

Chronic dietary *n*-3 PUFA intervention improves dyslipidaemia and subsequent cardiovascular complications in the JCR:LA-*cp* rat model of the metabolic syndrome

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Abstract

There is increasing interest in the potential chronic beneficial effects of dietary *n*-3 PUFA on the metabolic syndrome (MetS) and associated cardiovascular complications. We have recently established that increased dietary *n*-3 PUFA has a profound acute benefit on fasting lipids and the postprandial pro-inflammatory response in the JCR:LA-*cp* rat, a model of the MetS. However, it is unclear to what extent chronic dietary *n*-3 PUFA intervention can modulate the progression of end-stage metabolic and vascular complications. The present study aimed to determine the chronic effects of dietary *n*-3 PUFA supplementation on fasting and non-fasting dyslipidaemia, insulin resistance and vascular complications in the JCR:LA-*cp* rodent model. JCR:LA-*cp* rats were fed an isoenergetic lipid-balanced diet supplemented with 5% *n*-3 PUFA (w/w) of the total fat (fish oil-derived EPA/DHA) for 16 weeks. Fasting and non-fasting (postprandial) plasma lipid profile was assessed. Hepatic and adipose tissue was probed for the expression of lipogenic proteins (acyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and sterol regulatory element-binding protein-1 (SREBP-1)), while the activity of Jun N-terminal kinase (JNK) was assessed via Western blot to target phosphorylated JNK protein in primary enterocytes. The frequency of myocardial lesions was assessed by haematoxylin and eosin staining. Increased dietary *n*-3 PUFA improved both the fasting and postprandial lipid profiles (TAG, cholesterol and apoB48) in the JCR:LA-*cp* rat, potentially via the down-regulation of the hepatic or adipose tissue expression of lipogenic enzymes (ACC, FAS and SREBP-1). Rats fed the 5% *n*-3 PUFA diet had lower (58.2%; $P < 0.01$) enterocytic phosphorylated JNK protein and secreted less cholesterol (30%; $P < 0.05$) into mesenteric lymph compared with the control. The chronic metabolic benefits of dietary *n*-3 PUFA may underlie the potential to reduce vascular complications during the MetS, including the observed reduction in the frequency (approximately 80%) of late-stage 3 myocardial lesions.

Key words: CVD: Dietary interventions: Dyslipidaemia: Metabolic syndrome

The metabolic syndrome (MetS) is a pre-diabetic state, characterised by obesity, insulin resistance (IR) and dyslipidaemia⁽¹⁾. Lipid abnormalities of the MetS include low plasma HDL-cholesterol, increased LDL-cholesterol, abnormal apoB level and raised blood TAG (hypertriacylglycerolaemia)^(1,2). Emerging evidence also suggests that postprandial TAG and chylomicron (CM) concentrations are strongly correlated with cardiovascular risk⁽³⁾.

The long-term complications of the MetS include premature atherosclerosis and ischaemic myocardial lesion development^(1,4), and are often characterised in stages of

severity (i.e. stage 1 to stage 4^(5–7)) including early areas of cell lysis through to inflammatory cell infiltration and scarring.

n-3 PUFA, such as EPA (20:5) and DHA (22:6), have been reported to have beneficial effects in states of the MetS and cardiovascular complications^(8,9). Studies have reported that dietary fish oil (rich in *n*-3 PUFA) can reduce cardiovascular risk factors, including repression of plasma insulin, glucose and cholesterol levels^(10,11). Evidence from human clinical trials suggests that *n*-3 PUFA reduce acute atherosclerotic infarction, and hence lower the risk of CHD^(12,13). Furthermore, an investigation of the protective effect of *n*-3 PUFA

Abbreviations: CM, chylomicron; IR, insulin resistance; JNK, Jun N-terminal kinase; JNK-P, phosphorylated Jun N-terminal kinase; LBD, lipid-balanced diet; MetS, metabolic syndrome; SREBP, sterol regulatory element-binding protein.

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on isoproterenol-induced myocardial infarction in rats has reported a marked reduction in the level of lipid components (cholesterol, TAG and NEFA) in the plasma and heart tissue⁽¹⁴⁾. In addition, a human trial conducted by Metcalf *et al.*⁽¹⁵⁾ has reported dietary *n*-3 PUFA to be readily incorporated into cardiomyocyte phospholipids, potentially exerting beneficial outcomes by consequent effects on myocardial membrane function. Despite this, there is no comprehensive histological evidence regarding the improvement of dietary *n*-3 PUFA on myocardial lesions and the long-term beneficial vascular effects.

Recently, our group has reported that acute (3-week) dietary *n*-3 PUFA supplementation can reduce weight gain, and improve postprandial lipid metabolism and associated pro-inflammatory response in the obese JCR:LA-*cp* rat, a model of the MetS⁽¹⁶⁾. The JCR:LA-*cp* rat, homozygous for the corpulent trait (*cp/cp*), exhibits symptoms of the MetS, enhanced hepatic VLDL and intestinal CM overproduction, as well as end-stage complications such as ischaemic myocardial lesions^(5,6,17–22). Consequently, the aim of the present study was to (1) assess the long-term (chronic) effects of dietary *n*-3 PUFA supplementation on dyslipidaemia, IR and myocardial lesion development in the JCR:LA-*cp* rat and (2) investigate the putative mechanistic effects of dietary *n*-3 PUFA, specifically on intestinal enterocytes, in the obese JCR:LA-*cp* rat.

Materials and methods

Animal model and experimental procedures

Male obese (*cp/cp*) JCR:LA-*cp* rats were raised in our established breeding colony at the University of Alberta (Edmonton, AB, Canada), as described previously⁽⁵⁾. Rats were weaned at 6 weeks, and allowed to age until 8 weeks of age. Obese rats (*cp/cp*) were randomly allocated to either a control hypercholesterolaemic isoenergetic lipid-balanced diet (LBD; *n* 8) (1% (w/w) cholesterol; 15% (w/w) total fat; polyunsaturated:saturated fat ratio 0.4) or a LBD supplemented with 5% *n*-3 PUFA (of the total fat *n* 8; 1% (w/w) cholesterol, 15% (w/w) total fat; polyunsaturated:saturated fat ratio 0.4 and 5% (w/w) fish oil-derived EPA/DHA) (Table 1), for 3 or 16 weeks, as indicated throughout. Note that the equivalent amount of *n*-3 PUFA from 5% fish oil treatment in rats used in the present study equates to approximately 5 g fish oil/d for a 10 460 kJ/d (2500 kcal/d) human diet. Food consumption and body weight were recorded throughout the study. At 22 weeks of age, an oral fat challenge test was performed on all rats, as described previously⁽²²⁾. At 24 weeks of age, rats were fasted overnight and killed under isoflurane anaesthesia. Plasma and serum were collected via cardiac puncture. Liver, heart and inguinal fat pads were weighed and snap-frozen in liquid N₂ at –80°C for subsequent analysis. Animal care and experimental procedure were conducted in accordance with the Canadian Council on Animal Care (Ottawa, ON, Canada) and approved by the University of Alberta Animal Care and Use Committee (ACUC-Livestock).

Table 1. Nutrient and lipid summaries for both dietary groups*

	Control diet (LBD)	5% <i>n</i> -3 PUFA diet
Nutrient summary (per kg)		
Casein (g)	270.0	270.0
Starch (g)	214.0	214.0
Dextrose (g)	217.0	217.0
Non-nutritive cellulose (g)	80.0	80.0
Vitamin mixture (g)	9.5	9.5
Mineral mix (g)	48.0	48.0
Choline (g)	2.75	2.75
Inositol (g)	6.25	6.25
L-Met (g)	2.5	2.5
Linseed oil (g)	3.0	3.0
Tallow (g)	91.71	91.88
Sunflower oil (g)	55.29	40.13
Fish oil (g)	0	15.0
Cholesterol (g)	10.0	10.0
Lipid summary (% of fat)		
Total polyunsaturated fat	24.7	24.3
Total saturated fat	64.5	66.0
P:S ratio	0.4	0.4
Total <i>n</i> -6	23.4	17.4
Total <i>n</i> -3	1.2	6.9
Total EPA + DHA	0.0	5.3

LBD, lipid-balanced control diet; P:S, polyunsaturated:saturated fat.

* Fatty acid composition of the LBD and the diet supplemented with 5% fish oil containing EPA and DHA; 5% *n*-3 PUFA diet, as determined by GC as described previously⁽⁵⁶⁾.

Assessment of postprandial lipidaemia

At 22 weeks of age and following a 16 h overnight fast, rats were subjected to an oral fat challenge test in order to assess non-fasting lipid metabolism⁽²²⁾. In brief, all rats consumed a 5 g pellet prepared from 5001 laboratory chow, consisting of 49% carbohydrate, 24% crude protein, 10% moisture, 6.5% minerals, 6% fibre and 4.5% fat, and further supplemented with 25% (w/w) dairy fat from double cream (raising the total fat content of the 5 g meal to approximately 30% (w/w) of the total meal)^(16,22). Blood samples were collected (from the tail) into tubes containing Na₂EDTA at time intervals (0, 2, 4 and 6 h) following pellet consumption. Plasma and serum were separated by centrifugation (3901 g, 4°C, 10 min). Aliquots of plasma were immediately stored at –80°C for biochemical analyses.

Mesenteric lymph duct cannulation and nascent lymph collection

To determine the effect of *n*-3 PUFA on the secretion of CM in mesenteric lymph, the superior mesenteric lymph duct of obese JCR:LA-*cp* rats was cannulated following consumption of a control LBD (*n* 5) or a 5% *n*-3 PUFA diet (*n* 5) for 3 weeks⁽²³⁾. Mesenteric lymph was collected into EDTA-coated vacutainers for 5 h following infusion of intralipid (Kabi Pharmacia, Uppsala, Sweden). The concentrations of TAG and total cholesterol were measured as described below for the plasma.

Isolation of primary jejunal enterocytes and quantification of apoB48 and Jun N-terminal kinase protein

Primary jejunal enterocytes were isolated from the intestine of obese rats fed either a control LBD or a 5% *n*-3 PUFA diet for

3 weeks, as per the Weiser method⁽²⁴⁾ of isolation and fractionation, as described previously⁽²³⁾. The protein concentration of the isolated enterocyte fractions (no. 1–10) was determined, and immuno-Western blot analysis was used to probe the expression of apoB48 protein (as below) along the intestinal villus, from enterocyte fraction no. 1 (tip of the villus) to enterocyte fraction no. 10 (crypt of the villus). Pooled enterocyte fractions (no. 1–10) were assessed for Jun N-terminal kinase (JNK) activity by Western blotting, to target phosphorylated (active) JNK (JNK-P) protein. Briefly, cell extracts were prepared in Triton lysis buffer and examined (50 µg protein) by immunoblot analysis, to target JNK-P (catalogue no. 9252; Cell Signalling, Beverly, MA, USA) and JNK (catalogue no. 558268; Pharmingen, San Diego, CA, USA).

Plasma biochemical determination

The biochemical lipid profile of obese rats fed either a control LBD or a 5% *n*-3 PUFA diet was assessed using commercially enzymatic kits, as described previously⁽¹⁶⁾; including plasma TAG (catalogue no. 998-40 391/994-40 491; Wako Pure Chemicals, USA, Inc., Richmond, VA, USA), total cholesterol (catalogue no. 439-17 501; Wako), LDL-cholesterol (catalogue no. 993-00 404/999-00 504; Wako) and HDL-cholesterol (catalogue no. 258-20; Diagnostic Chemicals Limited, Charlotte, PE, Canada). Plasma glucose levels were assessed as per the glucose oxidase method (catalogue no. 220-32; Diagnostic Chemicals Limited). Insulin was analysed by a solid-phase, two-site enzyme immunoassay (catalogue no. 10-1137-01; Mercodia AB, Uppsala, Sweden). Plasma adiponectin (catalogue no. 44-ADPR-0434; Alpco Diagnostics, Salem, NH, USA) and leptin (catalogue no. 22-LEP-E06; ALPCO) concentrations were determined using commercially available rodent-specific immunoassays.

The concentration of intestinally derived CM particles was determined by quantification of plasma apoB48, using an adapted immuno-Western blotting procedure, as described previously^(25,26). Briefly, total plasma was separated by SDS-PAGE on a 3–8% Tris-acetate polyacrylamide NuPage[®] gel (Invitrogen, Camarillo, CA, USA). Separated proteins were transferred to a polyvinylidene fluoride membrane (0.45 µm, ImmobilonP[™]; Millipore, Billerica, MA, USA). Membranes were incubated with a goat polyclonal antibody to apoB (1:100; catalogue no. sc-11 795; Santa-Cruz Biotechnology, Inc., Santa Cruz, CA, USA), which recognises both apoB100 and apoB48 isoforms. Detection was achieved using an anti-goat secondary antibody (catalogue no. sc-2304; Santa-Cruz Biotechnology, Inc.) and chemiluminescence (Enhanced Chemiluminescence (ECL) advance; Amersham Biosciences, Little Chalfont, Bucks, UK); intensity was quantified using linear densitometric comparison with a known mass of purified rodent apoB48 protein.

Measurement of lipogenic gene targets

Total RNA was isolated from both hepatic and adipose tissue collected from obese (*cp/cp*) rats of both diet

groups (TRIzol; Invitrogen) and reverse-transcribed into complementary DNA using MultiScribe[™] Reverse transcriptase (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA). The expression of acyl-CoA carboxylase (*ACC*), fatty acid synthase (*FAS*), *PPARα*, *PPARγ* and sterol regulatory element-binding protein-1 (*SREBP-1*) mRNA, relative to the housekeeping gene *ACTB* (β -actin), was measured by quantitative real-time PCR, using the StepOne[™] Plus Real-Time PCR system (Applied Biosystems) and StepOne[™] Software (version 2). PCR contained complementary DNA template, 100 nm of commercially available, pre-mixed target-specific primers and Taqman[®] FAM[™]-labelled probe (Applied Biosystems) for *ACC* (reference sequence NM_022193.1; catalogue no. Rn01456582_m1), *ACTB* (NM_031144.2; catalogue no. Rn00667869_m1), *FAS* (NM_017332.1; catalogue no. Rn01463550_m1), *PPARα* (NM_013196.1; catalogue no. Rn00566193_m1), *PPARγ* (NM_001145366.1; catalogue no. Rn00440945_m1) and *SREBP-1* (XM_213329.5; catalogue no. Rn01495769). Thermal cycling conditions were as follows: 95°C for 20 s, followed by forty cycles of 95°C for 1 s and 60°C for 20 s. Gene quantification was assessed relative to *ACTB* mRNA, utilising the comparative $2^{-\Delta\Delta C_t}$ method.

Measurement of lipogenic-related proteins

Proteins from liver and adipose homogenates were separated by SDS-PAGE electrophoresis on 3–8% Tris-acetate polyacrylamide gels (Invitrogen), transferred to a polyvinylidene fluoride membrane (described previously) and incubated with antibodies for the following target proteins: anti-*ACC* goat polyclonal (1:20 000; catalogue no. sc-11 795; Santa-Cruz Biotechnology, Inc.); anti-*FAS* goat monoclonal (1:20 000; catalogue no. sc-55 580; New England BioLabs, Acton, MA, USA), anti-*SREBP-1* mouse monoclonal (1:20 000; catalogue no. sc-13 551; Santa-Cruz Biotechnology, Inc.); anti-actin goat polyclonal (1:20 000; catalogue no. A5441; Sigma-Aldrich, St Louis, MO, USA); antibodies. Detection was achieved using anti-goat and anti-murine secondary antibodies and the ECL advance detection system (described previously; Amersham Biosciences). Results are expressed as a ratio of target protein: β -actin protein.

Heart histology and myocardial lesion analysis

Hearts were fixed in formalin, embedded in a single paraffin block, sectioned and then stained with haematoxylin and eosin as described previously⁽⁶⁾. Heart sections were examined blindly by an experienced observer, and the number of ischaemic lesions was identified in each of the sections. Myocardial lesions were categorised as stage 1 through to stage 4, as described previously^(5–7,27); stage 1, necrotic areas; stage 2, cell lysis with long-term inflammatory infiltration; stage 3, nodules of long-term inflammatory infiltration; stage 4, old scarred lesions. The number of lesions determined from sections of individual hearts was aggregated, and the mean incidence for each group was calculated.

Table 2. Physical and fasting biochemical parameters of obese (*cp/cp*) male JCR:LA-*cp* rats fed a lipid-balanced control diet (LBD) or a 5% *n*-3 PUFA diet(Mean values with their standard errors, *n* 8)

Parameters	<i>cp/cp</i> LBD		<i>cp/cp</i> 5% <i>n</i> -3 PUFA	
	Mean	SEM	Mean	SEM
Food consumption (g)	32.5	2.7	31.9	1.8
Body weight (g)	668.5	8.2	584.0*	1.1
Fasting glucose (mg/l)	1780	99	1397	73
Fasting insulin (pmol/l)	2700.6	233.4	396.6*	45.6
Fasting cholesterol (mg/l)	1583	64	1249*	44
Fasting TAG (mg/l)	1563	224	1191*	103
HDL (mg/l)	652	41	549	27
LDL (mg/l)	460	16	431	30
Fasting apoB48 (μ g/ml)	57.1	7.5	20.1*	3.6
Leptin (ng/ml)	120.3	9.1	90.7*	5.2

* Mean values were significantly different from those of the obese control (*cp/cp*) group ($P < 0.05$).

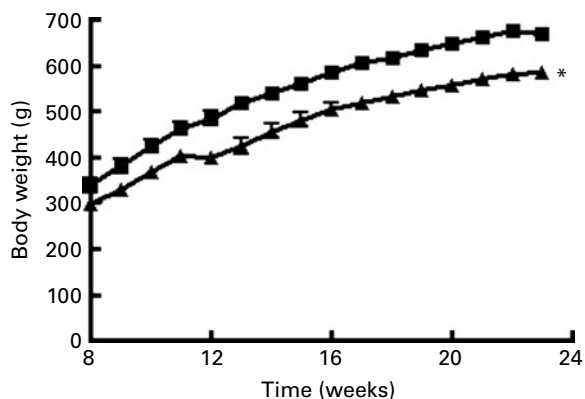
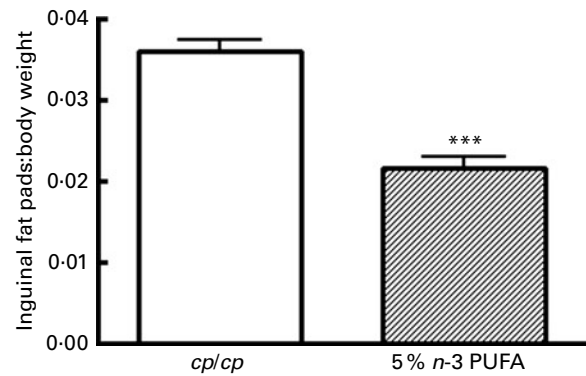
Statistical analysis

Statistical analysis was performed using GraphPad Prism software, version 4.0. Data were tested for normal distribution, and significant differences between the obese (*cp/cp*) LBD and obese (*cp/cp*) 5% *n*-3 PUFA groups were determined using Student's *t* test or repeated-measures ANOVA followed by Bonferroni *post hoc* tests, when appropriate. All results are expressed as means with their standard errors and the number of independent experiments as indicated in the figure legends. The level of significance was set at $P < 0.05$.

Results

Food intake, body fat and organ weight

Following a 16-week *n*-3 PUFA intervention, despite no reduction in food intake, 5% *n*-3 PUFA-supplemented obese rats had significantly lower body weight (12–17%; $P < 0.05$) compared with the obese control rats (Table 2; Fig. 1). Those rats supplemented with 5% *n*-3 PUFA (16 weeks) had a reduced ratio of inguinal fat-pad weight:body weight compared with the obese control rats ($P < 0.001$) (Fig. 2).

**Fig. 1.** Body weight of obese control (—■—, *cp/cp*) and 5% *n*-3 PUFA-fed (—▲—, 16 weeks) obese JCR:LA-*cp* rats. Values are means, with standard errors represented by vertical bars (*n* 8). * Mean values were significantly different from those of the lipid-balanced diet *cp/cp* control group ($P < 0.05$).**Fig. 2.** Ratio of the weight of inguinal fat pads:body weight of either obese (*cp/cp*) control JCR:LA-*cp* rats or JCR:LA-*cp* rats fed a 5% *n*-3 PUFA diet (16 weeks). Values are means, with standard errors represented by vertical bars (*n* 8). *** Mean value was significantly different from that of the lipid-balanced diet *cp/cp* control group ($P < 0.001$).

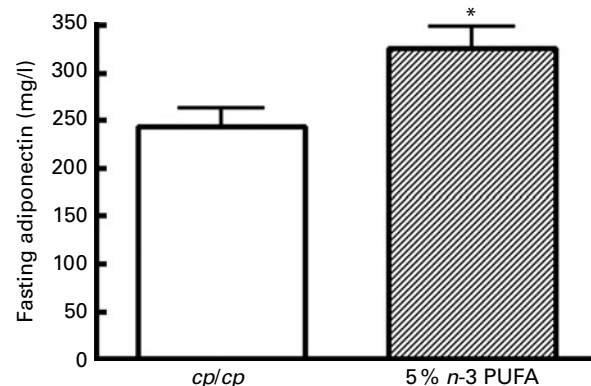
Furthermore, the weight of the liver isolated from obese rats supplemented with 5% *n*-3 PUFA (16 weeks) was significantly decreased ($P < 0.05$) compared with the obese control rats (data not shown). The weight of the heart isolated from rats of both groups was unaltered by dietary *n*-3 PUFA (data not shown).

Fasting biochemical profile

Treatment with 5% *n*-3 PUFA for 16 weeks significantly lowered fasting plasma leptin and insulin concentrations ($P < 0.05$; Table 2). In addition, treatment with 5% *n*-3 PUFA increased (improved) fasting plasma adiponectin concentration ($P < 0.05$; Fig. 3), but did not significantly reduce fasting glucose concentration (Table 2). Furthermore, fasting plasma cholesterol and TAG concentrations were reduced in rats fed the 5% *n*-3 PUFA diet (16 weeks), compared with the obese control rats ($P < 0.05$) (Table 2).

Non-fasting (postprandial) lipid response

Consistent with previous results from acute dietary *n*-3 PUFA supplementation in JCR:LA-*cp* rats, the postprandial lipid

**Fig. 3.** Fasting plasma adiponectin concentration of either obese (*cp/cp*) or 5% *n*-3 PUFA (16 weeks)-fed JCR:LA-*cp* rats. Values are means with standard errors represented by vertical bars (*n* 8). * Mean value was significantly different from that of the lipid-balanced diet *cp/cp* control group ($P < 0.005$).

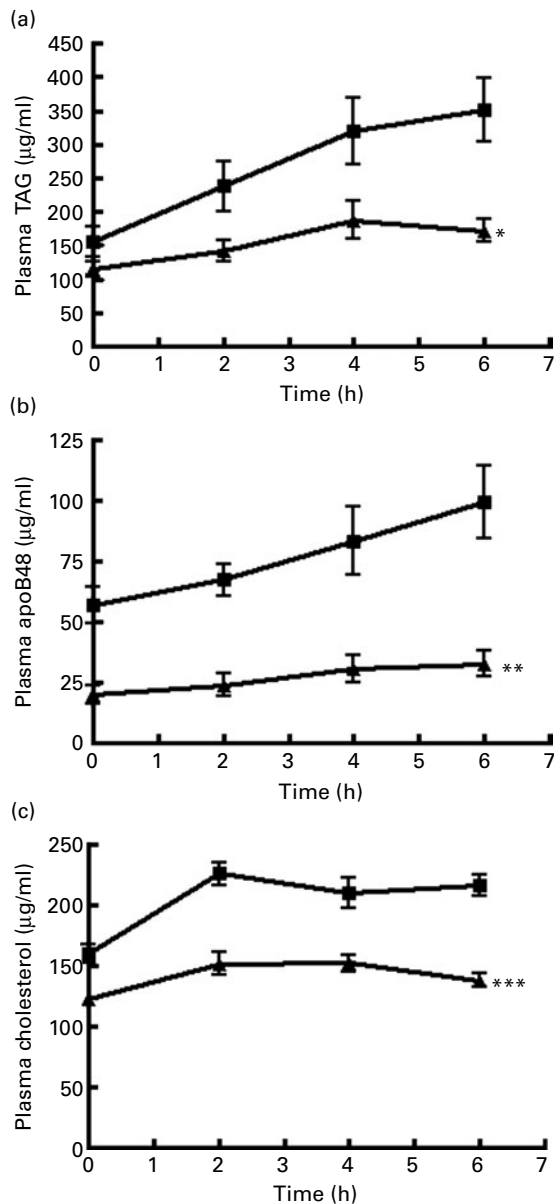


Fig. 4. Postprandial response of plasma TAG (a), apoB48 (b) and total cholesterol (c) (area under the curve) of control (—■—, *cp/cp*) and 5% *n*-3 PUFA-fed (—▲—), (16 weeks) JCR:LA-*cp* rats, following an oral fat challenge. Values are means with standard errors represented by vertical bars (*n* 8). Mean values were significantly different from those of the lipid-balanced diet *cp/cp* control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

response (measured as area under the curve) for TAG was significantly lower in obese rats fed a 5% *n*-3 PUFA diet chronically for 16 weeks compared with the obese control rats (54%; $P < 0.05$; Fig. 4(a)), apoB48 (69%; $P < 0.01$; Fig. 4(b)); and total cholesterol (38%; $P < 0.001$; Fig. 4(c)).

Expression of enterocytic apoB48 protein and secretion of chylomicron lymphatic cholesterol and TAG

The abundance of enterocyte-specific apoB48 (number of CM particles) was significantly lower (43.8%; $P < 0.05$) in obese JCR:LA-*cp* rats fed a 5% *n*-3 PUFA diet for 3 weeks, compared

with the obese control rats (Fig. 5). Obese JCR:LA-*cp* rats fed a 5% *n*-3 PUFA diet (3 weeks) also secreted 30% less CM-associated cholesterol ($P < 0.05$) into mesenteric lymph compared with rats fed the control diet (Fig. 6(a)). Interestingly, by comparison, the secretion of CM-TAG, into mesenteric lymph, was increased (1.7-fold; $P < 0.05$) in those rats fed a 5% *n*-3 PUFA diet (Fig. 6(b)).

Expression of genes involved in lipogenesis and fatty acid oxidation

The expression of both hepatic and adipose *ACC*, *FAS*, *SREBP-1*, *PPAR α* and *PPAR γ* mRNA is reported in Fig. 7(a) (hepatic) and Fig. 8(a) (adipose). Hepatic specific *ACC* (25%; $P < 0.05$) and *SREBP-1* (61.3%; $P < 0.01$) mRNA levels were significantly reduced in obese rats fed a 5% *n*-3 PUFA diet (16 weeks) compared with the control rats (Fig. 7(a)). Similarly, adipose-specific expression of *SREBP-1* mRNA was also significantly lower (37%; $P < 0.05$) in those obese rats fed a 5% *n*-3 PUFA diet (16 weeks) (Fig. 8(a)). The gene expression of *PPAR α/γ* and *ACC* was unaltered in the adipose tissue of obese rats fed a 5% *n*-3 PUFA diet, while *FAS* mRNA was significantly up-regulated (1.9-fold; $P < 0.05$) compared with the obese control rats (Fig. 8(a)).

Protein expression of lipogenic enzymes

The abundance of hepatic *ACC*, *FAS* and *SREBP-1* proteins is reported in Fig. 7(b) and (c). The abundance of hepatic *FAS* protein was significantly lower in rats fed a 5% *n*-3 PUFA diet for 16 weeks ($P < 0.01$; Fig. 7(b) and (c)), relative to the obese control rats. However, *ACC* protein was not significantly altered in livers from 5% *n*-3 PUFA-fed rats, relative to the obese control rats (Fig. 7(b) and (c)). Both

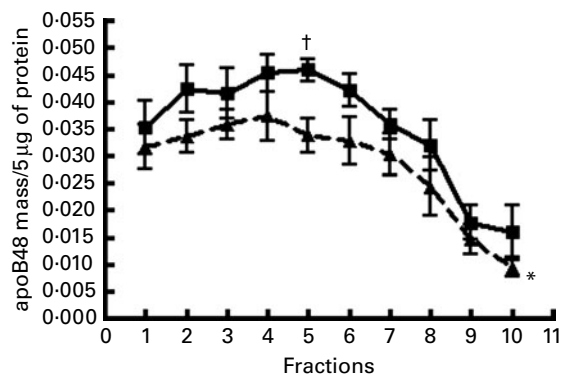


Fig. 5. Enterocyte-specific apoB48 protein expression in obese (—■—, *cp/cp*) JCR:LA-*cp* rats fed either a control lipid-balanced diet or a 5% *n*-3 PUFA (—▲—)-enriched diet for 3 weeks. Primary jejunal enterocytes were isolated as per the Weiser method of isolation and fractionation, as described previously⁽²³⁾. Immuno-Western blot analysis probed the expression of apoB48 protein (as per Methods) along the intestinal villus from enterocyte fraction 1 (tip of the villus) to enterocyte fraction 10 (crypt of the villus). Values are means, with standard errors represented by vertical bars (*n* 5). * Mean values were significantly different between total enterocyte and apoB48 mass of 5% *n*-3 PUFA *cp/cp* v. lipid-balanced diet (LBD) *cp/cp* control ($P < 0.05$). † Mean values were significantly different in enterocyte fraction 5-specific apoB48 protein of 5% *n*-3 PUFA *cp/cp* v. LBD *cp/cp* control ($P < 0.05$).

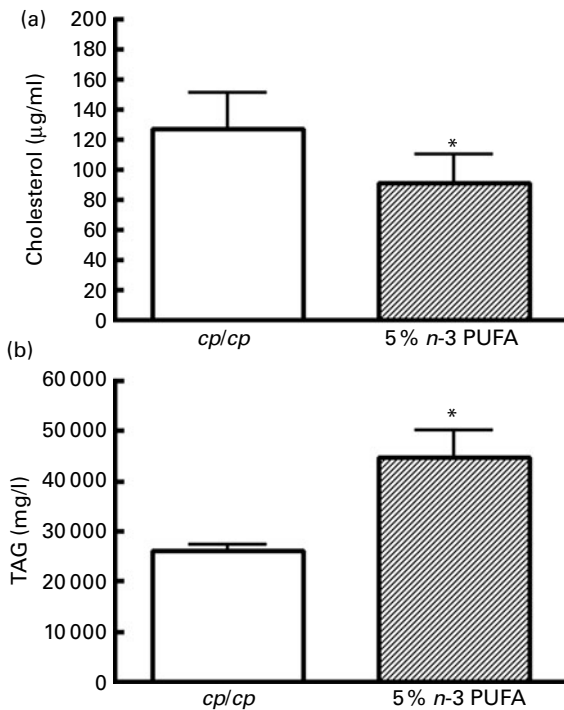


Fig. 6. Secretion of chylomicron-associated (a) cholesterol and (b) TAG into mesenteric lymph. Mesenteric lymph cannulation procedures were carried out as described previously⁽²³⁾. Cholesterol and TAG secretion ($\mu\text{g/ml}$) into mesenteric lymph was compared in obese (*cp/cp*) JCR:LA-*cp* rats fed a 5% *n*-3 PUFA diet v. those fed a control lipid-balanced diet (LBD) for 3 weeks. Values are means, with standard errors represented by vertical bars (n 5). * Mean value was significantly different from that of the LBD *cp/cp* control group in all cases ($P < 0.05$).

precursor (125 kDa) and mature (68 kDa) hepatic SREBP-1 proteins were reduced in the 5% *n*-3 PUFA diet group (16 weeks) compared with the obese control group ($P < 0.01$) (Fig. 7(b) and (c)). Adipose tissue-specific expression of ACC and SREBP-1 protein (125 kDa) was not significantly altered in rats fed a 5% *n*-3 PUFA diet (Fig. 8(b) and (c)). Furthermore, treatment with 5% *n*-3 PUFA significantly reduced FAS protein expression in the adipose ($P < 0.05$) compared with the obese control rats (Fig. 8(b) and (c)).

Enterocytic phosphorylated Jun N-terminal kinase protein

The protein expression of phosphorylated JNK (active) protein was significantly reduced (72.6%; $P < 0.01$) in the enterocytes of obese JCR:LA-*cp* rats fed a 5% *n*-3 PUFA diet for 3 weeks compared with the obese control group (Fig. 9).

Frequency of myocardial lesions

Representative images of the stages (stages 1–4) of myocardial lesions assessed are shown in Fig. 10. The frequency of stages of myocardial lesion development (stages 1–4) for all four treatment groups is reported in Fig. 11. Supplementation with *n*-3 PUFA for 16 weeks in the obese JCR:LA-*cp* rat had no effect on the frequency of early stage 1 lesions (Fig. 11). Most notably, the 5% *n*-3 PUFA diet significantly reduced

the number of late stage 3 (areas of chronic inflammatory infiltration) lesions (83.3%; $P < 0.05$) in obese rats compared with the control group (Fig. 11). Additionally, in the hearts of rats supplemented with a 5% *n*-3 PUFA diet, no stage 4 lesions were detected (Fig. 11).

Discussion

The main objective of the present study was to investigate the impact of chronic (16 weeks) dietary intervention with *n*-3

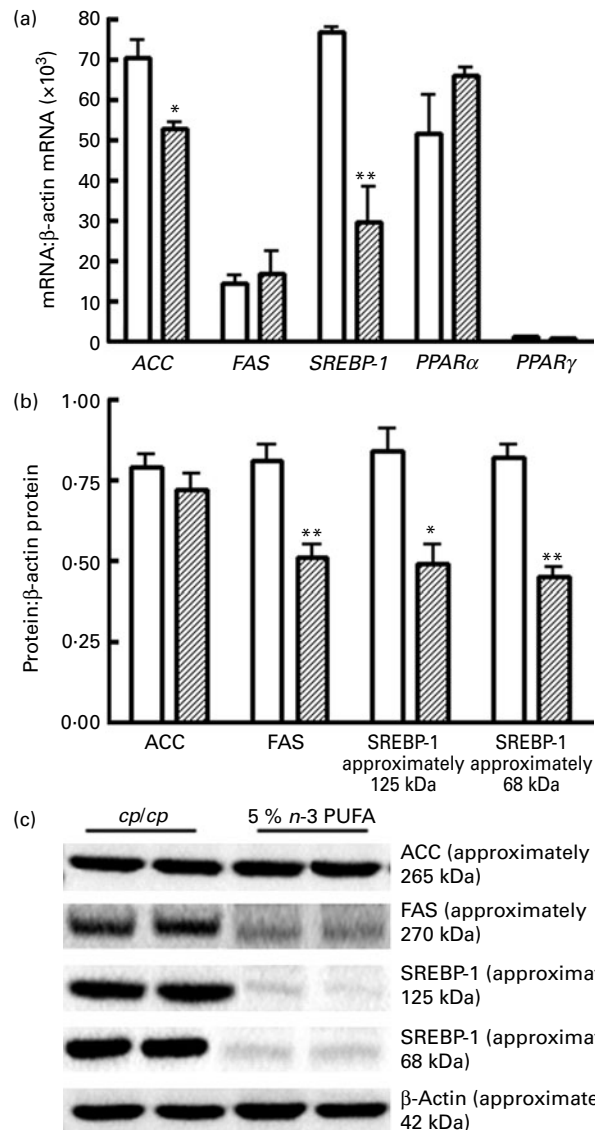


Fig. 7. Hepatic gene and protein expression of lipogenic enzymes in JCR:LA-*cp* rats in response to long-term feeding (16 weeks) of 5% *n*-3 PUFA (■). (a) The expression of ACC, FAS, SREBP-1, PPAR α and PPAR γ mRNA, relative to the housekeeping gene β -actin, in the livers of both obese (\square , *cp/cp*) and 5% *n*-3 PUFA-fed groups. ((b) and (c)) Protein abundance of ACC, FAS, precursor SREBP-1 (approximately 125 kDa) and mature SREBP-1 (approximately 68 kDa) protein, relative to β -actin protein expression, in the livers of the obese (*cp/cp*) control and 5% *n*-3 PUFA diet groups. Values are means, with standard errors represented by vertical bars (n 8). Mean values were significantly different from those of the lipid-balanced diet *cp/cp* control group: * $P < 0.05$, ** $P < 0.01$.

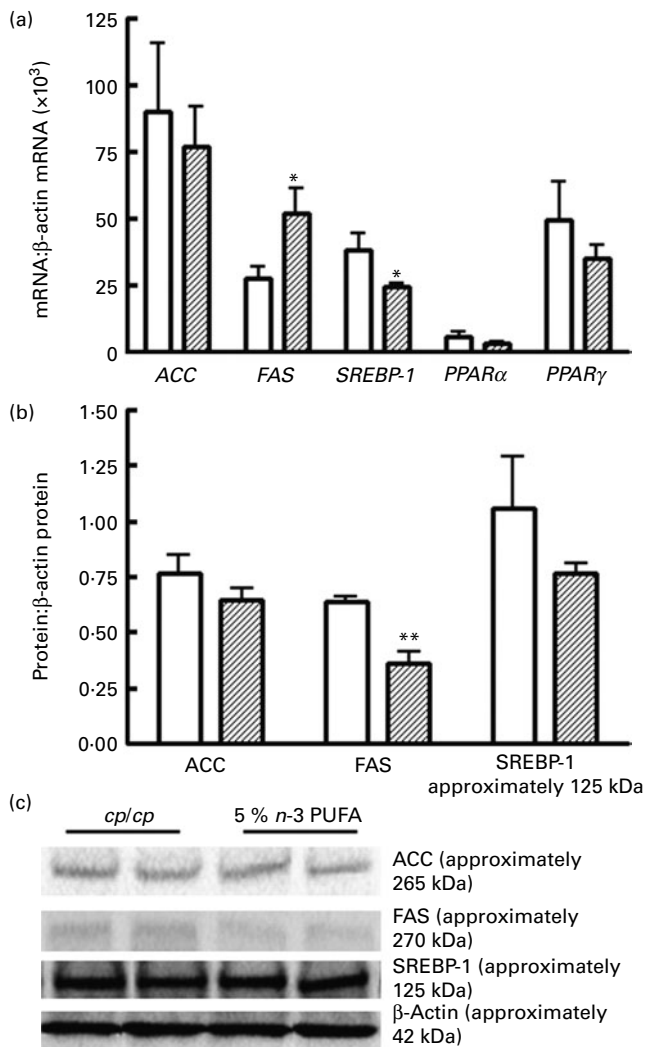


Fig. 8. Adipose-specific gene and protein expression of lipogenic enzymes in JCR:LA-*cp* rats in response to long-term feeding (16 weeks) of 5% *n*-3 PUFA (■). (a) Expression of ACC, FAS, SREBP-1, PPAR α and PPAR γ mRNA, relative to the housekeeping gene β -actin, in the adipose tissue of obese (\square , *cp/cp*) and 5% *n*-3 PUFA-fed JCR:LA-*cp* rats. (b) and (c) Protein abundance of ACC, FAS and precursor SREBP-1 (approximately 125 kDa) protein, relative to β -actin protein expression, in the adipose tissue of the obese (*cp/cp*) control and 5% *n*-3 PUFA diet groups. Values are means, with standard errors represented by vertical bars (n 8). Mean values were significantly different from those of the lipid-balanced diet *cp/cp* control group: * $P < 0.05$, ** $P < 0.01$.

PUFA on pre-existing hyperinsulinaemia, dyslipidaemia and ischaemic lesion development, in the JCR:LA-*cp* rat model. Our findings show that chronic feeding of a diet with increased *n*-3 PUFA can improve both metabolic parameters and vascular complications associated with the MetS.

The chronic effect of *n*-3 PUFA on body weight and fat deposition

While obesity is known to increase the risk of developing type 2 diabetes and cardiovascular complications, the long-term effect of dietary fat consumption in the process of

development of diabetes, IR and dyslipidaemia remains controversial. Thorsdottir *et al.*⁽²⁸⁾ demonstrated in a study of overweight men that the inclusion of fish oil (*n*-3 PUFA) in the diet induced a greater weight loss (1 kg) over 4 weeks, than those on a diet without fish oil⁽²⁸⁾. An additional study in overweight hypertensive subjects, which showed a weight-loss programme incorporating fish meals rich in *n*-3 PUFA, was more effective in reducing weight loss, serum lipids and glucose–insulin metabolism, than either treatment alone⁽²⁹⁾. Interestingly, and consistent with our previous finding in this rat model⁽¹⁶⁾, we observed that increased dietary *n*-3 PUFA reduced body-weight gain in the absence of any alteration in food intake.

Clinical data also suggest that type 2 diabetic patients subjected to a diet rich in *n*-3 PUFA have reduced abdominal subcutaneous fat, in addition to improved IR⁽³⁰⁾. Consistent with this, inguinal fat (intra-abdominal) depots were significantly lower in *n*-3 PUFA dietary groups in the present study. Additional animal studies have also shown that dietary supplementation with fish oil (*n*-3 PUFA) can make an impact upon fatty acid proportions and distribution in subcutaneous and visceral fat^(31,32). Indeed, it has been suggested previously that *n*-3 PUFA may not be as readily deposited, but more freely oxidised within the adipose tissue, implying that *n*-3 PUFA may at least have a partial protective effect against weight gain *per se*^(33–35).

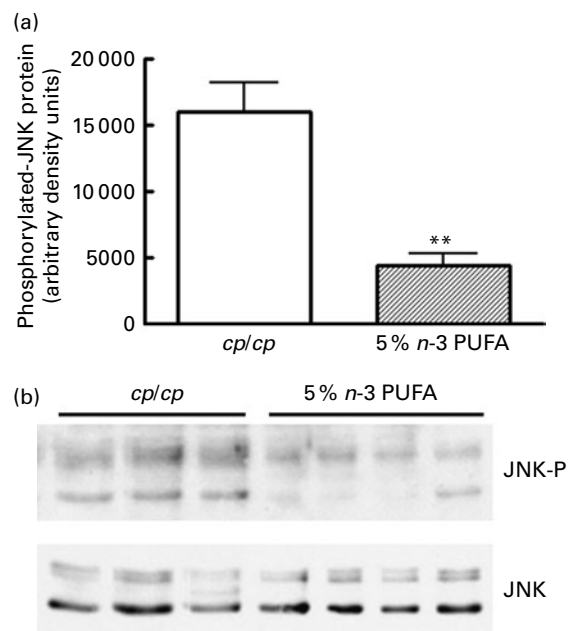


Fig. 9. Activity of Jun N-terminal kinase (JNK) was assessed via Western blot to target phosphorylated JNK (JNK-P) protein. Enterocyte extracts (50 μ g protein) were examined by immunoblot analysis, utilising antibodies from Cell Signalling (JNK-P) and Pharmingen (JNK). JNK-P protein was measured in obese (*cp/cp*) JCR:LA-*cp* rats fed either a 5% *n*-3 PUFA diet or a control lipid-balanced diet (LBD) for 3 weeks; a representative blot and graph, presented as a measure of arbitrary density units, are shown. Values are means, with standard errors represented by vertical bars (n 5). ** Mean value was significantly different from that of the LBD *cp/cp* control group in all cases ($P < 0.01$).

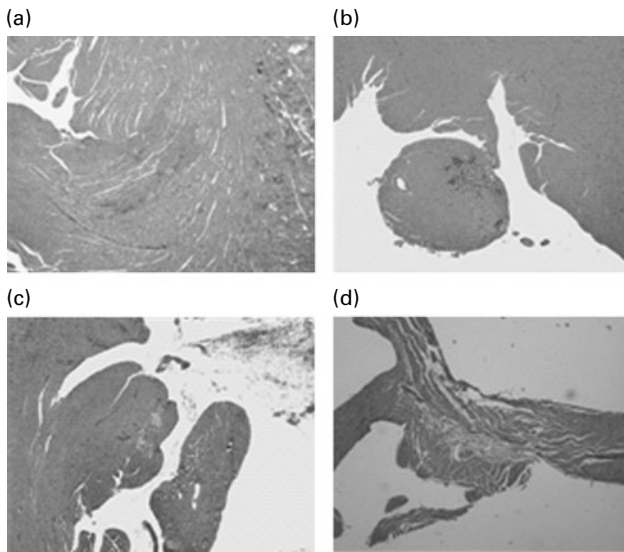


Fig. 10. Representative micrographs of ischaemic lesions in the hearts of obese JCR:LA-*cp* rats (age 24 weeks). (a) Stage 1: area of necrosis with no long-term inflammatory cell infiltration in the left ventricle. (b) Stage 2: area of long-term inflammatory cell infiltration, without visible cell lysis, in the trabecular muscle. (c) Stage 3: area of active inflammatory cell activity and cell lysis in the lower trabecular muscle. (d) Stage 4: early scarred lesion with a small number of inflammatory cells or fibroblasts in the upper perivalvular region of the heart. All images were captured at a magnification of 2 \times after haematoxylin and eosin staining of heart sections.

The chronic effect of *n*-3 PUFA on glucose, insulin resistance and adipokines

Studies have indicated that dietary fish oil (*n*-3 PUFA) may act to normalise and/or improve the storage of lipids and glucose oxidation within skeletal muscle. Evidence supports the notion that hypolipidaemic effects of *n*-3 PUFA act to reduce lipid utilisation within skeletal muscle, restore glucose oxidation and normalise insulin sensitivity⁽³⁶⁾. In the present study, JCR:LA-*cp* rats from the *n*-3 PUFA diet groups had significantly improved fasting plasma insulin (but only a trend towards improved glucose levels), implying improved IR, consistent with previous data⁽³⁷⁾.

Furthermore, *n*-3 PUFA supplementation significantly decreased plasma leptin levels. Existing evidence suggests leptin to be a contributor to the hypolipidaemic benefits of *n*-3 PUFA, reducing TAG biosynthesis and enhancing β -oxidation⁽³⁸⁾. While the effects of *n*-3 PUFA on plasma and tissue leptin levels remain controversial⁽³⁸⁾, studies have shown that *n*-3 PUFA supplementation may reduce leptin mRNA expression⁽³⁸⁾. Plasma leptin levels have also been shown to be reduced in rodents supplemented with dietary *n*-3 PUFA (fish oil), with corresponding decreases in visceral adipose tissue⁽³⁹⁾. The ability of *n*-3 PUFA to reduce plasma leptin levels in our IR rodent model may occur directly via suppression of leptin mRNA expression (not measured in the present study). Alternatively, it may be possible that plasma leptin could be modulated indirectly via the parallel reductions in plasma insulin, inguinal (intra-abdominal) fat weight and body weight.

It is well established that there is a negative correlation between the concentration of plasma adiponectin (an adipokine with anti-diabetic properties) and BMI⁽⁴⁰⁾. Consistent with this, fasting adiponectin concentration was significantly enhanced in rats supplemented with 5% *n*-3 PUFA, while body weight was profoundly reduced. It may be that *n*-3 PUFA influence the expression of adipokines (such as leptin and adiponectin) via direct interaction with transcription factors, or indirectly via mechanisms that control fatty acid oxidation, synthesis and/or storage⁽⁴¹⁾; but this remains to be defined.

Potential mechanisms of *n*-3 PUFA via regulation of lipogenic enzymes

SREBP are sterol-responsive transcription factors. SREBP are synthesised in their precursor form (approximately 125 kDa) in the endoplasmic reticulum, before cleavage to their active (nuclear) form (approximately 68 kDa), in response to low cellular sterol levels, regulating the expression of lipid-related genes, including lipogenic enzymes (FAS and ACC)⁽⁴²⁾. In particular, the SREBP-1 isoform is selective for genes involved in fatty acid synthesis: ACC, FAS; stearyl-CoA desaturase-1⁽⁴³⁾. In the present study, chronic dietary *n*-3 PUFA reduced both gene and protein expression of hepatic precursor and mature SREBP-1, also down-regulating lipogenic enzyme expression of hepatic ACC mRNA and FAS protein, consistent with the expression of SREBP-1⁽⁴⁴⁾. Studies have shown that PUFA can act as a competitive antagonist for the liver X receptor, a nuclear receptor responsive to endogenous oxysterols, *in vitro*⁽⁴⁵⁾. We also know that inhibition of the binding of the liver X receptor/retinoid X receptor heterodimer to the liver X receptor response element, in the promoter region of SREBP-1c, can suppress the expression of SREBP-1c⁽⁴⁵⁾. More recently, the same group has reported that the primary mechanism underlying PUFA-induced SREBP-1c suppression, in fact, occurs at the proteolytic processing level *in vivo*⁽⁴⁶⁾. The ability of PUFA to suppress SREBP-1 may also be

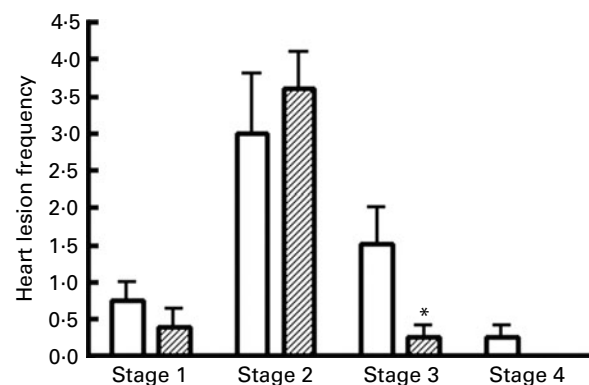


Fig. 11. Frequency of myocardial lesions in the hearts of JCR:LA-*cp* rats from obese control (□, *cp/cp*) and 5% *n*-3 PUFA (▨) diet groups (16 weeks). (a) Stage 1 lesions; (b) stage 2 lesions; (c) stage 3 lesions; (d) stage 4 lesions. Values are means, with standard errors represented by vertical bars (*n* 8). *Mean values were significantly different from those of the lipid-balanced diet *cp/cp* control group in all cases ($P < 0.05$).

dependent on the level of the incorporation of PUFA into cellular lipids, as recently suggested by Di Nunzio *et al.*⁽⁴⁷⁾.

The hypolipidaemic effect of *n*-3 PUFA may arise either via the reduced expression of SREBP-1, reducing lipogenesis and cholesterol biosynthesis, as discussed above, or via activation of the common PUFA-activated transcription factor, PPAR α ^(42,48,49), promoting fatty acid oxidation. However, in the present study, we found that hepatic- and adipose-specific expression of PPAR (α/γ) mRNA was not significantly regulated in response to chronic dietary *n*-3 PUFA.

Furthermore, while dietary *n*-3 PUFA induced a marked reduction in hepatic SREBP-1 gene and protein abundance, only a modest suppression of adipose-specific *SREBP-1* mRNA was observed. The differential PUFA-mediated regulation of SREBP-1 between tissue types in the JCR:LA-*cp* model is consistent with that observed in other rodent models^(50,51). In the present study, we observed a greater abundance of hepatic *SREBP-1* mRNA compared with adipose-specific *SREBP-1*. By contrast, we report greater *PPAR* γ mRNA in adipose compared with hepatic tissue. As reviewed by Kersten⁽⁵²⁾, data suggest that SREBP-1 regulates lipogenic genes in the liver, while PPAR γ is essential for the regulation of lipogenesis in the adipose. Thus, PUFA-induced regulation of lipogenesis may exhibit tissue specificity, via hepatic SREBP-1 and PPAR γ in the adipose tissue.

The acute effect of n-3 PUFA on intestinal enterocytes

We have shown that both acute⁽¹⁶⁾ and long-term *n*-3 PUFA intervention clearly exert beneficial effects specifically in our model to improve postprandial lipids, therefore suggesting that the ability of *n*-3 PUFA to improve postprandial metabolism, during conditions of IR, may occur via the direct action of *n*-3 PUFA on intestinal CM secretion (Figs. 5 and 6). For this reason, we initiated a subsequent acute (3-week) *n*-3 PUFA intervention to assess potential mechanistic effects of *n*-3 PUFA, directly on intestinal enterocyte CM production, associated lymphatic lipid secretion and JNK-associated insulin signalling. We observed that 3 weeks (short-term) of dietary intervention with *n*-3 PUFA was sufficient to reduce the intestinal production of apoB48 (CM) (Fig. 5), but also suppressed the subsequent lymphatic cholesterol secretion (Fig. 6). This is complementary to the observed improvement (reduction) to both fasting and postprandial plasma apoB48 (both acute and chronic) and cholesterol (chronic) reported in our previous acute study⁽¹⁶⁾ and the present long-term (chronic) study. Consistent with clinical observations, neither long-term nor acute⁽¹⁶⁾ intervention with *n*-3 PUFA lowered fasting plasma LDL concentrations, suggestive of preferential *n*-3 PUFA-induced improvement to intestinally derived lipoprotein fractions. Interestingly, while the *n*-3 PUFA diet lowered fasting and postprandial plasma TAG, we observed an increase in lymphatic TAG secretion from the intestine following acute *n*-3 PUFA supplementation (Fig. 6). We hypothesise that there may be an increase in CM particle size and/or enhanced clearance of TAG, although this remains to be elucidated.

Most recently, *n*-3 PUFA have been reported to exert potent anti-inflammatory effects that improve IR and other symptoms of the MetS in mice, via binding to G protein-coupled receptor 120⁽⁵³⁾. This study reported *n*-3 PUFA (DHA specifically) to block both NF- κ B and JNK pathways, reversing IR induced by a high-fat diet⁽⁵³⁾. Supportive of this notion, in the present study, *n*-3 PUFA appear to improve associated insulin signalling pathways in the intestine by reducing the activity of enterocyte-specific JNK (Fig. 9). Collectively, these data support the hypothesis that *n*-3 PUFA may act directly on the intestine to improve insulin signalling and lower non-fasting lipid contribution following a lipid excursion.

The chronic effect of n-3 PUFA on myocardial lesion development

Development of ischaemic lesions, secondary to vascular damage or dysfunction, is a major endpoint of CVD⁽⁵⁴⁾. JCR:LA-*cp* rats develop lesions spontaneously, as a result of the hyperinsulinaemic and hyperlipidaemic status of these rodents^(6,55). Rats from the present study were killed at a relatively early age, and may explain the low frequency of stage 4 (advanced scarred lesions) heart lesions compared with previous studies^(5,27). However, rats from the present study presented with a large number of early stage 2 lesions, probably due to the high lipid and cholesterol load, in the control diet. Dietary *n*-3 PUFA (5%) in the JCR:LA-*cp* rats resulted in fewer stage 3 myocardial lesions, consistent with the complete absence of stage 4 lesions in these animals, as well as an amelioration of myocardial histology; evidence that *n*-3 PUFA may inhibit CVD progression. It may be reasonable to suggest that the vascular improvement observed, in response to dietary *n*-3 PUFA, is due to chronic improvement to metabolic status, including non-fasting lipids, visceral fat, insulin sensitivity, or potentially via a direct effect of *n*-3 PUFA on the heart.

Concluding remarks

In conclusion, the present study provides evidence that chronic increased dietary *n*-3 PUFA has beneficial effects on both hepatic and intestinal lipid metabolism, IR, body weight and myocardial ischaemic lesion frequency in the obese JCR:LA-*cp* rat. Dietary *n*-3 PUFA may confer additional therapeutic potential to lower the risk of atherosclerotic progression in patients with metabolic disorders over the long term. Future directions should focus on additional animal and clinical studies to help verify a beneficial target dose for human therapy and to further define the mechanistic pathways behind the action of *n*-3 PUFA.

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throughout the present study. We also wish to acknowledge Anja Jaeschke (University of Cincinnati, Cincinnati, OH, USA) for her contribution to the JNK activity studies. S. D. P., J. L., D. F. V. and C. J. F. conceived and designed the experiments. J. L., Z. H., Y. W., R. M., M. R., D. S., F. B. and J. C. R. performed the experiments. J. L., S. D. P., J. C. R., F. B. and D. F. V. analysed the data. F. B., S. D. P. and J. L. prepared the manuscript.

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