**SUMMARY:** The aim of this study was to investigate the roles of the Acyl co-enzyme A oxidase (ACOX), carnitine acyl transferase I (CAT-1) and activating protein 1 (AP-1) in the reversal of hepatic steatosis with dietary change and \( \text{n-3} \) long chain polyunsaturated fatty acid (\( \text{n-3} \) LCPUFA) supplementation. Male C57BL/6J mice were given either a control diet (CD) or a high fat diet (HFD) for 12 weeks, and then continued with the CD or CD plus \( \text{n-3} \) LCPUFA for eight weeks. After this period, body and adipose visceral tissue weight were analyzed and liver samples were taken to measure ACOX, CAT-1 and c-jun levels. The dietary change from HFD to a norm caloric diet plus \( \text{n-3} \) LCPUFA supplementation significantly reduced liver steatosis and adipose tissue: body weight ratio, along with an increase in the hepatic ACOX and CAT-1 levels and normalization of AP-1 expression that could favor the fatty acid beta-oxidation over lipogenesis and regulate inflammation. These results provide new data on the enzymatic metabolism underlying dietary change to a norm caloric diet plus \( \text{n-3} \) LCPUFA supplementation.

**KEYWORDS:** ACOX (Acyl coenzyme A oxidase); AP-1 (activating protein 1); CAT-1 (carnitine acyl transferase I); HFD (high fat diet); NAFLD (non-alcoholic fatty liver disease); \( \text{n-3} \) LCPUFA (\( \text{n-3} \) long chain polyunsaturated fatty acid); Reversion

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**RESUMEN:** El objetivo de este estudio fue investigar el rol de las enzimas Acil coenzima A oxidasa (ACOX) y Acil carnitina transferasa 1 (CAT-1), además del factor de transcripción, Proteína activadora 1 (AP-1) en la reversión de la esteatosis hepática mediante cambio de dieta más suplementación con Ácidos grasos poliinsaturados de cadena larga omega tres (AGPICL n-3). Ratones macho de la cepa C57BL/6J fueron alimentados con dieta control (DC) o alta en grasas (DAG) durante 12 semanas, luego continuaron con DC con o sin suplementación de AGPICL n-3 durante 8 semanas. Después de este periodo, se analizó el peso corporal y del tejido adiposo visceral; en las muestras hepáticas se evaluaron los niveles de ACOX, CAT-1 y AP-1. El cambio a dieta control más suplementación con AGPICL n-3 reduce significativamente la esteatosis hepática y la relación tejido adiposo/peso corporal, acompañado de un incremento en los niveles hepáticos de ACOX y CAT-1 y normalización de la expresión de AP-1; regulando la inflamación y favoreciendo la beta-oxidación sobre la lipogénesis. Estos resultados proveen nuevos datos sobre el metabolismo enzimático cuando se realiza cambio a dieta control más suplementación con AGPICL n-3.

**PALABRAS CLAVE:** ACOX (Acil coenzima A oxidasa); AGPICL n-3 (ácidos grasos poliinsaturados de cadena larga omega tres); AP-I (Proteína activadora 1); CAT-1 (Acil carnitina transferasa 1); DAG (dieta alta en grasa); Enfermedad del hígado graso no alcohólico (EHGNA); Esteatosis hepática; Reversión
1. INTRODUCTION

The non-alcoholic fatty liver disease (NAFLD) is considered to be the hepatic expression of the metabolic syndrome, defined as the clustering of risk factors for cardiovascular disease and type II diabetes, which include hyperglycemia, insulin resistance, hypertriglyceridemia and obesity (Bellentani et al., 2009; Malaguarnera et al., 2009). It is the most prominent cause of chronic liver disease and it is characterized by an intra-hepatic triacylglycerol (TG) content higher than 5% of the liver weight (steatosis) in the absence of significant alcohol consumption (10–20 g/day in women; 20–30 g/day in men) (Musso et al., 2009), which can progress to inflammation (steatohepatitis), fibrosis, and cirrhosis (Valenzuela et al., 2011). The pathogenic mechanisms involved in the development of hepatic steatosis are not completely understood, but it is known that it results from an imbalance between lipid availability, either from enhanced uptake and/or de novo lipogenesis, and lipid disposal, either from decreased mitochondrial and peroxisomal fatty acid oxidation and/or reduced lipid output by the liver (Musso et al., 2009). The establishment of liver steatosis leads to the production of free radicals with a lipid peroxidation response, pro-inflammatory cytokine release (Aronis et al., 2005), and n-3 long-chain polyunsaturated fatty acid (n-3 LCPUFA) depletion, with enhancement in the n-6/n-3 LCPUFA ratio favoring a pro-inflammatory state. This n-3 LCPUFA depletion induces changes in the DNA binding activity of the peroxisome proliferator-activated receptor Alfa (PPAR-α) (Valenzuela et al., 2011), with a decrease in PPAR-α phosphorylation thus eliciting low transcription levels of the target genes for carnitine acyl transferase 1 (CAT-1) and acyl coenzyme A oxidase (ACOX), favoring lipogenesis over fatty acid oxidation (Araya et al., 2004; Araya et al., 2010). CAT-1 is an enzyme that regulates the process of beta oxidation in mitochondria, while ACOX is responsible for fatty acid dehydrogenation to promote the beta oxidation process in the peroxisomes (Barlett et al., 2004; Poirier et al., 2006).

Secondly, it has been shown that in NAFLD the DNA binding of c-jun (AP-1 subunit) is increased, enhancing the production of pro-inflammatory cytokines, which promote an inflammation state and facilitate the development and progression of the disease (Dorn et al., 2014).

The transcriptional factor AP-1 is a protein complex of two subunits from families Jun (c-jun, junB and junD) and Fos (c-Fos, FosB, among others) forming homo-dimers or hetero-dimers between them (Halazonetti et al., 1988). AP-1 is capable of promoting cytokine expressions such as TNF-α (tumor necrosis Alfa factor), IL (interleukin)-1 and 2; which are involved in the recruitment and activation of Kupffer cells and the progression of NAFLD (Baffy, 2009).

Because of the increasing prevalence of NAFLD, numerous studies have focused on therapeutic strategies for this liver disease which have included weight loss, physical activity, and therapies based on insulin sensitizers, lipid-lowering agents, antioxidant drugs and n-3 LCPUFAs (Nobili et al., 2012). Previous studies by our group have demonstrated that n-3 LCPUFA supplementation prevents the pro-steatotic and pro-inflammatory effects of a high-fat diet (HFD) at the hepatic level (Valenzuela et al., 2012). Furthermore, the dietary change from HFD to a norm caloric diet with n-3 LCPUFA supplementation significantly reduced insulin resistance and liver steatosis when compared to switching HFD to a norm caloric diet alone (Dossi et al., 2014).

The aim of this study was to investigate the role of ACOX, CAT-1 and AP-1 in the reversal of hepatic steatosis with dietary change and n-3 LCPUFA supplementation. Parameters related to liver morphological characteristics (lipid vesicles), metabolic syndrome (visceral adipose tissue), liver total fat content, enzymes involved in lipid metabolism as ACOX and CAT-1 (western blot and PCR), and the levels of the AP-1 c-jun subunit (immunohistochemistry) were determined.

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 to 14 g were obtained from the Animal Facility at the Faculty of Medicine, University of Chile, Santiago,
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) the control diet containing (wt/wt) 10% fat, 20% protein, and 70% carbohydrate or ii) a high-fat diet (HFD) containing (wt/wt) 60% fat, 20% protein, and 20% carbohydrate (D12492, Research Diets, NJ, USA) from days 1 to 84 (12 weeks). 60% of the fat in the HFD was provided by the addition of lard (saturated fatty acids and cholesterol) in significantly higher amounts compared to the control diet. After 12 weeks, the animals given the control diet were divided into two diet groups (n=9): a) control diet, b) control diet plus n-3 LCPUFA; similarly, the animals subjected to the HFD were divided into two diet groups (n=9): c) change to control diet and d) change to control diet plus n-3 LCPUFA; all groups continued during 8 weeks to complete 20 weeks of total observation. The n-3 LCPUFA supplemented groups received fish oil (encapsulated fish oil containing 200 mg·kg^{-1} [108 mg·kg^{-1} of EPA and 92 mg·kg^{-1} of DHA]; Acolest) through oral administration; the control groups were given iso-volumetric amounts of saline. Weekly controls of body weight and diet intake were carried out throughout the entire period. At the end of the 20th week, the animals were fasted (6-8 h) and then given an anesthesia with tiletamine and zolacepam (Zoletil®, Virbac Laboratories). The visceral adipose tissue was extracted from the epididymal area, weighed and stored at −20 °C.

2.3. Tissue and blood samples

Liver samples were frozen in liquid nitrogen and stored at −80 °C, or fixed in phosphate-buffered formalin, embedded in paraffin, sectioned using a microtome and stained with haematoxylin-eosin. Blood samples were taken from a cardiac puncture, and then centrifuged, and the serum was stored at −80 °C, or fixed in phosphate-buffered 4 °C. The presence of both steatosis and inflammation were graded as absent, mild, moderate or severe (Brunt et al., 1999).

2.4. Western blot analysis of CAT-1 and AP-1

Liver samples (100–500 mg) frozen in liquid nitrogen were homogenized and suspended in a buffer solution pH 7.9 containing 10mM HEPES, 1mM EDTA, 0.6% Nonidet p-40, 150mM NaCl and protease inhibitors (1mM phenylmethylsulfonyl fluoride, 1 μg·mL^{-1} aprotinin, 1 μg·mL^{-1} leupeptin, and 1mM orthovanadate). Soluble protein fractions (10 mg) were separated on 12% polyacrylamide gel electrophoresis using SDS-PAGE, transferred to nitrocellulose membranes, and blocked with TBS containing 5% non-fat dry milk for 1 h at room temperature. The blots were washed with TBS-containing 0.1% Tween 20 and hybridized with rabbit polyclonal antibodies for human CAT-1 (Santa Cruz Biotechnology, CA, USA) and anti-c-Jun (Fitzgerald Industries International, Acton, MA, U.S.A.). Mouse monoclonal antibody for rat β-actin (ICN Biomedicals, Inc., Aurora, OH) was used as internal control in all determinations. After extensive washing, the antigen-antibody complexes were detected using labeled horseradish peroxidasa, and a Super Signal West Pico Chemiluminescence kit detection system (Pierce, Rockford, IL, USA).

2.5. RT-PCR assay of ACOX mRNA expression

The expression of ACOX was assessed by RT-PCR. Total RNA was isolated from 15–25 mg of frozen liver using an E.Z.N.A. total RNA Kit (Omega Biotek, Norcross, Georgia, 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 a Thermo Script 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 (Fwd: 5’-ACCTTCAGGGCCAAATGTAGTTG-3’ and Rv: 5’-GAGCCCCGTGTAGTGATGTTCC-3’ ) and the control 18S rRNA as internal control (Quantum RNA 18S Internal Standards, Ambion Inc., Austin TX, EE.UU). The amplification (TECHNE TC-5000, Bibby Scientific Ltd., United Kingdom) was initiated after 5 min de-naturation at 94 °C, followed by 34 cycles (94 °C for 30s, 62 °C for 30 s at 72 °C for 30 s) and finalizing with 72 °C for 5 min. The expected outputs for each amplification correspond to 249 pb for ACOX. 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 for densitometry using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT, USA).

2.6. Immunohistochemistry studies

For AP-1 staining a specific antibody was used against the c-jun subunit followed by deparaffinization and rehydration. The sections were then incubated with EDTA for 45 minutes at 95 °C. Then, blocking was carried out for 30 minutes followed
by incubation with primary antibody anti-(c-jun) (Fitzgerald Industries International, Acton, MA, U.S.A) according to the manufacturer's instruction. For nuclear contrast the Hematoxilin of Mayer was used (Modified solution according to Lillie, ScyTek Laboratories, Utah, USA). An analysis of positive nuclei was performed under light microscope in a blind fashion in 10 adjacent (400×) per slide. Then a percentage of positive nuclei per slide was calculated.

2.7. Statistical analyses

Statistical analysis was performed with GraphPad Prism™ version 5.0 (GraphPad Software, Inc. San Diego, CA, USA). Values shown represent the mean ± SEM for the number of separate experiments indicated. One-way ANOVA and the Newman-Keuls test for unpaired data assessed the statistical significance of differences between mean values as indicated, considering P<0.05 as significant.

3. RESULTS

3.1. Reduction in adipose tissue weight and corporal weight ratio, liver steatosis and morphological alterations induced by a HFD with change to the control diet with n-3 LCPUFA supplementation

The weights of the animals subjected to the control diet or HFD with and without n-3 LCPUFA supplementation were not significantly different. However, the visceral adipose tissue/body weight ratio was enhanced (P<0.05) in the mice subjected to HFD+CD compared with CD+CD, CD+CD/n-3 LCPUFA, and HFD+CD/n-3 LCPUFA (Fig. 1A). In all groups, liver histology was characterized by the absence of architectural distortion, lobular inflammation, necrotic foci, or fibrosis (Figure 1B), and those given CD with and without n-3 LCPUFA did not show lipid infiltration [Fig. 1B, (a) and (b)]. However, HFD+CD exhibited macro and micro-vesicular steatosis (30% lipid vesicles infiltration) (Fig. 1B (c)), whereas HFD+CD/n-3 LCPUFA elicited 8% fat infiltration (Fig. 1B (d); Fig. 1C; P<0.05).

3.2. Change to control diet with n-3 LCPUFA supplementation reduces the liver expression of enzymes involved in fatty oxidation

Measurement of the enzymes involved in fatty liver oxidation CAT-1 and ACOX (Fig. 2) showed that mice subjected to the change from HFD to CD had lower expressions in their mRNA (ACOX, Fig. 2A) and protein levels (CAT-1) (Fig. 2B) with respect to the CD group (P<0.05), whereas the change from HFD to CD/n-3 LCPUFA increased the expression of these enzymes to control values (Fig. 2A y B).

3.3. Change to control diet with n-3 LCPUFA supplementation normalizes the AP-1 levels

The presence of liver AP-1 in nuclear fractions, assessed by immunohistochemistry, (Fig. 3A), revealed that the mice subjected to CD+CD and CD+CD/n-3 LCPUFA exhibited a percentage of 36.53% and 33.13% of c-jun positive nuclei, respectively; whereas animals given HFD+CD showed 63.30% (P<0.05). The HFD+CD/n-3 LCPUFA group shows a diminished c-jun liver positive nuclei (P<0.05) with respect to HFD+CD group, with a 44.30% of c-jun liver positive nuclei (Fig. 3B). However, when analyzed by western blot, there were no significant differences at the c-jun protein level among all the groups (Fig. 3C).

4. DISCUSSION

NAFLD is a condition that requires multiple factors for development (Adams et al., 2005), which are induced by excessive carbohydrate or lipid dietary ingestion (Leamy et al., 2013). Once this condition is acquired, it is important to slow its progress to steatohepatitis; later the condition is essentially irreversible (Dowman et al., 2010).

Mice with the HFD intake developed glucose intolerance and insulin resistance, with higher visceral adiposity and hepatic steatosis when compared with animals subjected to a norm caloric diet (Dossi et al., 2014). Previously published data show that a norm caloric diet plus n-3 LCPUFA supplementation reverses the HFD-induced prosteatotic and pro-inflammatory state in mice livers. This reversal was evidenced by a reduction in insulin resistance, suppression of liver oxidative stress and inflammatory cytokine expression (IL-1β and TNF-α). These findings were 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. This situation favors catabolism over anabolism and that effect could be mediated by SREBP-1c and PPAR-α (Dossi et al., 2014). This view is supported by the significant correlation between the reduction observed in the hepatic levels of the PPAR-α-regulate enzymes ACOX and CAT-1 (Figure 2A y 2B). From a mechanistic point of view, down-regulation of liver enzymes ACOX and CAT-1 may be ascribed to hepatic n-3 LCPUFA depletion (Araya et al., 2004; Araya et al., 2010; Valenzuela et al., 2012) observed in experimental and human obesity. Different PPAR-α agonist factors are able to lower the number of available triglycerides through different pathways (Araya et al., 2004; Surwit et al., 1988). In this respect, here we observed a significant increase in both mRNA ACOX levels and CAT-1
FIGURE 1. Effect of n-3 long-chain polyunsaturated fatty acid (n-3 LCPUFA) and dietary change on (A) relation between weight of visceral adipose tissue (g) and corporal weight (g), (B) liver histology (magnification × 100) and (C) hepatocyte lipid infiltration in mice. Animals were given either (a) control diet (CD), (b) CD plus n-3 LCPUFA (CD+n-3 LCPUFA), (c) high fat diet (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±SEM for 9 animals per experimental group. Letters above the bars indicate statistically significant differences (P<0.05) compared to the respective animals given the control diet assessed by one-way ANOVA and the Newman-Keuls.
protein levels in those animals that underwent the norm caloric diet change and supplementation with n-3 LCPUFA.

The effects of n-3 LCPUFA supplementation prevented an HFD-induced increase in liver lipid content (Nobili et al., 2012; Valenzuela et al., 2012). The increase in visceral adipose tissue content induced by the HFD produces an increment in the pro-inflammatory cytokine (adipokines) secretion such as IL-6, IL-1β and TNF-α (Fernández-Sánchez et al., 2011). In this respect, the significant increase in the visceral adipose tissue/corporal body weight ratio and hepatic lipid infiltration in the HFD+CD group was normalized in the HFD+CD/n-3 LCPUFA group, meaning that n-3 LCPUFAs are involved in lipid content changes and thus could be also involved in the cytokine expression decrease. The decrease in the cytokine expression could be explained by the reduction in the nuclear c-jun content. Previous studies support the participation of c-jun (as a subunit of AP-1) in developing NAFLD, supporting the results found in this study (Dorn et al., 2014). The increase in AP-1 expression could be in part responsible for the pro-inflammatory cytokine (IL-1β and TNF-α) expression observed in HFD (Dossi et al., 2014). It has been observed that increasing c-jun, and therefore AP-1, leads to an increased transcription of the c-jun, in an auto-regulatory cycle, indicating that the c-jun increase in NAFLD is a consistent indicator of the AP-1 dimer increase, and it could participate in increasing the transcription of pro-inflammatory cytokines, which are elevated in the development of this pathology (Hasenfuss et al., 2014). In another aspect, the action of AP-1 factor has been linked with the sensor lipid PPAR, postulating an inhibitory role to the AP-1 factor, acting in the PPAR-DNA binding (Ye et al., 2002); thus, performing a protective role. In mice with HFD-induced hepatic steatosis an upregulation of PPAR-γ levels was evident (Shapiro et al., 2011) and is regulated differently depending on the AP-1 dimer formed: c-jun joined Fra-1 and Fra-2 (members of the Fos family) inhibit the expression of PPAR-γ resulting in a liver steatotic reduction, but the dimer formed by c-jun and c-Fos has the opposite effect (Fernández-Sanchez et al., 2011).

To increase the reliability of the results obtained in this work, we analyzed the subunit c-jun levels using two techniques based on the antigen-antibody union: immunohistochemistry and western blotting: both techniques aim to determine the relative levels of the protein. The immunohistochemical analysis allowed for determination of the AP-1 levels through the measurement of the subunit c-jun (Halazonetis et al., 1988; Hong et al., 2003). The analysis showed that dietary change associated with n-3 LCPUFA supplementation is effective in reducing AP-1 levels, although it does not show significant differences in the measurement by western blot. Regarding the differences obtained with both techniques, it is important to mention that tissue processing is a relevant factor in the discrepancy found in the results of each technique. Immunohistochemistry is set to maintain...
the morphology of the living body, which involves maintaining the three dimensional structure of protein; while in the western blotting, the tissue is homogenized and then treated with de-naturating agents such as SDS (sodium dodecyl sulfate) and β-mercaptoethanol, causing a loss of the three-dimensionality of proteins, which could directly affect the antigen-antibody binding agents. The loss of the tertiary structure of c-jun may be the reason for the differences in the results between the two techniques as antibody binding can occur in the different structures that make up a protein (primary, secondary, tertiary or quaternary).

Considering these results together, we conclude that n-3 LCPUFA supplementation associated with diet change reverses the HFD-induced hepatic steatosis, along with a recovery of ACOX and CAT-1 levels and normalization of the AP-1 nuclear subunit c-jun levels.

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