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Reversal of high-fat diet-induced hepatic steatosis by *n-3* LCPUFA: role of PPAR-α and SREBP-1c☆

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Abstract

Nonalcoholic fatty liver disease is characterized by an abnormal accumulation of triacylglycerides in the liver in absence of significant alcohol consumption. Under these conditions, it has been observed an impaired bioavailability of hepatic *n-3* long-chain polyunsaturated fatty acids (LCPUFAs). The aim of this study was to test the reversion of the prosteatotic and proinflammatory effects of high-fat diet (HFD) in the mouse liver by changing to normocaloric diet and *n-3* LCPUFA supplementation. Male C57BL/6J mice were given either control diet (CD) or HFD for 12 weeks. Control and HFD groups were divided into subgroups that continue with CD or subjected to CD plus *n-3* LCPUFA for 8 additional weeks. After this time, blood and liver samples were taken and metabolic, morphologic, oxidative stress, inflammatory and signaling parameters were analyzed. The dietary change from HFD to a normocaloric diet with *n-3* LCPUFA supplementation significantly reduced insulin resistance and liver steatosis when compared to switching HFD to normocaloric diet alone. In addition, HFD-induced increases in adiposity, adipocyte enlargement and liver oxidative stress and inflammatory cytokine expression were suppressed by *n-3* LCPUFA to control values. Importantly, *n-3* LCPUFA supplementation abolish HFD-induced enhancement in hepatic SREBP-1c/PPAR-α ratios, suggesting a change in the metabolic status of the liver from a lipogenic condition to one favoring fatty acid oxidation and steatosis attenuation. These findings may provide the rational basis for the use of normocaloric diets supplemented with *n-3* LCPUFA in patients with liver steatosis.

Keywords: *n-3* Long-chain polyunsaturated fatty acid; High fat diet; Liver steatosis; PPAR-α; SREBP-1c

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most important cause of chronic liver disease that is characterized by an abnormal accumulation of triacylglycerides (TG) in the liver in absence of significant alcohol consumption (>20 g/day) [1]. NAFLD encompasses a spectrum of diseases from asymptomatic and reversible steatosis, with or without elevated aminotransferases, to cirrhosis with different complications and hepatocellular carcinoma [2,3]. Hepatic steatosis is established when the liver accumulates more than 5% of its weight as fat [1], which may result from an imbalance between lipid availability (enhanced blood uptake and/or *de novo* lipogenesis) and lipid disposal (decreased fatty acid (FA) β-oxidation and lipoprotein synthesis inability) [2,4,5]. The establishment of liver steatosis underlies both free radical generation with a lipid peroxidation response and proinflammatory cytokine release [6], which may trigger progression to nonalcoholic steatohepatitis (NASH) [2]. NAFLD is considered the hepatic manifestation of the metabolic syndrome that is characterized by insulin resistance, atherogenic dyslipidemia and hypertension, in association with proinflammatory and prothrombotic states [7]. Under these conditions, patients with metabolic syndrome have a higher risk of developing type-2 diabetes and cardiovascular diseases [8].

One of the characteristics of NAFLD is the impaired bioavailability of liver *n-6* and *n-3* long-chain polyunsaturated fatty acids (LCPUFAs) [9]. In this regard, eicosapentaenoic acid (C20:5 *n-3*, EPA) and docosahexaenoic acid (C22:6 *n-3*, DHA) are the most important *n-3* LCPUFA due to their key roles in different physiological functions, including the regulation of hepatic lipid metabolism [10]. EPA and/or DHA (i) decrease the expression of transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) along with inhibition of lipogenic genes transcription and consequent *de novo* lipogenesis reduction; (ii) activate peroxisome proliferator-activated receptor α (PPAR-α), which favors FA oxidation [11,12]; and (iii) activate PPAR-γ leading to enhanced liver uptake and transport of free FAs for further oxidation and/or disposal [13,14]. In addition, EPA and DHA have cytoprotective actions through up-regulation of antioxidant enzyme and down-regulation of proinflammatory gene expression [15].

Owing to the increasing prevalence of NAFLD, numerous studies have focused on therapeutic strategies for this liver disease. These include (i) weight loss and physical activity and (ii) therapies based

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on pathogenic mechanisms such as lipid-lowering agents, insulin sensitizers, antioxidants and *n-3* LCPUFA [16]. Previous studies by our group have demonstrated that *n-3* LCPUFA supplementation decreases the prosteatotic and proinflammatory effects of a high-fat diet (HFD) at hepatic level [17]. HFD is a validated experimental model to induce both systemic and liver-specific insulin resistance, with development of hyperglycemia, hyperinsulinemia, dyslipidemia, higher liver lipid content and obesity [18]. The aim of this study was to test the reversion of the prosteatotic and proinflammatory effects of HFD in the mouse liver by changing to normocaloric diet and *n-3* LCPUFA supplementation. For this purpose, C57BL/6J mice were given either control diet (CD) or HFD for 12 weeks, after which control animals and mice given HFD were divided into groups that continue with CD and groups subjected to CD plus *n-3* LCPUFA, for 8 additional weeks. Under these conditions, blood and liver samples were taken to assess parameters related to metabolism, morphology, oxidative stress, inflammation [tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β)] and signaling (PPAR-α and SREBP-1c).

2. Materials and methods

2.1. Ethics statement

Experimental animal protocols and animal procedures complied with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 6-23, revised 1985) and were approved by the Bioethics Committee for Research in Animals, Faculty of Medicine, University of Chile (CBA 0386 FMUCH).

2.2. Animal preparation and supplementation with n-3 LCPUFA (EPA plus DHA)

Weaning male C57BL/6J mice weighing 12–14 g were obtained from the Animal Facility at the Faculty of Medicine, University of Chile, Chile. Room temperature was kept constant at 21°C and light was maintained on a 12:12-h light–dark cycle. At 20 days of age, mice were randomly divided into two diet groups: (i) CD containing 10% fat, 20% protein and 70% carbohydrate (expressed as % total calories) with a caloric value of 3.85 kcal/g, free of EPA and DHA and contained 0.7 g of α-linolenic acid (ALA)/100 g of diet or (ii) HFD containing 60% fat, 20% protein and 20% carbohydrate with a caloric value of 5.24 kcal/g, free of EPA and DHA and contained 0.7 g of ALA/100 g of diet (Research Diets Inc, rodent diet, product data D12450B and D12492, USA) from days 1 to 84 (12 weeks). After 12 weeks, the animals given the CD were divided into two diet groups (n=9): (a) CD and (b) CD plus *n-3* LCPUFA; similarly, the animals subjected to the HFD were divided into two diet groups (n=9): (c) change to CD and (d) change to CD plus *n-3* LCPUFA; all groups continue during 8 weeks to complete 20 weeks of total treatment. The *n-3* LCPUFA supplemented groups received daily doses of 0.2 ml of fish oil [encapsulated fish oil, 84.3% of EPA and DHA, containing 200 mg/kg (108 mg/kg of EPA and 92 mg/kg of DHA) corresponding to 0.18 g/day of fat, increasing in a 0.5% the calories as *n-3* LCPUFA] (Acolest TG Product, Procaps, Colombia), through oral administration. The control groups were given isovolumetric amounts of saline. Weekly controls of body weight and diet intake were performed through the whole period. At the end of the 20th week, animals were fasted (6–8 h) and then anesthetized with ketamine and xylazine (150 and 10 mg/kg, respectively) ip.

2.3. Tissue and blood samples

Liver samples (left lobe) were frozen in liquid nitrogen and visceral fat tissue was removed from epididymal area. Both tissues samples were weighed and stored at −80°C or fixed in phosphate-buffered formalin, embedded in paraffin, sectioned using a microtome and stained with hematoxylin–eosin (HE). Adipose and liver slides stained with HE were analyzed by optical microscopy (Olympus CX31, Japón) for morphology analysis in a blind fashion. Presence of both steatosis and inflammation were both graded as absent, mild, moderated or severe [19].

Blood samples were taken from cardiac puncture, and then were centrifuged, and serum was stored at −20°C.

2.4. Glucose tolerance test and biochemical determinations

Glucose tolerance test was performed on all animals at 12 weeks of treatment. Mice were subjected to a 6-h fast and blood glucose (mg/dl) was measured at time zero by means of a glucometer (Johnson and Johnson, One Touch Glucometer) following manufacturer's instructions, and glucose (1.5 mg/g of body weight) was injected ip. Determinations of blood glucose levels were measured at 15, 30, 90 and 120 min post glucose intake. The data obtained were analyzed and plotted, and the area under the curve was calculated.

Plasma insulin concentration (U/ml) was determined by a commercially available immunoassay specific for mice (Mercodia, Uppsala, Sweden). Insulin resistance was estimated by the homeostasis model assessment method (HOMA) [fasting insulin (mU/ml)×fasting glucose (mM)/22.5] [20].

2.5. RT-PCR assay of TNF-α and IL-1β mRNA expression

The expression of cytokines regulated by NF-κB (TNF-α and IL-1β) was assessed by RT-PCR. Total RNA was isolated from 15 to 25 mg of frozen liver using an E.Z.N.A. total RNA Kit (Omega Biotek, Norcross, GA, USA) according to the manufacturer's instructions. Quantification of total RNA was performed spectrophotometrically (A260/A280 ratio) and RNA quality was checked by electrophoresis on 1.2% agarose gels, using a molecular size marker. The resulting DNAse free RNA was reverse-transcribed to cDNA with ThermoScript reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions using random hexamer primers (Promega, Madison, WI, USA). The resulting cDNA was amplified in a PCR reaction using Taq DNA polymerase recombinant (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's instructions. Nucleotide sequences for sense and antisense primers used in this study were as follows: 5′- ACT GAA CTT CGG GGT GAT CG -′3 and 5′- GCA GCC TTG TCC CTT GAA GA -′3 for TNF-α; 5′- AGA TGA AGG GCT GCT TCC AA -3′ and 5′- GCC GTC TTT CAT TAC ACA GG -3′ for IL-1β; and control 18S rRNA as internal control (Classic II QuantumARN 18S InteARNl Standards, Ambion Inc., Austin, TX, USA). The amplification (TECHNE TC-5000, Bibby Scientific Ltd., United Kingdom) was initiated after 3 min denaturation at 94°C, followed by 36 cycles (94°C for 90 s, 59.4°C for 30 s and 72°C for 30 s for TNF-α; 94°C for 90 s, 60°C for 30 s and 72°C for 30 s for IL-1β) and finalizing with 72°C for 10 min. All amplification products were stored at 4°C. PCR products were electrophoresed on 1.2% agarose gels containing ethidium bromide, visualized by UV-induced fluorescence and analyzed by densitometry using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT, USA).

2.6. Immunohistochemistry studies

Staining for SREBP-1c and PPAR-α was performed after deparaffination, rehydration and antigen retrieval in citrate buffer pH 6.1 (10 mM sodium citrate and 0.05% Tween-20) for 30 min at 95°C, endogenous biotin was blocked with Biotin Blocking System Solution (Dako, California, USA). SREBP-1c and PPAR-α polyclonal antibodies (Thermo Scientific, Illinois, USA) were used according to the manufacturer's instruction. A negative control without the primary antibody was included. Analysis was performed under light microscope in a blind fashion in 10 adjacent (×400) per slide.

2.7. Adipocyte area

Adipocyte area (size in μm^2) was assessed with ImageJ 1.46r (National Institutes of Health, EE.UU.) software in HE-stained slides, and these were observed under light microscope at high-power field in at least five adjacent fields.

2.8. Oxidative protein damage

Oxidized protein contents were determined in frozen tissue and treated with 2,4- dinitrophenylhydrazine to form a Schiff base. Production of the corresponding hydrazone was measured spectrophotometrically between 350 and 390 nm to determine concentration of carbonyls and at 280 nm to determine total protein concentration [21]. Values were expressed as nanomoles of carbonyls per milligram of protein.

2.9. Liver total fat content

Total lipids were extracted from whole-liver homogenates using a modified Bligh and Dyer extraction procedure [22]; liver samples were homogenized in distilled water and the lipid components were extracted with a 1:2 chloroform:methanol solution, followed by centrifugation (2000*g* for 10 min at room temperature). After extraction of the chloroformic phase, the solvent was allowed to evaporate and the samples were stored at −20°C [23]. Values were expressed as grams of fat per 100 g of liver.

2.10. Statistical analyses

Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Values shown represent the mean±S.E.M. for the number of separate experiments indicated. One-way analysis of variance (ANOVA) and the Newman–Keuls test or Student's *t* test for unpaired data assessed the statistical significance of differences between mean values as indicated by the letters identifying each experimental group, with $P<.05$ being considered significant. The Pearson coefficient was used to assess associations between variables.

3. Results

3.1. Change to CD with n-3 LCPUFA supplementation reduces visceral fat and enhanced adipocyte size induced by HFD

The weight of the animals subjected to CD or HFD for 12 weeks was not significantly different [CD, 21.05±0.9 g (n=18); HFD, 22.74±0.9 g (n=18)]. Under these conditions, glucose tolerance test revealed significantly increased blood glucose levels (P<.05) in mice subjected to HFD compared to those given CD (Fig. 1A), with the consequent 18% increase (P<.05) in the respective area under the curve (Fig. 1B).

Following 8 weeks of diet change to CD without or with *n-3* LCPUFA supplementation, body weight was not significantly altered [(a) CD, 25.57±0.3 g (n=9); (b) CD+*n-3* LCPUFA, 25.19±0.3 g (n=9); (c) HFD, 25.0±0.5 g (n=9); and (d), HFD+*n-3* LCPUFA, 25.57±0.4 g (n=9)]. However, the visceral fat/body weight ratio was enhanced by 67%, 10% and 21% (P<.05) in mice subjected to HFD+CD compared with CD+CD, CD+CD/*n-3* LCPUFA and HFD+CD/*n-3* LCPUFA (Fig. 2A). Measurement of adipocyte area as white fat cell hypertrophy marker (Fig. 2B) showed that mice subjected to change from HFD to CD had higher mean area with respect to CD groups without (78% enhancement; P<.05) or with *n-3* LCPUFA supplementation (30% increase; P<.05), whereas change from HFD to CD/*n-3* LCPUFA reduced adipocyte area to control values (Fig. 2C).

3.2. Change to CD with n-3 LCPUFA supplementation reduces liver steatosis, morphological alterations and the elevated liver inflammatory parameters induced by HFD

In all groups, liver histology was characterized by the absence of architectural distortion, lobular inflammation, necrotic foci or fibrosis (Fig. 3A), and those given CD without and with *n-3* LCPUFA did not show lipid infiltration [Fig. 3A, (a) and (b); Fig. 3B]. However, HFD+CD exhibited macrovesicular and microvesicular steatosis (30%) (Fig. 3A (c); Fig. 3B), whereas HFD+CD/*n-3* LCPUFA elicited 8% fat infiltration [Fig. 3A (d); Fig. 3B; P<.05]. In addition, liver fat content in HFD+CD fed mice was significantly higher with respect to groups CD+CD (78%) and CD+CD/*n-3* LCPUFA (76%), a value that was reduced by 47% when HFD is changed to CD+CD/*n-3* LCPUFA (P<.05), the latter group exhibiting hepatic fat levels 59% and 55% higher than animals fed CD without and with CD/*n-3* LCPUFA postadministration, respectively (Fig. 3C; P<.05). Concomitantly, liver mRNA levels of proinflammatory cytokines IL-1β (Fig. 3D) and TNF-α (Fig. 3E) in HFD mice showed 115% enhancement (P<.05) over values in animal given CD without or with *n-3* LCPUFA supplementation. This increase was reverted to control values when HFD was changed to CD/*n-3* LCPUFA, representing 40% and 54% decrease in IL-1β and TNF-α mRNA levels with respect to HFD+CD, respectively (Fig. 3D and E; P<.05).

3.3. Change to CD with n-3 LCPUFA supplementation decreases HOMA-IR index and liver protein oxidation induced by HFD

Mice subjected to HFD and change to CD exhibited a significantly enhancement in HOMA index with respect to control animals given CD either without (240%; P<.05) or with *n-3* LCPUFA supplementation (181%; P<.05) (Fig. 4A). This enhancement was diminished by 41% (P<.05) when HFD was changed to CD/*n-3* LCPUFA (Fig. 4A). In addition, hepatic protein carbonylation as an oxidative-stress-related parameter in mice subjected to change from HFD to CD showed a significant 156% enhancement, with 64% increase upon change to CD/*n-3* LCPUFA (P<.05) (Fig. 4B). However, this effect was significantly reduced by 41% (P<.05) when HFD was changed to CD/*n-3* LCPUFA, reaching values comparable to controls (Fig. 4B).

3.4. Change to CD with n-3 LCPUFA supplementation recovers HFD-induced liver PPAR-α depletion and SREBP-1c enhancement

Liver PPAR-α presence in nuclear fractions assessed by immunohistochemistry studies (Fig. 5A) revealed that mice subjected to CD/*n-3* LCPUFA after CD alone exhibited 17% (P<.05) enhancement in the percentage of PPAR-α liver positive nuclei compared to those maintained with CD, whereas animals given HFD with CD/*n-3* LCPUFA supplementation showed 40% (P<.05) increase in nuclear PPAR-α over HFD+CD mice (Fig. 5B). Immunohistochemistry of hepatic nuclei for SREBP-1c presence (Fig. 5C) revealed 98% decrease (P<.05) in the CD+CD/*n-3* LCPUFA group over that given only CD, with animals subjected to HFD+CD showing 352% (P<.05) increases compared to the control group (Fig. 5D). This effect of HFD was reduced by 97% (P<.05) when animals were changed to CD supplemented with *n-3* LCPUFA (Fig. 5D). Liver SREBP-1c and PPAR-α levels were inversely correlated in the studied groups (r=−0.76; P<.002). Under these conditions, calculation of the SREBP-1c/PPAR-α ratios representing the relationship between liver lipogenesis and FA oxidation indicated 5-fold (P<.05) increase in the HFD+CD group over that given only CD, with *n-3* LCPUFA supplementation achieving suppression (P<.05) in animals subjected to CD or HFD (Fig. 5E). Furthermore, insulin resistance (HOMA-IR) (r=0.77; P<.002) and liver steatosis (grams of fat per 100 g of liver) (r=0.67; P<.01) were significantly associated with hepatic SREBP-1c/PPAR-α ratios, respectively.

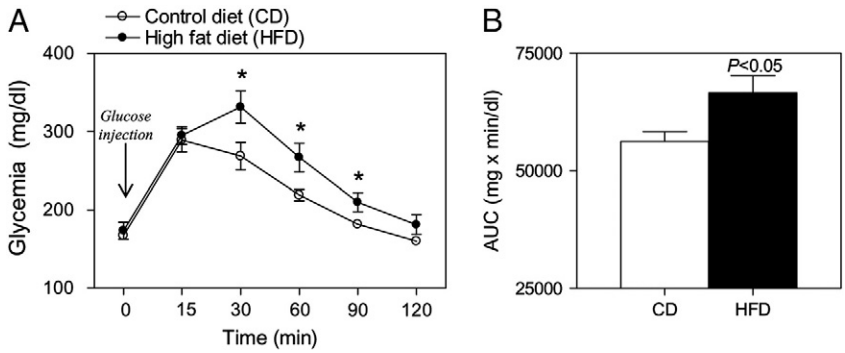

Fig. 1. Influence of HFD on glucose homeostasis in mice. (A) Glucose tolerance test and (B) respective areas under the curve (AUC). Animals were given either a CD or HFD for 12 weeks. Values are expressed as mean±S.E.M. for 9 animals per experimental group. Statistical differences (*P<.05) between animals given CD or HFD were assessed by one-way ANOVA and the Newman–Keuls test (A) or by Student's t test for unpaired data (B).

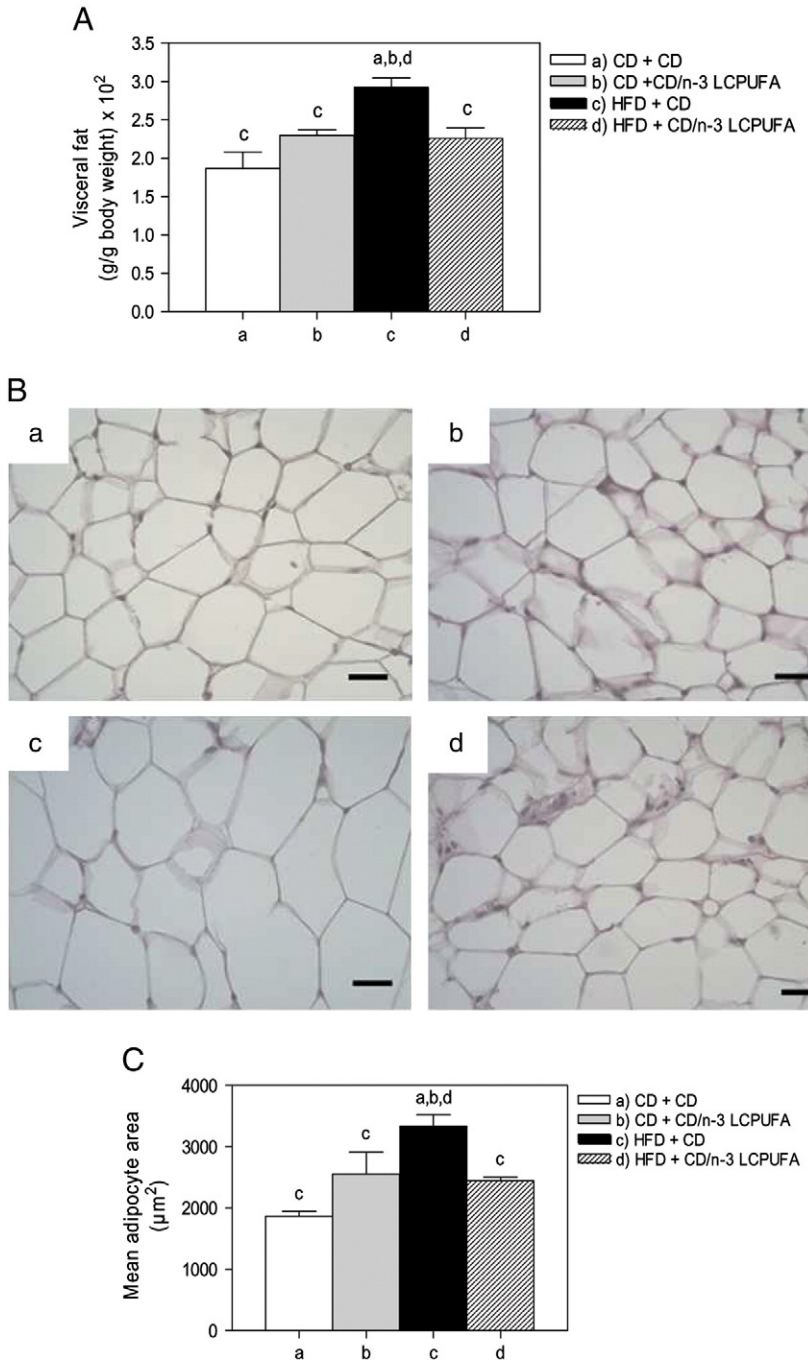

Fig. 2. Effect of *n-3* LCPUFA and dietary change on (A) visceral fat, (B) adipose tissue histology (magnification ×400) and (C) adipocyte area in mice. Animals were given either (a) CD, (b) CD plus *n-3* LCPUFA (CD+CD/*n-3* LCPUFA), (c) HFD followed by CD (HFD+CD) or (d) HFD plus CD supplemented with *n-3* LCPUFA (HFD+CD/*n-3* LCPUFA). Values are expressed as mean±S.E.M. for 9 animals per experimental group. Letters above the bars identifying each experimental group indicate statistically significant differences ($P<.05$; one-way ANOVA and the Newman–Keuls test).

4. Discussion

NAFLD underlies a complex array of metabolic alterations in the setting of insulin resistance and oxidative stress development, which affect several organs including liver and adipose tissue [1,2,4]. HFD intake in mice develops glucose intolerance and insulin resistance, with higher adiposity (visceral fat) and hepatic steatosis, compared with animals subjected to a normocaloric diet. These metabolic alterations were previously demonstrated to be decreased by a dietary change from HFD to a normocaloric diet [24,25], whereas *n-3* LCPUFA (EPA plus DHA) supplementation prevented HFD-induced increase in liver lipid content [17,26]. Here, reversal of the prosteatotic and proinflammatory effects of HFD in mouse liver by both changing to normocaloric diet and *n-3* LCPUFA supplementation was achieved, as evidenced by reduction in HFD-induced insulin resistance and liver steatosis, with suppression of enhanced adiposity, liver oxidative stress and inflammatory cytokine expression. The latter findings are associated with major changes in hepatic nuclear abundance of the lipid metabolism-related transcription factors PPAR-α and SREBP-1c, with abolishment of the HFD-induced enhancement in SREBP-1c/PPAR-α ratios by combined HFD and *n-3* LCPUFA supplementation.

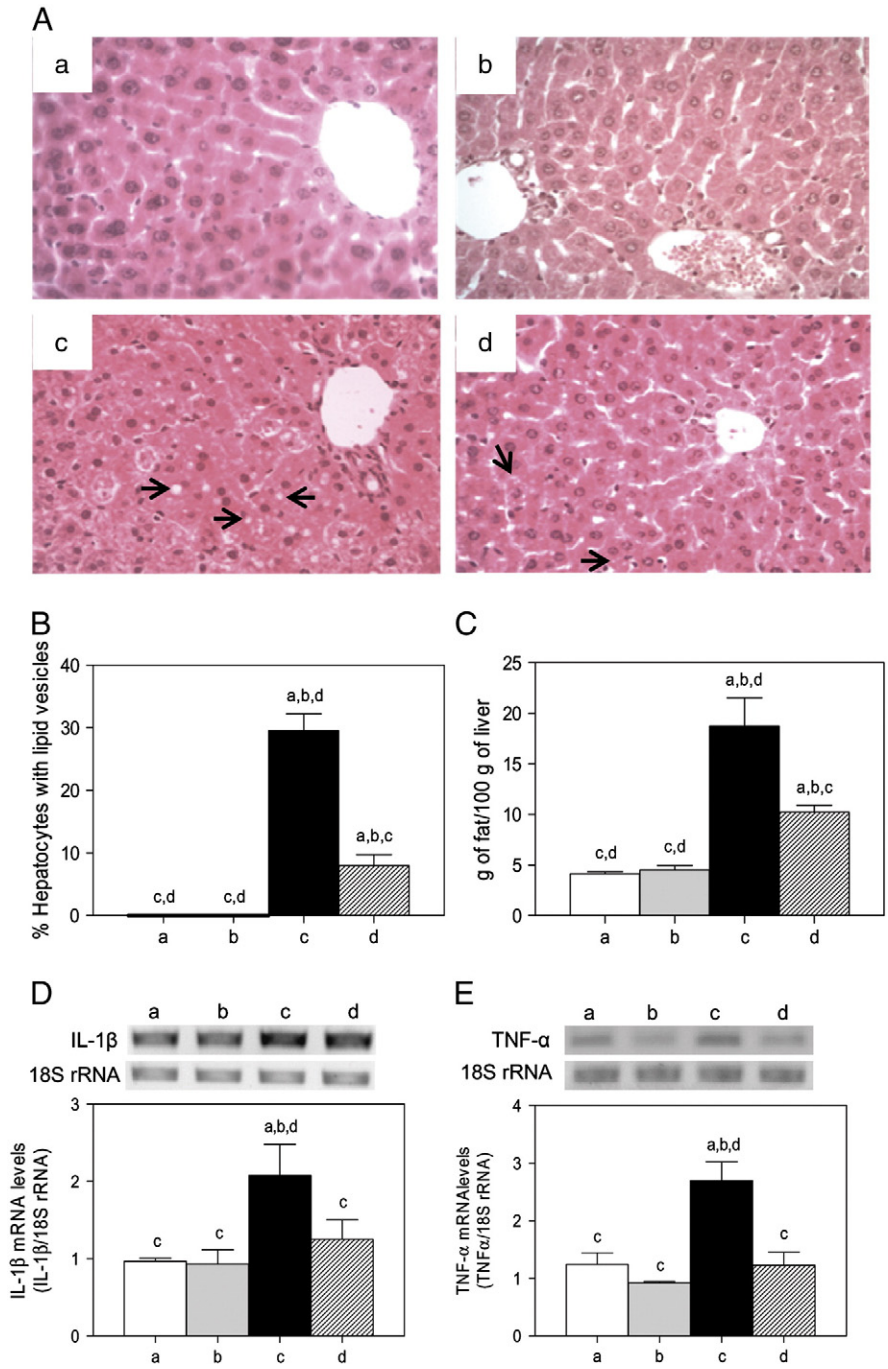

Fig. 3. Effect of *n-3* LCPUFA and dietary change on (A) liver histology (magnification ×100), (B) hepatocyte lipid infiltration, (C) total liver fat content, (D) liver interleukin 1β (IL-1β) and (E) tumor necrosis factor α (TNF-α) mRNA levels in mice. Animals were given either (a) CD, (b) CD plus *n-3* LCPUFA (CD+CD/*n-3* LCPUFA), (c) HFD followed by CD (HFD+CD) or (d) HFD plus CD supplemented with *n-3* LCPUFA (HFD+CD/*n-3* LCPUFA). Values are expressed as mean±S.E.M. for 9 animals per experimental group. Letters above the bars identifying each experimental group indicate statistically significant differences ($P<.05$; one-way ANOVA and the Newman–Keuls test).

n-3 LCPUFA has an important role in membrane fluidity and, consequently, in the behavior of membrane-bound receptors and enzymes [27,28]. It has been described important effects of fish oil administration in proximal insulin signaling steps such as increases of protein mass level of IRS-1 in skeletal muscle from rats fed with sucrose-rich diet and, furthermore, fish oil and produces incorporation of 20:5 *n-3* and 22:6 *n-3* FAs into the phospholipids of the skeletal muscle affecting the membrane composition [29]. *n-3* LCPUFA supplementation produces incorporation of *n-3* PUFA in triglycerides and phospholipids liver, muscle and adipose tissue. IR and IRS-1 tyrosine phosphorylation insulin-dependently occurred in rat fed with *n-3* PUFA at difference with rat fed with *n-6* PUFA in whom insulin pathway was not activated [30]. Also, AKT and PI3K activities were improved after fish oil administration to rats fed with high sucrose diet [31]. On the other hand, it has been reported that *n-3* PUFA is involved in gene expression changes related with insulin signaling, Glut-4 mRNA is recovered after fish oil supplementation in insulin resistance rats, but only in adipose tissue [32]. Apparently, changes in membrane composition induced by *n-3* PUFA could influence the activity of insulin signal molecules but are needed more direct evidence to prove it.

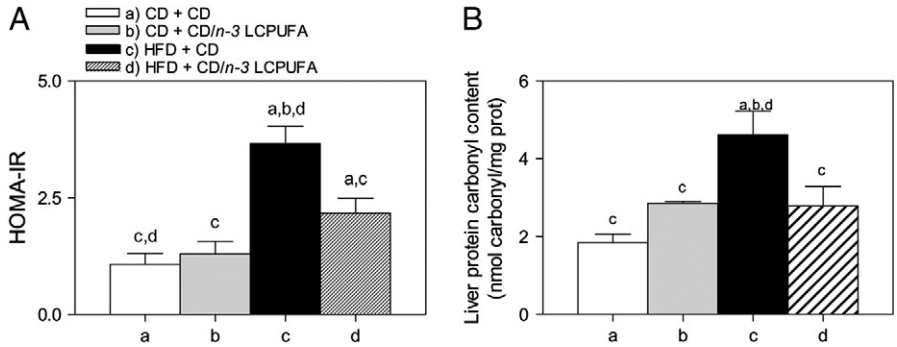

Fig. 4. Effect of *n-3* LCPUFA and dietary change on (A) homeostasis model assessment index (HOMA-IR) and (C) liver protein carbonyl content in mice. Animals were given either (a) CD, (b) CD plus *n-3* LCPUFA (CD+CD/*n-3* LCPUFA), (c) HFD followed by CD (HFD+CD) or (d) HFD plus CD supplemented with *n-3* LCPUFA (HFD+CD/*n-3* LCPUFA). Values are expressed as mean±S.E.M. for 9 animals per experimental group. Letters above the bars identifying each experimental group indicate statistically significant differences ($P<.05$; one-way ANOVA and the Newman–Keuls test).

Accordingly, HFD-induced increase in visceral fat, as a suitable index sensing alterations in high-fat intake-dependent insulin resistance [33], was significantly diminished in animals subjected to HFD plus *n-3* LCPUFA supplementation. Furthermore, enhancement in the hepatic expression of proinflammatory cytokines such as TNF-α and IL-1β by HFD was also blunted by concomitant *n-3* LCPUFA administration, cytokines are able to generate reactive oxygen species (ROS) that have a causal role in multiple forms of insulin resistance

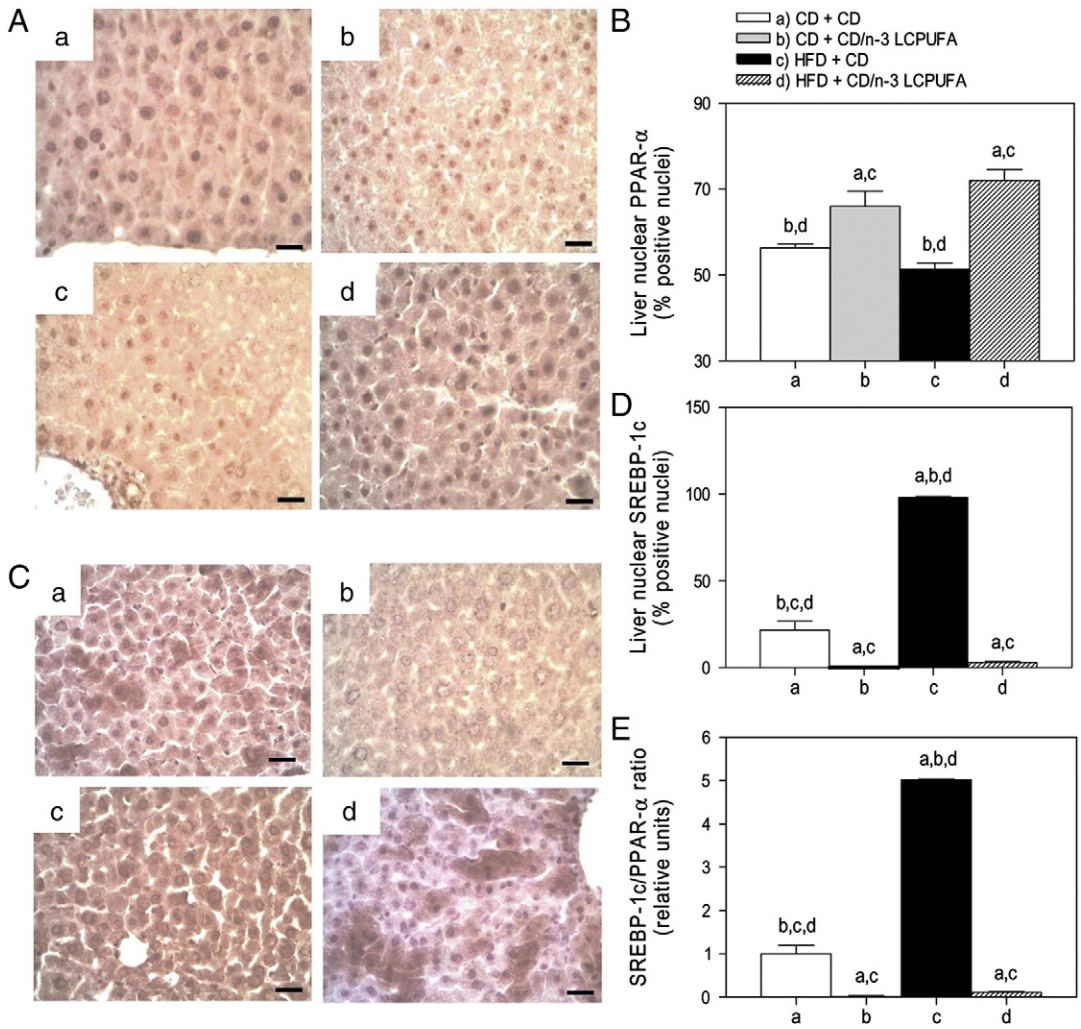

Fig. 5. Effect of *n-3* LCPUFA and dietary change on hepatic (A) inmmunohistochemical determination of nuclear peroxisome proliferator-activated receptor α (PPAR-α) mRNA and percentage of positive nuclei for PPAR-α (B), (C) inmmunohistochemical assessment of nuclear sterol responsive element-binding protein 1c (SREBP-1c) and percentage of positive nuclei for SREBP-1c (D) and (E) respective SREBP-1c/PPAR-α ratios in mice. Animals were given either (a) CD, (b) CD plus *n-3* LCPUFA (CD+CD/*n-3* LCPUFA), (c) HFD followed by CD (HFD+CD) or (d) HFD plus CD supplemented with *n-3* LCPUFA (HFD+CD/*n-3* LCPUFA). Values are expressed as mean±S.E.M. for 9 animals per experimental group. Letters above the bars identifying each experimental group indicate statistically significant differences ($P<.05$; one-way ANOVA and the Newman–Keuls test).

[34]. *n-3* LCPUFA-dependent reversion of HFD-induced liver oxidative stress, as evidenced by the higher content of carbonylated proteins, has been related to the induction of antioxidant enzymes [15], including heme oxygenase-1, glutamate cysteine ligase, glutathione peroxidase, glutathione reductase, glutathione-*S*-transferase and catalase, with enhancement in glutathione levels and lipid peroxidation reduction [35,36]. The antioxidant response induced by *n-3* LCPUFA was related to their spontaneous lipid peroxidation, with generation of cyclopentenone-containing J-ring isoprostanes that activate nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [36], a factor controlling the expression of antioxidant enzymes and other cytoprotective proteins [37]. Mice fed HFD followed by a normocaloric diet supplemented with *n-3* LCPUFA showed a significant diminution in total liver fat content over that in nonsupplemented animals, a finding that may be related to changes in the pattern of lipid metabolism in the liver. This contention is supported by the significant increase in liver nuclear PPAR-α abundance and suppression in that of SREBP-1c found after HFD plus CD/*n-3* LCPUFA intake compared to HFD plus CD feeding. The resulting abrogation of HFD-induced increase in liver SREBP-1c/PPAR-α ratio by the switch to CD with *n-3* LCPUFA supplementation suggests a change from a lipogenic condition to one favoring FA oxidation. From the mechanistic point of view, *n-3* LCPUFA (i) represses SREBP-1c down-regulating FA synthase, stearoyl desaturase-1 and acetyl-CoA carboxylase with concomitant reduction in *de novo* lipogenesis and (ii) activates PPAR-α up-regulating the expression of carnitine palmitoyl transferase-1 and acyl-CoA oxidase with parallel enhancement in FA oxidation [11,12,38,39]. In agreement with the effect of HFD reported, the liver of NAFLD patients with steatosis exhibiting *n-3* LCPUFA depletion shows a drastic significant increase in the SREBP-1c/PPAR-α ratio, as a major steatotic mechanism [40].

Liver steatosis in both NAFLD patients and in animals subjected to HFD is accompanied by a proinflammatory condition that is characterized by Kupffer-cell activation, increased number of hepatic neutrophils and higher levels of TNF-α, IL-1β and IL-6 in serum [41–44]. In agreement with these findings, data presented here show that liver TNF-α and IL-1β mRNA levels were higher in HFD-fed mice compared to controls, a condition that was reverted upon switching from HFD to normocaloric diet plus *n-3* LCPUFA supplementation. Interestingly, studies in PPAR-α-null mice fed a HFD exhibit increased steatosis, oxidative stress and inflammation over the respective control groups, suggesting a critical role of PPAR-α in preventing fat-induced NASH [45]. The antiinflammatory effects of *n-3* LCPUFA supplementation are related to at least two mechanisms of action, namely, (i) *n-3* LCPUFA-induced PPAR-α activation and direct interaction with the proinflammatory transcription factors nuclear factor-κB (NF-κB) and activating protein 1 (AP-1) and (ii) *n-3* LCPUFA metabolism with production of antiinflammatory mediators [15]. The former mechanism is based on the findings that PPAR-α activation by *n-3* LCPUFA promotes its interaction with the p65 component of NF-κB and the c-Jun component of AP-1, which interferes with NF-κB and AP-1 transactivation of inflammatory genes [46]. This primary negative cross-talk mechanism may be contributed by either PPAR-α-induced higher inhibitor of κB-α (IκB-α) expression and lower IκB-α degradation, determining a diminished NF-κB DNA binding activity, and/or *n-3* LCPUFA-induced antioxidant status blunting the redox activation of NF-κB and AP-1 and the inflammatory cytokine production [15]. Alternatively, inactivation of redox-sensitive NF-κB signaling and inflammatory outcome may be achieved through *n-3* LCPUFA-induced Nrf2 activation [36], which reduces ROS levels by up-regulation of the expression of antioxidant enzymes [37]. Metabolism of *n-3* LCPUFA generates several potent antiinflammatory mediators including (i) E-series and D-series of resolvins produced by the action of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) [47], (ii) 5-LOX-dependent protectin D1 production [48], (iii) 17*S*-hydroxy-DHA [14] and (iv) formation of epoxyeicosaquatraenoic acid and epoxydocosapentaenoic acid regioisomers catalyzed by cytochrome P450 NADPH-dependent epoxygenases [47,49].

In conclusion, using a well-established animal model of liver steatosis induced by a HFD, we demonstrate that (i) the dietary change from HFD to a normocaloric diet with *n-3* LCPUFA supplementation significantly reduced insulin resistance and liver steatosis when compared to switching HFD to normocaloric diet alone, parameters that remained marginally elevated over control values; and (ii) HFD-induced increases in adiposity, adipocyte enlargement and liver oxidative stress and inflammatory cytokine expression were suppressed by *n-3* LCPUFA to values comparable to those in control groups. Under the studied conditions, *n-3* LCPUFA did not modify the metabolic parameters assessed in mice subjected to normocaloric diet, except for the significant elevation in the hepatic nuclear abundance of PPAR-α and reduction in that of SREBP-1c. More important, it is demonstrated that *n-3* LCPUFA supplementation abolished HFD-induced enhancement in hepatic SREBP-1c/PPAR-α ratios, thus switching the metabolic status of the liver from a lipogenic condition to one favoring FA oxidation and steatosis attenuation. The findings reported may provide the rational basis for the use of normocaloric diets supplemented with *n-3* LCPUFA in patients with liver steatosis, FAs that additionally improved circulating liver function markers, serum TG and TNF-α levels, and hepatic microcirculatory function in patients with NASH (for specific references, see Ref. [15]). *n-3* LCPUFA effects on liver inflammation and fibrosis are being currently addressed by several clinical trials [50].

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