on replicates and significance is reported in the figure legends.

Sample size calculations were made using G*Power 3.1 software. As we planned to analyse our data using non-parametric t tests (for experiments with two groups) and one/ two-way ANOVA (for experiments with four and three groups, respectively), group sizes of 8–10 per condition were calculated, reaching a power level of at least 0·95.

Results

Effect of corn oil and fish oil diets on blood parameters

All data are reported in Table 3. At Day 0 (D0), before the exposure to CO and FO diets, blood glucose of ZO rats was not

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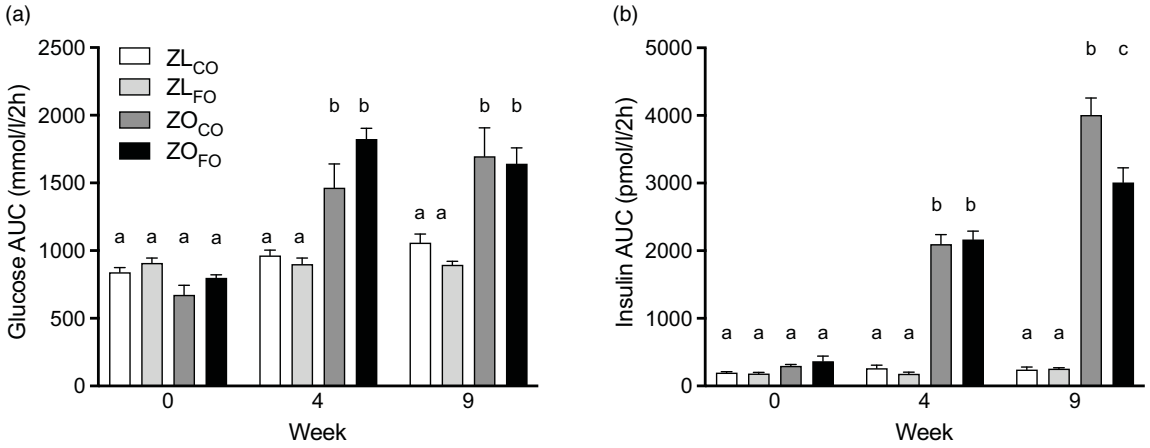

Fig. 3. AUC of blood glucose (a) and insulin (b) responses to an oral glucose challenge (3 g/kg BW) in male obese and lean Zucker rats fed either corn oil diet (CO) or fish oil diet (FO) from Day 0. Oral glucose tolerance test was performed in the morning after overnight fasting. Results are expressed as mean values with their standard error of the mean (n 6 /group). [a,b,c] Parameters with differing superscripts differ from each $P < 0{\cdot}05$ level by Tukey's post hoc test after significant intergroup differences were found by the two-way ANOVA with repeated measures (factors: time and group).

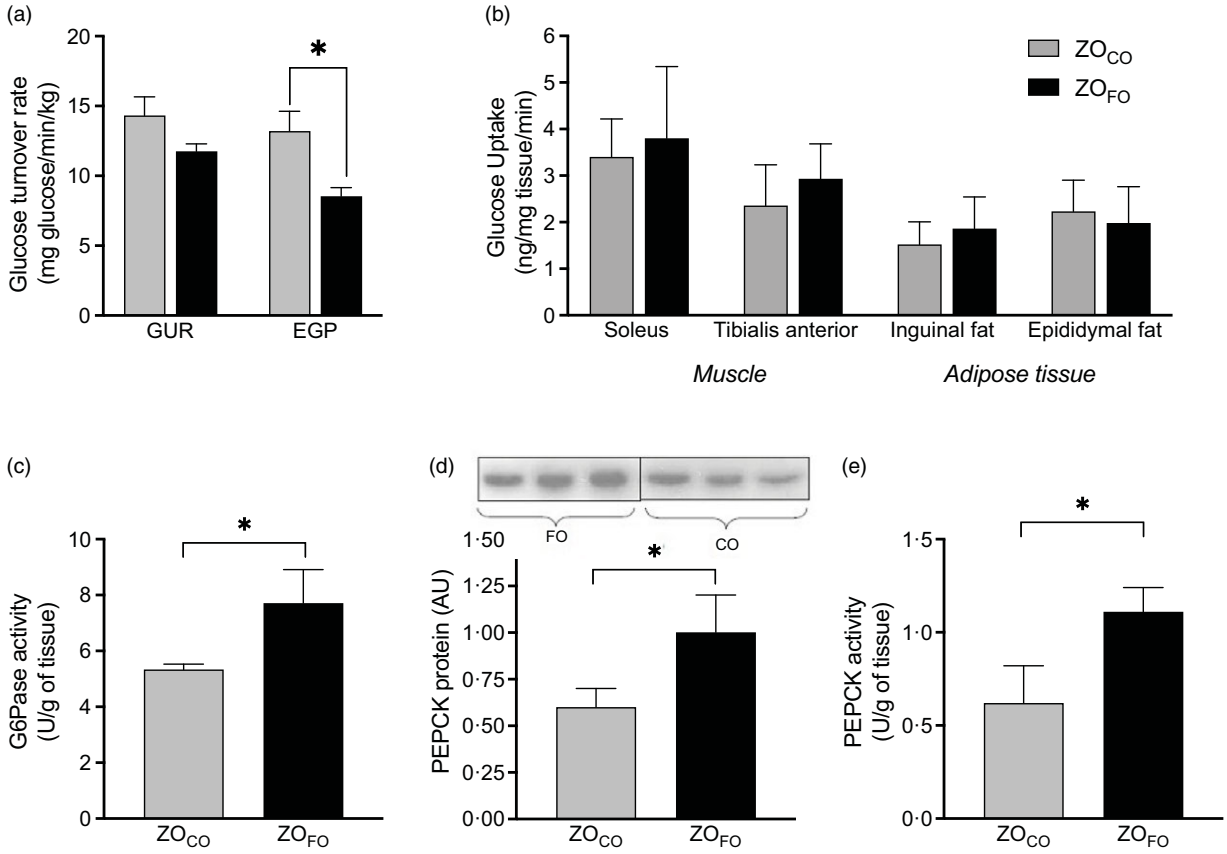

Fig. 4. Glucose turnover rate (a) and *in vivo* glucose uptake in muscle and adipose tissue (b) performed in male obese and lean Zucker rats fed either corn oil diet (CO) or fish oil diet (FO) for 9 weeks. The top graph (a) displays the glucose utilisation rate (glucose Rd) and the endogenous glucose production (glucose Ra). The bottom graph (b) displays *in vivo* glucose uptake in soleus (oxidative muscle), tibialis anterior (oxidative/glycolytic muscle), inguinal fat and epididymal fat. Assessment of liver gluconeogenic enzymes: glucose-6-phosphatase activity (c), (d) PEPCK protein content estimated from western blot of frozen liver samples. Three representative liver samples of obese rats fed fish oil (FO, on the left) compared with three representative liver samples of obese rats fed corn oil (on the right) were analysed (quantification shown in lower panel was performed as previously described; Mutel E, Gautier-Stein A, Abdul-Wahed A, Amigó-Correig M, Zitoun C, Stefanutti A, Houberdon I, Tourette JA, Mithieux G, Rajas F. Control of blood glucose in the absence of hepatic glucose production during prolonged fasting in mice: induction of renal and intestinal gluconeogenesis by glucagon. Diabetes. 2011 Dec;60(12):3121 31.) and (e) PEPCK activity assayed in frozen liver samples of male obese Zucker rats fed either corn oil diet (CO) or fish oil diet (FO) for 9 weeks. Results are expressed as mean values with their standard error of the mean (n 4–5/group), $^{*}P < 0{\cdot}05$ after Mann–Whitney non-parametric test.

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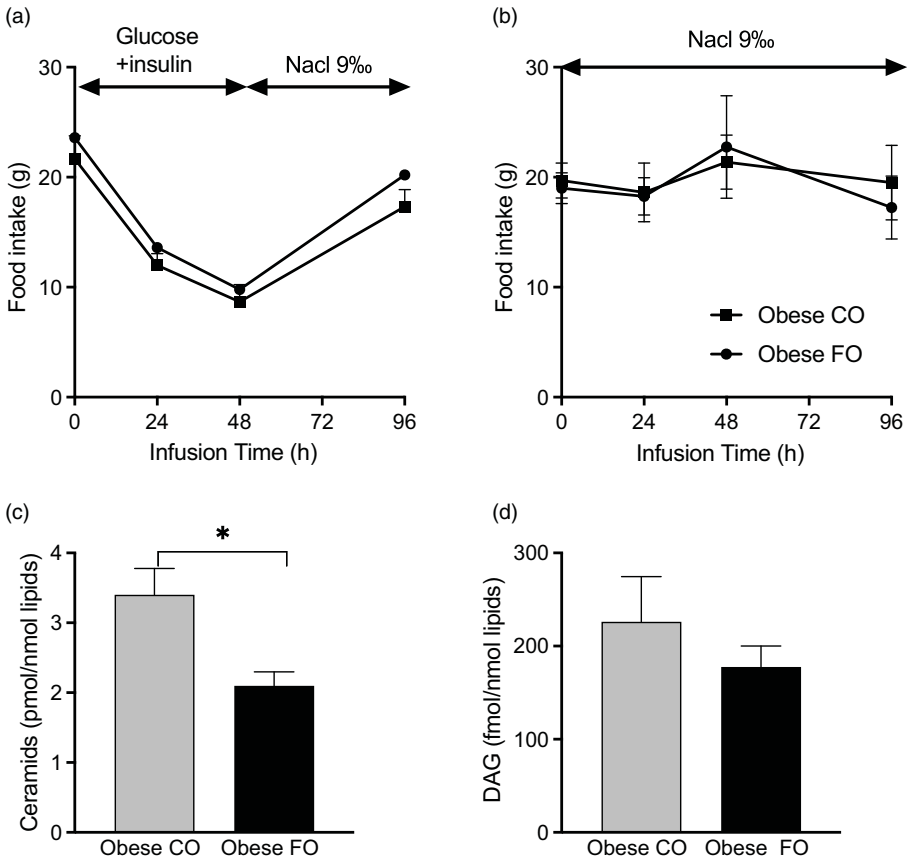

Fig. 5. Feed intake in response to successive central infusion of insulin plus glucose (a) and NaCl 9‰ (b) in male obese Zucker rats paired fed either corn oil diet (CO) or fish oil diet (FO) for 9 weeks. Quantification of hypothalamic ceramides (c) and diacylglycerol (DAG) (d) in fasted male obese Zucker rats fed either corn oil diet (CO) or fish oil diet (FO) for 9 weeks. Results are expressed as mean values with their standard error of the mean (*n* 5/group), $^{*}P < 0{\cdot}05$ after Mann–Whitney non-parametric test.

different from that of ZL, but plasma insulin (+116 %; $P < 0{\cdot}05$) and NEFA (+111%; $P < 0{\cdot}05$) levels were markedly higher than those of ZL rats ($P < 0{\cdot}05$). IR is not systematically associated with a higher basal or even post-OGTT hyperglycaemia. Hyperglycaemia only appears when a higher insulin secretion is no more able to compensate for IR as in obese humans[30]. It should be noted that in the absence of any effect of FO and CO on basal glycaemia and insulinaemia, we did not measure leptin and adiponectin in these animals, considering that glucose homoeostasis was not modified by the diet.

Once exposed to the diets, both ZO_{FO} and ZO_{CO} rats, blood glucose, insulin and leptin increased until the end of the study (D0 *v* week 9; ANOVA $P < 0{\cdot}0001$). Conversely, plasma adiponectin decreased in both groups (D0 *v* week 9; ANOVA $P < 0{\cdot}0001$). At week 4, no difference between glucose, NEFA, leptin and adiponectin plasma concentrations was observed between ZO_{FO} and ZO_{CO} rats. Blood glucose and insulin did not change over the 9 weeks in ZL rats. After 9 weeks of diet, ZO_{FO} rats had a lower plasma insulin (–11 %; $P < 0{\cdot}05$) and NEFA (–23 %; $P < 0{\cdot}05$) concentrations compared with ZO_{CO} rats. Similarly, ZL_{FO} rats had a 27 % lower insulin ($P < 0{\cdot}05$) than ZL_{CO} rats.

At week 9, insulin levels were 37 % lower in ZL_{FO} than in ZL_{CO} rats ($P < 0{\cdot}05$). Blood glucose and NEFA were not different between these two groups.

Note that in the absence of any effect of FO and CO on basal blood glucose and insulin levels and AUC in the LZ (see Fig. 3), we did not measure leptin and adiponectin in these animals.

Effect of fish oil diet on feed intake and weight gain

FI was similar between ZO and ZL rats during the first 2 weeks (Fig. 2(a)). After 2 weeks, FI was higher in ZO than in ZL rats (+ 14 %, $P < 0{\cdot}05$) (Fig. 2(a)**)**. FI was 11 % ($P < 0{\cdot}05$) lower in ZO_{FO} than in ZO_{CO} rats from week 4 to week 9 (Fig. 2(a)). FI was not different between ZL_{FO} and ZL_{CO} rats all over the 9 weeks (Fig. 2(a)).

BW was lower in ZO_{FO} than in ZO_{CO} rats from week 4 to week 9 ($P < 0{\cdot}05$) (Fig. 2(b)). Similarly, BW gain was lower in ZO_{FO} than in ZO_{CO} rats from week 4 to week 9 (–9 %; $P < 0{\cdot}05$) (Fig. 2(c)). BW and BW gain were not different between ZL_{CO} and ZL_{FO} rats all over the study (Fig. 2(b) and (c)). From week 4 to week 9, ZO rats had a higher BW (+22 % ZO_{CO}, +14 % ZO_{FO}; $P < 0{\cdot}05$) than ZL rats (Fig. 2(b)). Similarly, from week 4 to week 9, ZL rats maintained a lower BW gain than ZO rats (≈ –14 %; $P < 0{\cdot}05$) (Fig. 2(c)).

Effect of fish oil diet on glycaemic and insulinaemic responses to an oral glucose tolerance test

At D0, the AUC of blood glucose and insulin following oral glucose was not different between ZL and ZO rats (Fig. 3(a) and (b)). At weeks 4 and 9, AUC of both blood glucose and insulin

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was higher in ZO rats than in ZL rats ($P < 0·05$), regardless of the diet (Fig. 3(a) and (b)). At week 9, AUC of blood glucose was not different between ZO_{FO} and ZO_{CO} rats (FO = 1643 (SEM 116) *v.* CO = 1698 (SEM 210) mmol/l/2 h) (Fig. 3(a)), whereas the AUC of plasma insulin was 25 % lower in ZO_{FO} than in ZO_{CO} rats (3009 (SEM 216) *v.* 4006 (SEM 253) ng/ml/2 h, respectively, $P < 0·05$) (Fig. 3(b)).

Effect of fish oil diet for 9 weeks on insulin sensitivity as assessed by an euglycaemic–hyperinsulinaemic clamp

Euglycaemic–hyperinsulinaemic clamps were only performed in ZO_{FO} and ZO_{CO} rats. Indeed, although we could not formally exclude a difference in insulin sensitivity between ZL_{FO} and ZL_{CO} rats, insulin and glycaemia values during OGTT were identical between the two groups (Fig. 3) and we hypothesised that insulin sensitivity was not altered, even if OGTT is not the best means to assess insulin sensitivity. Thus, the lack of differential effect of CO *v* FO diets on glucose homoeostasis in ZL rats prompted us to focus our efforts on ZO rats. For this reason, we performed clamps only on these animals. During euglycaemic–hyperinsulinaemic conditions, endogenous glucose production of ZO_{FO} rats was inhibited by 35 % more than in ZO_{CO} rats (8·54 (SEM 0·62) *v.* 13·21 (SEM 1·42) mg/kg per min; $P < 0·05$) (Fig. 4(a)). Conversely, whole body blood glucose utilisation assessed from glucose utilisation rate was not different between ZO_{FO} and ZO_{CO} rats (11·77 (SEM 0·52) *v.* 14·32 (SEM 1·33) mg/kg per min, respectively). In both groups, we showed similar insulin-induced glucose uptake in muscles (soleus and tibialis anterior) as well as in white adipose tissue (inguinal, perieipididymal) (Fig. 4(b)). Adiponectin decreased significantly in ZO rats at week 4 and then remained stable at week 9, lower than at D0; this was probably mainly related to a whole body decrease in insulin sensitivity probably reaching its maximum at week 4. Adiponectin was not different between ZO_{FO} and ZO_{CO} rats. We can speculate that the lack of effect of FO on adiponectin is explained by the fact that only liver insulin sensitivity was altered by FO.

Effect of fish oil diet for 9 weeks on specific hepatic enzyme activities

The control of gluconeogenesis depends on key gluconeogenic enzymes such as PEPCK and G6Pase. Liver enzyme analysis showed that G6Pase was more active (+45 %; $P < 0·05$) (Fig. 4(a)) in ZO_{FO} diet at 9 weeks compared with ZO_{CO} rats. In ZO_{FO} rats, PEPCK protein abundance in liver was increased by 45 % ($P < 0·05$) (Fig. 4(b)) and PEPCK activity tended to be increased (+49 %; $P = 0·1$) at 9 weeks compared with ZO_{CO} rats (Fig. 4(c)).

Feed intake in response to intra-carotid infusion of glucose plus insulin

This experiment was designed to evaluate FI in pair-fed ZO rats in response to glucose + insulin infusion, in order to assess whether the lower FI observed in ZO_{FO} rats could be explained by an increase in hypothalamic sensitivity to glucose plus insulin. Infusions were performed via the carotid artery cannulation directed towards the brain. In response to carotid infusion of glucose plus insulin, systemic concentrations of glucose, insulin and leptin remained unchanged (data not shown), while FI decreased by 42 % and 58 % after 24 h and 48 h of infusion, respectively, in ZO_{FO} and also by 42 % and 58 % in ZO_{CO} rats (Fig. 5(a)). This response was fully reversible since FI returned to baseline within 24 h when the glucose and insulin infusion was switched to saline (Fig. 5(a)). FI was not altered either in ZO_{FO} or ZO_{CO} rats with saline infusion (Fig. 5(b)).

Effect of fish oil and corn oil diet on ceramides and diacylglycerol content and Akt phosphorylation in the hypothalamus

In fasted rats, hypothalamic ceramide level was lower in ZO_{FO} than in ZO_{CO} rats ($P < 0·05$) (Fig. 5(c)). In contrast, DAG level was not different between the two groups (Fig. 5(d)).

To test the activation of the insulin pathway in the hypothalamus, Akt serine^{473} phosphorylation was assessed in fasting state in the ventromedial and lateral hypothalamus areas. Akt serine^{473} phosphorylation was similarly stimulated by insulin administration in ZO_{FO} and ZO_{CO} rats (data not shown).

Discussion

The present study was designed to evaluate whether a low dose of LC *n*-3 PUFA given as FO could prevent the genetic predisposition of ZO rats to develop IR, as they do in several other models of nutritionally induced IR in rodents[3–6]. As awaited, in response to an OGTT, ZO rats displayed, in response to an OGTT, a higher AUC glycaemia and insulinaemia than ZL rats, which translates both glucose intolerance and IR. After 9 weeks of diet, AUC of insulinaemia in ZO_{FO} rats was lower following than in ZO_{CO} rats, while AUC of glycaemia was not different. This observation suggests that FO improved insulin sensitivity in ZO rats.

The improvement of insulin sensitivity by FO in ZO rats was confirmed by the euglycaemic–hyperinsulinaemic clamp. However, only liver IR was reduced, while peripheral insulin sensitivity in muscle and adipose tissue remained unaffected. A prevention of liver and muscle IR was reported previously in rodents with a low[5,31] or high dose of FO[3]. It is possible that the lack of effect on muscle insulin sensitivity in our study was due to the different rodent model.

In rats, the decrease in hepatic glucose production during a hyperinsulinaemic–euglycaemic clamp, as well as after refeeding, is mainly related to a decrease in overall hepatic gluconeogenic flux, which is obviously associated with a decrease in flux through PEPCK and G6Pase[32–34]. Here, we show that both enzyme activities, assayed *in vitro* at Vmax (thus corresponding to the total amount of both enzymes), are higher in ZOFO rats than in ZOCO rats. This may seem contradictory. However, *in situ* G6Pase activity and hepatic glucose production depend primarily on insulin's additional post-translational regulation (here, inhibition) of enzyme activity, not just on Vmax[32–34]. These post-translational regulatory processes could occur either at the level of enzyme activities or upstream at the level of insulin signalling, as has been proposed for the hepatic insulin-sensitising effect of

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metformin[33–37]. Therefore, we speculate that the increase in G6Pase and PEPCK Vmax in ZOFO rats observed here could be a response of liver cells in an attempt to compensate for the decrease in hepatic glucose production due to increased liver insulin sensitivity.

A lesser activation of gluconeogenesis has been shown in Fat1 mice, a model of endogenous increase in LC *n*-3 PUFA[38]. Gluconeogenesis is stimulated by liver NEFA flux, which is driven by plasma NEFA concentrations[39]. In our study, the lesser plasma NEFA concentrations observed in ZO_{FO} rats may have contributed to a lesser stimulation of liver gluconeogenesis.

An alleviation of liver lipotoxicity, as previously observed in rats and mice fed a high-fat diet[1,40–46], has also probably contributed to the alleviation of liver IR in our study. It must be noted that in our study we did not explore these molecular mechanisms, in particular fatty acid oxidation. Further studies should explore the expression levels of these genes to better understand the beneficial impact of FO on the liver IR in ZO rats.

Beside a direct effect of LC *n*-3 PUFA on liver, it cannot be completely excluded that the lower BW of FO-fed obese rats has contributed for a part to the lesser liver IR. We observed, in ZO_{FO} only, a lower weight gain associated with a lower FI than in ZO_{CO} rats. However, we did not assess body composition. This was a limitation to our study. We measured plasma leptin concentration as a marker of adipose tissue mass in ZO rats in order as an estimate of an effect of FO on adiposity as has been done in other studies[14,15]. It can also be hypothesised that FO supplementation may also reduce intestinal absorption and bioavailability of fatty acids, as has been shown in mice[47]. So, FO supplementation could also have a global effect on metabolism and energy balance including insulin sensitivity. We can speculate that changes in FI that we observed in ZO_{FO} rats could also suggest an effect on hypothalamus. Indeed, LC *n*-3 PUFA can modulate the expression of gene controlling FI such as pro-opiomelanocortin, neuropeptide Y, leptin receptor and other genes involved in the control of fatty acid metabolism[48–52].

We can also speculate from studies in mice and rats that have shown that LC *n*-3 PUFA reduced brain inflammation[53–55] and consequently IR. Indeed, over the last years, hypothalamic inflammation not only impairs energy balance but also contributes to the development and progression of obesity and associated IR[56].

In addition, it is of special interest to note that in two randomised clinical trials *v* placebo performed in obese patients, LC *n*-3 PUFA reduced energetic intake[57,58], increased fullness and decreased hunger sensations[59]; in the latter study comparing a low *v* higher dose of LC *n*-3 PUFA in obese patients, hunger sensation was reduced following the high dose. This could be related to changes in plasma parameters such as hormones (leptin, ghrelin), which in turn could act on hypothalamus as described in preclinical models[49,50]. Although an effect of LC *n*-3 PUFA on obese patients has been reported, the Zucker rat model is a very specific rodent model, so that caution must be taken in extrapolation of our date to human obesity.

In the present study, we first hypothesised that FO increased hypothalamic insulin sensitivity by minimising hypothalamic lipotoxicity. In another study, we demonstrated that the ceramide content of the hypothalamus was high in Zucker rats and could partly explain their metabolic dysfunction[60]. We evidenced that hypothalamic ceramide content was lower in ZO_{FO} compared with ZO_{CO}, suggesting that hypothalamic lipotoxicity was at least partially alleviated. Ceramides and DAG alter insulin signalling by activating several species of protein kinase C (PKC): PKCθ, PKCε, PKC ζ, nPKC[61–64], leading to a lesser activation of phosphoinositide 3'-kinase-Akt (PI3K/Akt) and Akt[64]. However, Akt serine 473 phosphorylation was identical in both ZO_{CO} and ZO_{FO} rats. In addition, we performed an intra-carotid infusion of glucose and insulin in order to assess inhibitory effect of insulin on FI using a previously published protocol[15]. Intra-carotid infusion of both glucose and insulin decreased FI to the same extent in both ZO_{CO} and ZO_{FO}, which is consistent with the activation of the insulin signalling pathway. Thus, it is unlikely that the decreased hyperphagia observed in FO-fed obese rats was due to a lower hypothalamic IR.

However, as ceramide level in hypothalamus was reduced by FO in ZO rats, a lower lipotoxicity could have been responsible and/or associated with the known hypothalamic anti-inflammatory effect of LC *n*-3 PUFA and/or to their ability to stimulate expression of anorexigenic peptides and to inhibit that of orexigenic ones[52–55,65].

Concerning the difference in saturated, monounsaturated and *n*-6 fatty acids, composition of the two diets was unable to calculate statistical differences, as this information was supplied by the manufacturer. There are indeed small differences in SFA (+9 % FO *v* CO) and greater differences in MUFA (–44 % FO *v* CO), but we cannot say whether they are significant. Although an effect of an imbalance between types of fatty acids between the two diets cannot be completely excluded, it is very improbable that they had an effect towards IR and FI. Indeed, the amount of saturates is slightly higher in the CO diet and so if they had an effect, it could have aggravated IR and not minimised it. The MUFA are at a lower amount in CO than in the FO diet and that have not been shown to induce IR.

The difference in *n*-6 content (–62 % FO *v* CO) is high, but *n*-6 is neutral towards insulin sensitivity[66]. *n*-6 fatty acids do not induce IR[67], and they do not alter the effects of *n*-3[68]. In addition, they are associated with a lower risk of type 2 diabetes[69].

For all these reasons, although it cannot be completely excluded, we assume that the differences observed in the phenotypes measured are mainly if not only due to *n*-3.

Conclusion

We showed in this study that a low dose of FO introduced in a normoenergetic normolipidic diet during 9 weeks prevented liver IR and decreased FI and weight gain in obese Zucker rats genetically deficient for leptin receptor (*fa/fa*), without effect in lean Zucker counterparts (*Fa/Fa*). The amelioration of liver insulin sensitivity can be attributed to an alleviation of lipotoxicity by a direct effect on liver lipogenesis and/or an indirect effect secondary to the lesser weight gain. In pair-fed rats, intra-carotid co-administration of glucose and insulin decreased FI similarly in ZO_{FO} and ZO_{CO} rats, without alteration of activation of phosphorylation of Akt protein in hypothalamic VMH and LH nuclei, which suggests that other mechanisms than hypothalamic IR or leptin modulation explained the inhibitory effect of FO on FI. A possible mechanism

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could be a decrease in hypothalamic lipotoxicity strongly suggested by a lower ceramide concentration in hypothalamus of ZO_{FO} rats. A lower lipotoxicity could be responsible and/or associated with the known hypothalamic anti-inflammatory effect of LC *n*-3 PUFA and/or to their ability to stimulate expression of anorexigenic peptides and to inhibit that of orexigenic ones. Although extrapolation of our results to human obesity is not appropriate, it is of interest to highlight that several studies performed in obese patients have shown the ability of LC *n*-3 PUFA to decrease energetic intake and sensation of fullness and hunger.

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The authors have no conflict of interest related to this paper.

Supplementary material

For supplementary materials referred to in this article, please visit https://doi.org/10.1017/S0007114523002404

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