RESEARCH ARTICLE

# *N-3* long-chain PUFA supplementation prevents high fat diet induced mouse liver steatosis and inflammation in relation to PPAR-α upregulation and NF-κB DNA binding abrogation

*Gladys Tapia[1], Rodrigo Valenzuela[1], Alejandra Espinosa[2], Pamela Romanque[3,4], Camila Dossi[1], Daniel Gonzalez-Mañán[1], Luis A. Videla[1] and Amanda D'Espessailles[1]*

[1] Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile
[2] Department of Medical Technology, Faculty of Medicine, University of Chile, Santiago, Chile
[3] Department of Physiopathology Program, Faculty of Medicine, University of Chile, Santiago, Chile
[4] Faculty of Medicine, Diego Portales University, Santiago, Chile

Received: June 25, 2013
Revised: November 25, 2013
Accepted: December 3, 2013

**Scope:** Dietary *n-3* long-chain PUFAs *(n-3* LCPUFAs) supplementation was studied in an HFD-induced (HFD is high-fat diet) steatosis and inflammation in relation to peroxisome proliferator-activated receptor alpha (PPAR-α) and nuclear factor κB (NF-κB) signaling.
**Methods and results:** Male C57BL/6J mice received (i) control diet (10% fat, 20% protein, 70% carbohydrate), (ii) control diet plus *n-3* LCPUFAs (daily doses of 108 mg/kg body weight of eicosapentaenoic acid plus 92 mg/kg body weight of docosahexaenoic acid), (iii) HFD (60% fat, 20% protein, 20% carbohydrate), or (iv) HFD plus *n-3* LCPUFAs for 12 wk. PPAR-α, tumor necrosis factor alpha (TNF-α), and IL-1β mRNA expression, acyl-CoA oxidase 1 (ACOX1), and carnitine-acyl-CoA transferase 1 (CAT-I) protein contents, and NF-κB DNA binding activity were measured. HFD significantly decreased liver PPAR-α, ACOX1, and CAT-I levels with NF-κB activation, higher TNF-α and IL-1β expression, and steatosis development. These changes were either reduced or normalized to control values in animals subjected to HFD plus *n-3* LCPUFAs, with establishment of an inverse association between NF-κB activation and PPAR-α mRNA expression ($r = -0.66$, $p < 0.0001$).
**Conclusion:** Data presented indicate that *n-3* LCPUFAs supplementation prevents liver steatosis and inflammation induced by HFD, with underlying mechanisms involving enhanced PPAR-α signaling and diminished NF-κB activation.

**Keywords:**
High-fat diet / Liver steatosis / *n-3* long-chain PUFA / Nuclear factor κB / Peroxisome proliferator-activated receptor alpha

Additional supporting information may be found in the online version of this article at the publisher's web-site

## 1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is a clinical–pathological condition that encompasses a wide spectrum

**Correspondence:** Amanda D'Espessailles, Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Independencia 1027, Independencia, Santiago, Chile
**E-mail:** adespessailles@ug.uchile.cl
**Fax:** +56-2-7372783

**Abbreviations: ACOX1**, acyl-CoA oxidase 1; **CAT-I**, carnitine-acyl-CoA transferase 1; **DHA**, docosahexaenoic acid; **EPA**, eicosapentaenoic acid; **FA**, fatty acid; **HFD**, high-fat diet; ***n*-3 LCPUFA**, *n*-3 long-chain PUFAs; **NAFLD**, nonalcoholic fatty liver disease; **NASH**, nonalcoholic steatohepatitis; **NF-κB**, nuclear factor κB; **PPAR-α**, peroxisome proliferator-activated receptor alpha; **SREBP1c**, sterol regulatory element-binding protein 1c; **TNF-α**, tumor necrosis factor alpha

 

of pathologies characterized by abnormally high accumulation of fat in hepatocytes without any other disease related to liver steatosis, ranging from mild asymptomatic liver steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis [1]. NAFLD is considered the hepatic expression of the metabolic syndrome, usually defined as the clustering of risk factors for cardiovascular disease and type II diabetes, which include hyperglycemia, insulin resistance, hypertriglyceridemia, obesity, and other metabolic alterations [2, 3]. 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 blood uptake and/or de novo lipogenesis, and lipid disposal, either from decreased mitochondrial and peroxisomal fatty acid (FA) β-oxidation and/or reduced lipid output by the liver [4]. The establishment of liver steatosis leads to the production of free radicals with a lipid peroxidation response and pro-inflammatory cytokine release [5], which may trigger progression to the more severe state of NASH [6].

One of the characteristics of NAFLD is the depletion of liver *n-6* and *n-3* long-chain PUFAs (*n*-3 LCPUFAs) [7, 8], which is particularly important since LCPUFAs constitute crucial components for membrane functions. For example, establishing adequate membrane fluidity and regulatory functions by acting as second messengers in signal transduction processes, with a minor contribution to cellular energy expenditure [9]. Alterations in liver LCPUFA status in NAFLD include significant depletion of *n-3* LCPUFA content and enhancement in the *n-6*/*n-3* LCPUFA ratio [7,8]. These changes also occur in cardiovascular disease [10], obesity [11], and diabetes type II [12], in association with the inflammatory response component of these pathologies [13].

Eicosapentaenoic acid (C20:5 *n-3*, EPA) and docosahexaenoic acid (C22:6 *n-3*, DHA) are the most important *n-3* LCPUFAs due to their critical roles in different physiological functions, leading to positive health effects [14, 15], that support their use for the prevention of nontransmissible chronic diseases [16]. Relevant functions of EPA and DHA include the regulation of hepatic lipid metabolism, which is accomplished through downregulation of the expression and processing of transcription factor sterol regulatory element-binding protein 1c (SREBP1c) leading to depressed lipogenic and glycogenic capacity, and upregulation of peroxisome proliferator-activated receptor alpha (PPAR-α), which favors FA oxidation [15,17,18]. In addition to being considered as PPAR-α agonists, EPA and DHA are effective inhibitors of nuclear factor-κB (NF-κB) DNA binding activity, which limits inflammatory gene responses [19].

The aim of this study was to test the hypothesis that dietary *n-3* LCPUFA supplementation decreases the prosteatotic and proinflammatory effect of a high-fat diet (HFD) at hepatic level by upregulation of PPAR-α and abrogation of NF-κB DNA binding, with concomitant prevention of HFD-induced liver steatosis in mice. PPAR-α-controlled genes for acyl-CoA oxidase 1 (ACOX1) and carnitine-acyl-CoA transferase-1 (CAT-I) were measured by Western blot, whereas those for tumor necrosis factor alpha (TNF-α) and IL-1β regulated by NF-κB were assessed by RT-PCR.

## 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 ($n = 36$) weighing $13.3 \pm 0.2$ g (Bioterio Central, ICBM, Faculty of Medicine, University of Chile) were randomly assigned to each experimental group and allowed free access to specially formulated control diets or HFDs. The composition of the control diet (expressed as percentage total calories) was 10% fat, 20% protein, and 70% carbohydrate, with a caloric value of 3.85 kcal/g, free of EPA and DHA, and contained 0.7 g of α-linolenic acid per 100 g of diet. The composition of the HFD was 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 α-linolenic acid per 100 g of diet (Research Diet INC, Rodent Diet, Product data D12450B and D12492, USA). The ingredients and FAs composition of each diet are shown in detail in Supporting Information Tables 1 and 2. Animals received water ad libitum and were housed on a 12-h light/dark cycle. From days 1 to 84 (12 wk), the *n-3* LCPUFA supplemented groups received encapsulated fish oil containing 379 mg [EPA + DHA]/g (Acolest TG Product. Procaps, Colombia) through oral administration and the control groups isovolumetric amounts of saline. FA profile for the encapsulated fish oil is provided in Supporting Information Table 3. There were four experimental groups: (i) control diet, (ii) control diet plus *n-3* LCPUFAs, (iii) HFD, and (iv) HFD plus *n-3* LCPUFAs. Under these conditions, the *n-3* LCPUFA groups received daily doses of 108 mg/kg body weight of EPA and 92 mg/kg body weight of DHA. Weekly controls of body weight and diet intake were performed through the whole period. At the end of the 12th week, animals were fasted (6–8 h) and then anesthetized with ketamine and xylazine (150 and 10 mg/kg, respectively). 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 hematoxylin-eosin. For morphology, assessment sections were assessed by optical microscopy in a blind fashion and the presence of steatosis and inflammation were both graded as absent, mild, moderated, or severe [20]; the hepatic lipid accumulation was also evaluated as percentage of cells

 

with lipid vesicles infiltration per field counted in a blinded fashion in ten fields at higher magnification (400×).

### 2.3 Assessment of NF-κB p65 DNA binding activity

Nuclear extracts from hepatic tissue (left lobe) were obtained using a commercial extraction kit (Cayman Chemical Company, Item 10011223, Ann Arbor, MI, USA). NF-κB binding activity was assessed with a commercial ELISA kit (Cayman Chemical Company, Item 10009277), according to the manufacturer's instructions. Values were expressed as percentage of NF-kB p65 binding to DNA, with respect to a positive control provided in the kit.

### 2.4 RT-PCR assay of PPAR-α, TNF-α, and IL-1β mRNA expression

The expression of PPAR-α and cytokines regulated by NF-κB (TNF-α and IL-1β) were assessed by RT-PCR. Total RNA was isolated from 15 to 25 mg of frozen liver (left lobe tissue) 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, 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 PlatinumH Taq (Invitrogen), according to the manufacturer's instructions. Nucleotide sequences for sense and antisense primers used in this study were 5′- GAG ATT TCT CAG TCC ATC GG -3′ and 5′- CCG AAT CTT TCA GGT CGT GT -3′ for PPAR-α; 5′- TGC CTA TGT CTC AGC CTC TT -′3 and 5′- GTA TAT GGG CTC ATA CCA GG -′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 (Classic II QuantumARN 18S InteARNl Standards, Ambion, Austin TX, USA). The amplification (Biometra H thermocycler T, Goettingen, Germany) was initiated after 5-min denaturation at 94°C, followed by 40 cycles (94°C for 4 min, 37°C for 30 s, 57°C for 30 s, 72°C for 1 min, and 72°C for 10 min) for TNF-α and PPAR-α and 44 cycles (94°C for 4 min, 37°C for 30 s, 55°C for 30 s, 72°C for 1 min, and 72°C for 10 min) for IL-1β, and 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, Orem, UT, USA).

### 2.5 Western blot analysis of ACOX1 and CAT-I

Left liver lobe samples (100–500 mg) frozen in liquid nitrogen were homogenized in buffer solution (pH 7.9) containing 10 mM HEPES, 1 mM EDTA, 0.6% Nonidet P-40, 150 mM NaCl, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM orthovanadate). Soluble protein fractions (10 μg) were separated on 12% polyacrylamide gels by SDS-PAGE [21] and transferred to nitrocellulose membranes [22], which were blocked for 1 h at room temperature with TBS containing 5% nonfat dry milk. The blots were washed with TBS containing 0.1% Tween 20 and incubated with rabbit polyclonal antibodies for mouse ACOX1 (1:500) or CAT-I (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). In all determinations, a mouse monoclonal antibody for β-actin (ICN Biomedicals, Aurora, OH, USA) was used as internal control. After extensive washing, the antigen–antibody complexes were detected using horseradish peroxidase-labeled goat anti-rabbit IgG (1:5000) or goat anti-mouse IgG (1:5000) and a Super-Signal West Pico Chemiluminescence kit detection system (Pierce, Rockford, IL, USA). Densitometric analysis of the bands was performed using UN-SCAN-IT software (Silk Scientific, Orem, UT, USA).

### 2.6 Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Values shown represent the mean ± SEM for the number of separate experiments indicated. Two-way ANOVA and Bonferroni's posthoc test assessed the statistical significance of differences between mean values of columns, with a $p < 0.05$ being considered significant. The Pearson's coefficient was used to assess associations between variables.

## 3 Results

### 3.1 *N-3* LCPUFA supplementation reduces HFD-induced body weight gain, liver weight gain, visceral fat accumulation, liver steatosis, and morphological alterations

Following 12 wk of treatment, body weight in HFD mice (44.7 ± 0.8 g ($n = 9$)) was significantly higher ($p < 0.05$) than in animals subjected to control diet (35.1 ± 0.3 g ($n = 9$); 27%), control diet plus *n-3* LCPUFAs (30.0 ± 0.7 g ($n = 9$); 49%), and HFD plus *n-3* LCPUFAs (33.3 ± 1.2 g ($n = 9$); 34%). Under these conditions, liver weight in HFD animals (1.88 ± 0.05 g ($n = 9$)) was significantly higher ($p < 0.05$) than in mice that received the control diet (1.30 ± 0.05 g ($n = 9$); 45%), control diet plus *n-3* LCPUFA (1.25 ± 0.03 g ($n = 9$) 50%), and HFD plus *n-3* LCPUFAs (1.49 ± 0.05 g ($n = 9$); 26%). In addition, visceral fat/body weight ratios in HFD-treated animals were enhanced by 40% compared with animals that received control diet. This change was suppressed in mice given the HFD and *n-3* LCPUFA, in the absence of significant changes elicited by *n-3* LCPUFA over controls (visceral fat/body weight ratio (%): (i) control diet, 5.4 ± 0.6 ($n = 9$); (ii) control diet

 

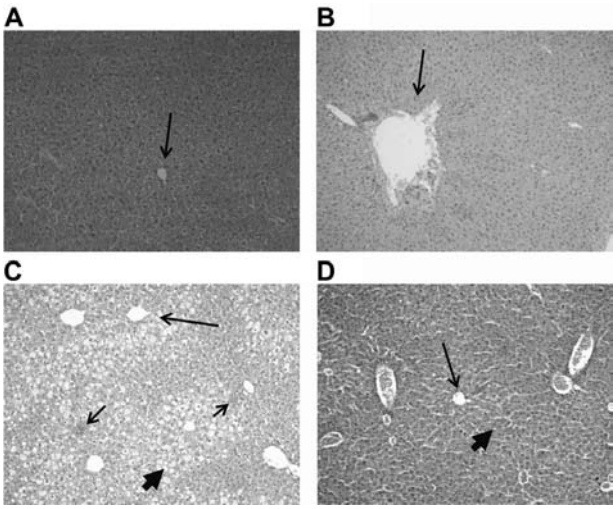


**Figure 1.** Effect of *n*-3 LCPUFA supplementation on liver histology in HFD-fed mice. Representative liver sections from mice subjected to (A) control diet, (B) control diet plus *n-3* LCPUFA, (C) HFD, and (D) HFD plus *n-3* LCPUFA. Hematoxylin–eosin liver sections from a total of nine animals per experimental group. Thin arrows indicate central vein branches; short thick arrows indicate lipid vesicles and short arrows indicate lobular inflammation foci. Original magnification: 10×.

plus *n-3* LCPUFAs, 4.4 ± 0.3 ($n = 9$); (iii) HFD, 7.5 ± 0.9 ($n = 9$); (iv) HFD plus *n-3* LCPUFA, 4.7 ± 0.4 ($n = 9$); (iii) versus (i), (ii), and (iv), $p < 0.05$). The above changes were induced in the absence of significant alterations in the food intake of the animals (control diet, 5.1 ± 0.2 g/day ($n = 9$); control diet plus *n-3* LCPUFA, 4.8 ± 0.2 g/day ($n = 9$); HFD, 4.7 ± 0.4 g/day ($n = 9$); HFD plus *n-3* LCPUFA, 4.4 ± 0.5 g/day ($n = 9$); $p > 0.05$). Mice subjected to control diet (Fig. 1A) and control diet plus *n-3* LCPUFA (Fig. 1B) exhibited normal liver histology, whereas those administered the HFD showed macro and microvesicular steatosis (62 ± 1.0% cells with lipid vesicles per field), arquitectural distortion with moderate lobular inflammation, and necrosis foci (Fig. 1C). *N-3* LCPUFA supplementation significantly decreased hepatic steatosis (8 ± 1.7% cells with lipid vesicles per field), with persistence of few steatotic foci, absence of inflammation, and modest arquitectural distortion (Fig. 1D).

### 3.2 *N-3* LCPUFA supplementation recovers HFD-induced diminution in liver PPAR-α mRNA contents and associated enzyme expression levels to control values

Mice subjected to control diet plus *n-3* LCPUFA showed significantly higher liver PPAR-α mRNA expression levels compared to control diet without supplementation (108%; Fig. 2A), concomitantly with enhancement in the protein content of ACOX1 (119%; Fig. 2B) and CAT-I (57%; Fig. 2C) regulated by PPAR-α. HFD significantly reduced hepatic PPAR-α mRNA (Fig. 2A), ACOX1 (Fig. 2B), and CAT-I (Fig. 2C) protein levels by 64, 82, and 55%, respectively, compared to control values, which were normalized in animals fed the HFD plus *n-3* LCPUFA (Fig. 2). Under these conditions, liver PPAR-α mRNA expression levels were significantly correlated with the protein levels of either ACOX1 ($r = 0.67$, $p < 0.0002$) or CAT-I ($r = 0.60$, $p < 0.001$)).

### 3.3 *N-3* LCPUFA supplementation decreases HFD-induced enhancement in liver NF-κB DNA binding and related proinflammatory cytokine expression levels to control values

Hepatic NF-κB DNA binding was significantly higher in the HFD group compared to the control diet (15-fold increase) and control diet plus *n-3* LCPUFA groups (19-fold enhancement), an effect that was suppressed in mice given HFD plus *n-3* LCPUFA (Fig. 2D). Also, mRNA expression levels of cytokine genes regulated by NF-κB such as TNF-α (Fig. 2E) and IL-1β (Fig. 2F) showed a similar pattern to NF-κB DNA binding activity, whereas the HFD group showed a high expression of both proinflammatory cytokines, which returned to control values upon *n-3* LCPUFA supplementation. Under these conditions, values for liver NF-κB DNA binding were significantly correlated with the mRNA levels for either TNF-α ($r = 0.62$, $p < 0.002$) or IL-1β ($r = 0.76$, $p < 0.0001$), whereas an inverse association was established between NF-κB activation and PPAR-α mRNA expression ($r = -0.66$, $p < 0.0001$; Fig. 2G). Furthermore, NF-κB/PPAR-α ratios in HFD-treated mice were 42-fold and 116-fold ($p < 0.05$) higher than those in animals given control diet or control diet plus *n-3* LCPUFA, respectively, an effect that was diminished by 85% due to the combined HFD plus *n*-3 LCPUFA protocol compared to animals fed the HFD (Fig. 2H).

## 4 Discussion

Obesity in experimental animals and humans underlies a complex array of metabolic alterations in the setting of oxidative stress and insulin resistance development, which affect several organs including the liver [6, 23]. Under these conditions, derangement of cell biochemistry and function is known to involve misregulation of key components responsible for cell signaling, such as membrane receptors, kinases, phosphatases, and transcription factors [24, 25]. In agreement with these views, data presented in this study indicate that HFD-induced obesity in mice occurs concomitantly with major changes in transcription factors, namely, decreased liver mRNA expression of the metabolic factor PPAR-α and activation of the proinflammatory factor NF-κB, which are associated with the respective steatotic and inflammatory responses found in obesity. This view is supported by the significant correlation between the (i) reduction in the levels of hepatic ACOX1 and CAT-I regulated by PPAR-α that may lead to a

 

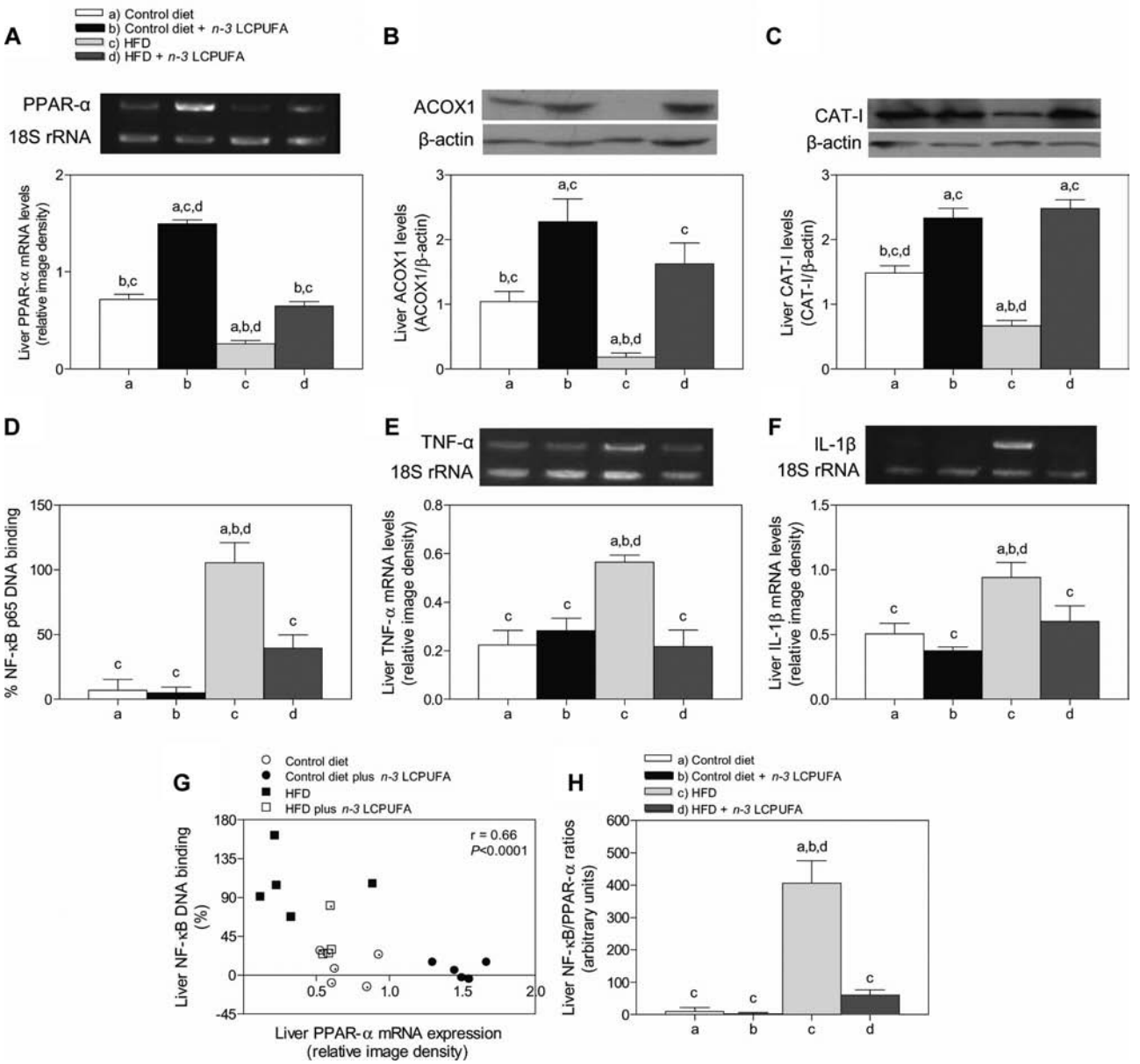


**Figure 2.** Effect of *n-3* LCPUFA supplementation on expression of liver PPAR-α mRNA and NF-κB DNA binding activity, and associated enzymes and cytokines in HFD-fed mice. (A) PPAR-α (RT-PCR); (B) ACOX1; (C) CAT-I (Western blot); (D) NF-κB DNA binding assay; (E) TNF-α; (F) IL-1β expression evaluated by RT-PCR; (G) correlation between liver NF-κB DNA binding and PPAR-α mRNA expression; and (H) NF-κB/PPAR-α ratios in mice given (a) control diet, (b) control diet plus *n-3* LCPUFA, (c) HFD, and (d) HFD plus *n-3* LCPUFA. Values are expressed as mean ± SEM for five to six animals per experimental group. Significance studies ($p < 0.05$) are indicated by the letters identifying each experimental group.

lower FA oxidation status and (ii) enhancement in the mRNA expression of TNF-α and IL-1β controlled by NF-κB. In the latter case, enhanced TNF-α and IL-1β mRNA expression in the liver induced by HFD agrees with previous reports of elevated serum cytokines [26].

From the mechanistic point of view, downregulation of liver PPAR-α signaling may be ascribed to at least three major factors, namely, repression of liver PPAR-α transcription (Fig. 2A) [27], hypoadiponectinemia [28–30], and hepatic *n-3* LCPUFA depletion [7, 8, 26], observed in experimental and human obesity. Although the mechanisms involved in the HFD-induced reduction in PPAR-α mRNA expression require further studies, low serum levels of adiponectin may explain the reduced binding of the adipokine to hepatic receptors AdipoR1 and/or AdipoR2 [31], leading to diminished activation of AMP-activated protein kinase and p38

 

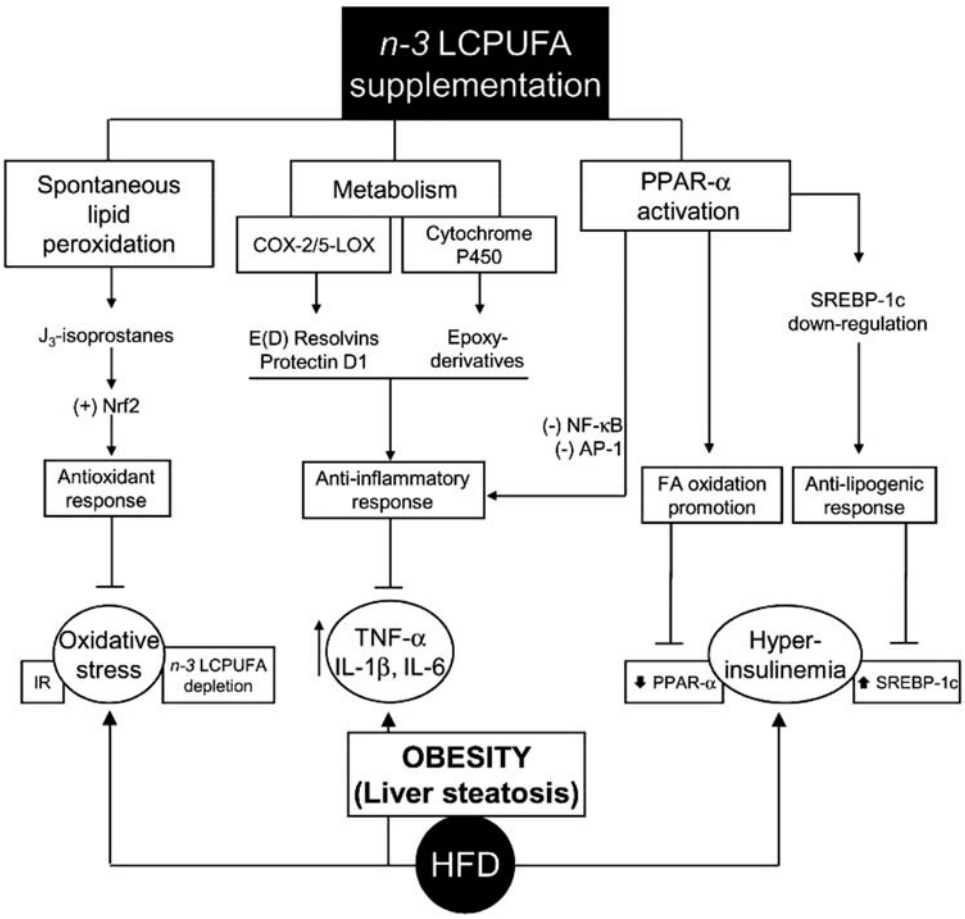


**Figure 3.** Molecular mechanisms involved in the antisteatotic actions of *n-3* LCPUFA supplementation. COX-2, cyclooxygenase 2; 5-LOX, 5-lipoxygenase; Nrf2, nuclear factor erythroid 2 related factor 2.

mitogen-activated kinase [32, 33], with depressed PPAR-α phosphorylation, thus eliciting low transcription levels of the target genes for ACOX1 and CAT-I (Fig. 2B and C). Furthermore, target gene transcriptional activation by PPAR-α would be also compromised by the substantial depletion of hepatic *n-3* LCPUFA reported [7, 8, 26, 30], as loss of these FAs and their oxidized derivatives can promote failure in the expression of genes encoding for proteins involved in FA oxidation at mitochondrial, peroxisomal, and microsomal levels, as well as lipoprotein assembly and transport [15, 17, 18, 34]. In relation to HFD-induced liver NF-κB DNA binding, activation is associated with redox signaling, a process that involves generation of ROS eliciting modifications in components of the signaling cascade [24, 25], with the consequent upregulation of the expression and release of TNF-α and IL-1β at the Kupffer cell level [6]. The HFD-induced liver NF-κB activation reported here is in agreement with data in obese NASH patients [35, 36], who also exhibited activation of the alternate proinflammatory factor activating protein 1 (AP-1) [36]. NF-κB activation by ROS occurs through stimulation of the canonical inhibitor of κB (IκB) kinase (IKK) complex pathway involving NF-κB essential modulator and kinases IKK-α and IKK-β, which can be reinforced by TNF-α and IL-1β binding to their respective receptors TNF-R1 and IL-1R1 [37]. Interestingly, mouseliver NF-κB and PPAR-α signaling exhibited a significant inverse correlation among the groups studied, with NF-κB/PPAR-α ratios being enhanced by 42-fold by HFD over control values, a parameter proposed to represent a proinflammatory indicator that is also increased in obese patients [38]. This contention is supported by the normal formation of transcriptionally inactive NF-κB p65-PPAR-α complexes [19] that is promoted in the liver by *n-3* LCPUFA supplementation [39], which otherwise may contribute to inflammatory responses in the case of HFD administration.

Dietary *n-3* LCPUFA supplementation in mice subjected to HFD for 12 wk significantly reduced HFD-induced visceral fat accumulation and macro- and microvesicular hepatic steatosis, in agreement with studies in *ob/ob* mice [40]. The antisteatotic effect of *n-3* LCPUFA could be ascribed to different molecular mechanisms. These include diminution in FAs and glycerol mobilization from peripheral tissue lipolysis to the liver that is associated with HFD-induced insulin resistance, as *n-3* LCPUFA supplementation was shown to normalize HOMA values in mice [26]. In addition, *n-3* LCPUFAs enhance the antioxidant potential of the liver, acting through direct (ROS scavenging) and/or indirect (nuclear transcription factor erythroid 2 related factor 2 (Nrf2) activation) mechanisms, a condition proposed to improve insulin

 

sensitivity [23, 26, 41]. Enhancement in liver FA oxidation may represent an alternate antisteatotic mechanism [15] afforded by *n-3* LCPUFA supplementation, considering that EPA and DHA are intracellular signals regulating lipid metabolism in the liver [17, 18]. In fact, *n-3* LCPUFA may favor FA oxidation through PPAR-α signaling associated with (i) the substantial increase in PPAR-α mRNA expression achieved over basal values, (ii) upregulation of adiponectin expression and release [42] promoting PPAR-α phosphorylation and target gene transcription [32, 33], and (iii) higher liver *n-3* LCPUFA availability [26] activating PPAR-α, which would allow recovery of PPAR-α signaling to control levels after reduction due to HFD (Fig. 3). Consequently, *n-3* LCPUFA triggered the recovery of mouse liver ACOX1 levels and the upregulation of those of CAT-I over control values, key enzymes of hepatic FA oxidation. In agreement with this view, creatine supplementation prevents liver steatosis in rats fed a high-fat liquid diet through a mechanism involving upregulation of the mRNA expression of PPAR-α, CAT-I, and long-chain acylCoA dehydrogenase (LCAD) [43]. Diminution in hepatic de novo lipogenesis may also contribute to *n-3* LCPUFA-induced antisteatotic effects in the liver, through downregulation of transcription factor SREBP-1c expression and activation, leading to low levels of FA synthase (Fig. 3) [23, 44].

An important finding previously reported by our group is that EPA and DHA hepatoprotection against HFD is accompanied by a substantial consumption of these FAs, along with a significant diminution in the *n-6*/*n-3* LCPUFA ratio with respect to HFD without supplementation [26], features that may have a direct impact on the attainment of anti-inflammatory responses. In fact, *n-3* LCPUFAs are known to be metabolized by several routes, generating *n-3* LCPUFA-derived mediators targeting inflammation (Fig. 3). These include (i) epoxy derivatives generated by cytochrome P450 NADPH-dependent epoxygenases [45, 46], (ii) E-series and D-series resolvins formed by the action of the cycooxygenase-2 (COX-2)/5-lipoxygenase (5-LOX) pathway or protectin D1 by 5-LOX [47], and (iii) $J_3$-isoprostanes from EPA and DHA spontaneous lipid peroxidation [48], which activate Nrf2 and antioxidant gene expression that abrogate NF-κB redox activation and TNF-α/IL-1β expression (Fig. 3) [49]. In addition to these mechanisms, *n-3* LCPUFA-activated PPAR-α may negatively crosstalk with NF-κB and AP-1 [19, 49], contributing to the anti-inflammatory responses observed. Alternate mechanisms triggered by PPAR-α activation include upregulation of IκB-α mRNA and protein expression [50] and/or reduced IκB-α degradation [51], thus diminishing NF-κB DNA binding. In agreement with these views, augmentation in hepatic NF-κB/PPAR-α ratio, as an indicator of the inflammatory status achieved by HFD, was suppressed by *n-3* LCPUFA supplementation, which also abrogated liver TNF-α and IL-1β mRNA upregulation and enhancement in the serum levels of both cytokines [26].

In conclusion, *n-3* LCPUFA supplementation prevents HFD-induced liver steatosis and inflammation by enhancing PPAR-α signaling and suppressing that of NF-κB, parameters that exhibit a significant inverse correlation and negative crosstalk. In agreement with the proposed antisteatotic mechanisms outlined in Fig. 3, clinical studies have reported that *n-3* LCPUFA supplementation reduces hepatic lipid content in adult [52–55] and pediatric NAFLD patients [56], while the effects on liver inflammation and fibrosis require further assessment [57].

*This work was supported by grant 1110043 from FONDECYT (National Fund for Scientific and Technological Development) to G. T., Chile. The authors thank Dr. Patricia Cogram for kindly reviewing the manuscript.*

*The authors have declared no conflict of interest.*

## 5 References

[1] Bellentani, S, Marino, M., Epidemiology and natural history of non-alcoholic fatty liver disease (NAFLD). *Ann. Hepatol.* 2009, *8*, S4–S8.

[2] Alberti, K. G., Zimmet, P., Shaw, J., IDF Epidemiology Task Force Consensus Group. The metabolic syndrome – a new worldwide definition. *Lancet* 2005, *366*, 1059–1062.

[3] Poudyal, H., Panchal, S. K., Diwan, V., Brown, L., Omega-3 fatty acids and metabolic syndrome: effects and emerging mechanisms of action. *Prog. Lipid Res.* 2011, *50*, 372–387.

[4] Musso, G., Gambino, R., Cassader, M., Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Prog. Lipid Res.* 2009, *48*, 1–26.

[5] Aronis, A., Madar, Z., Tirosh, O., Mechanism underlying oxidative stress-mediated lipotoxicity: exposure of J774.2 macrophages to triacylglycerols facilitates mitochondrial reactive oxygen species production and cellular necrosis. *Free Radic. Biol. Med.* 2005, *38*, 1221–1230.

[6] Videla, L. A., Rodrigo, R., Araya, J., Poniachik, J., Insulin resistance and oxidative stress interdependency in non-alcoholic fatty liver disease. *Trends Mol. Med.* 2006, *12*, 555–558.

[7] Araya, J., Rodrigo, R., Videla, L. A., Thielemann, L. et al., Increase in long-chain polyunsaturated fatty acid n-6/n-3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin Sci.* 2004, *106*, 635–643.

[8] Araya, J., Rodrigo, R., Pettinelli, P., Araya, A. V. et al., Decreased liver fatty acid Δ-6 and Δ-5 desaturase activity in obese patients. *Obesity* 2010, *18*, 1460–1463.

[9] Brenner, R. R., Hormonal modulation of Δ6 and Δ5 desaturases: case of diabetes. *Prostaglandins Leukot. Essent. Fatty Acids* 2003, *68*, 151–162.

[10] Warensjö, E., Sundström, J., Vessby, B., Cederholm, T. et al., Markers of dietary fat quality and fatty acid desaturation as predictors of total and cardiovascular mortality: a population-based prospective study. *Am J Clin Nutr.* 2008, *88*, 203–209.

[11] Zhou, Y. E., Kubow, S., Dewailly, E., Julien, P., Egeland, G. M., Decreased activity of desaturase 5 in association with obesity and insulin resistance aggravates declining long-chain n-3 fatty acid status in Cree undergoing dietary transition. *Br. J. Nutr.* 2009, *102*, 888–894.

 

[12] Delarue, J., LeFoll, C., Corporeau, C., Lucas, D., N-3 long-chain polyunsaturated fatty acids: a nutritional tool to prevent insulin resistance associated to type 2 diabetes and obesity? *Rep. Nutr. Develop.* 2004, *44*, 289–299.

[13] Martinelli, N., Girelli, D., Malerba, G., Guarini, P. et al., FADS genotypes and desaturase activity estimated by the ratio of arachidonic acid to linolenic acid are associated with inflammation and coronary artery disease. *Am. J. Clin. Nutr.* 2008, *88*, 941–949.

[14] Simopoulos, A. P., The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp. Biol. Med.* 2008, *233*, 674–688.

[15] Valenzuela, R., Videla, L. A., The importance of the long-chain polyunsaturated fatty acid n-6/n-3 ratio in development of non-alcoholic fatty liver associated with obesity. *Food Funct.* 2011, *2*, 644–648.

[16] Kim, W., McMurray, D. N., Chapkin, R. S., N-3 polyunsaturated fatty acids: physiological relevance of dose. *Prostaglandins Leukot. Essent. Fatty Acids* 2010, *82*, 155–158.

[17] Clarke, S. D., The multi-dimensional regulation of gene expression by fatty acids: polyunsaturated fats as nutrient sensors. *Curr. Opin. Lipidol.* 2004, *15*, 13–18.

[18] Goto, T., Kim, Y.-I., Takahashi, N., Kawada, T., Natural compounds regulate energy metabolism by the modulating activity of lipid-sensing nuclear receptors. *Mol. Nutr. Food Res.* 2013, *57*, 20–33.

[19] Delerive, P., De Bosscher, K., Besnard, S., Vanden Berghe, W. et al., Peroxisome proliferator-activated receptor-alpha negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-kappaB and AP-1. *J. Biol. Chem.* 1999, *274*, 32048–32054.

[20] Brunt, E. M., Janney, C. G., Di Biscegle, A. M., Neuschwander-Tetri, B. A., Bacon, B. R., Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am. J. Gastroenterol.* 1999, *94*, 2467–74.

[21] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, *227*, 680–685.

[22] Towbin, H., Staehlin, T., Gordon, J., Electroforetic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 1979, *76*, 4350–4354.

[23] Videla, L. A., Pettinelli, P., Misregulation of PPAR functioning and its pathogenic consequences associated with nonalcoholic fatty liver disease in human obesity. *PPAR Res.* 2012, 107434. DOI:10.1155/2012/107434.

[24] Leonarduzzi, G., Sottero, B., Testa, G., Poli, G., New insights into redox-modulated cell signaling. *Curr. Pharmt. Design* 2011, *17*, 3994–4006.

[25] Brigelius-Flohé R., Flohé L., Basic principles and emerging concepts in the redox control of transcription factors. *Antioxid. Redox Signal.* 2011, *15*, 2335–2381.

[26] Valenzuela, R., Espinosa, A., González-Mañán, D., D'Espessailles, A. et al., N-3 long-chain polyunsaturated fatty acid supplementation significantly reduces liver oxidative stress in high fat induced steatosis. *PLoS One* 2012, *7*, e46400.

[27] Pettinelli, P., del Pozo, T., Araya, J., Rodrigo, R. et al., Enhancement in liver SREBP-1c/PPARα ratio and steatosis in obese patients: correlation with insulin resistance and *n-3* long-chain polyunsaturated fatty acid depletion. *Biochim. Biophys. Acta* 2009, *1792*, 1080–1086.

[28] Naitoh, R., Miyawaki, K., Harada, N., Mizunoya, W. et al., Inhibition of GIP signaling modulates adiponectin levels under high-fat diet in mice. *Biochem. Biophys. Res. Commun.* 2008, *376*, 21–25.

[29] Pagano, C., Soardo, G., Esposito, W., Fallo, F. et al., Plasma adiponectin is decreased in nonalcoholic fatty liver disease. *Eur. J. Endocrinol.* 2005, *152*, 113–118.

[30] Pettinelli, P., Videla, L. A., Up-regulation of PPAR-γ mRNA expression in the liver of obese patients: an additional reinforcing lipogenic mechanism to SREBP-1c induction. *J. Clin. Endocrinol. Metab.* 2011, *96*, 1424–1430.

[31] Czaja, M. J., Liver injury in the setting of steatosis: crosstalk between adipokine and cytokine. *Hepatology* 2004, *40*, 19–22.

[32] Kadowaki, T., Yamauchi, T., Kubota, N., The physiological and pathophysiological role of adiponectin and adiponectin receptors in the peripheral tissues and CNS. *FEBS Lett.* 2008, *582*, 74–80.

[33] Yoon, M. J., Lee, G. Y., Chung, J. J., Ahn, Y.-H. et al., Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor α. *Diabetes* 2006, *55*, 2562–2570.

[34] Kersten, S., Desvergne, B., Wahli, W., Roles of PPARs in health and disease. *Nature* 2000, *405*, 421–424.

[35] Ribeiro, P. S., Cortez-Pinto, H., Solá, S., Castro, R. E. et al., Hepatocyte apoptosis, expression of death receptors, and activation of NF-κB in the liver of nonalcoholic and alcoholic steatohepatitis patients. *Am. J. Gastroenterol.* 2004, *99*, 1708–1717.

[36] Videla, L. A., Tapia, G., Rodrigo, R., Pettinelli, P. et al., Liver NF-κB and AP-1 DNA binding in obese patients. *Obesity* 2009, *17*, 973–979.

[37] Gloire, G., Legrand-Poels, S., Piette, J., NF-κB activation by reactive oxygen species: fifteen years later. *Biochem. Pharmacol.* 2006, *72*, 1493–1505.

[38] Videla, L. A., Liver NF-κB and AP-1 activation and PPAR-α expression are negatively correlated in obese patients: pro-inflammatory implications. *Clin. Nutr.* 2010, *29*, 687–688.

[39] Zúñiga, J., Cancino, M., Medina, F., Varela, P. et al., N-3 PUFA supplementation triggers PPAR-α activation and PPA-α/NF-κB interaction: anti-inflammatory implications in liver ischemia-reperfusion injury. *PLoS One* 2011, *6*, e28502.

[40] González-Périz, A., Horrillo, R., Ferré, N., Gronert, K. et al., Obesity-induced insulin resistance and hepatic steatosis are alleviated by ω-3 fatty acids: a role for resolvins and protectins. *FASEB J.* 2009, *23*, 1946–1957.

 

[41] Houstis, N., Rosen, E. D., Lander, E. S., Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* 2006, *440*, 944–948.

[42] Oster, R. T., Tishinsky, J. M., Yuan, Z., Robinson, L. E., Docosahexaenoic acid increases cellular adiponectin mRNA and secreted adiponectin protein, as well as PPARγ mRNA, in 3T3-L1 adipocytes. *Appl. Physiol. Nutr. Metab.* 2010, *35*, 783–789.

[43] Deminice, R., da Silva, R. P., Lamarre, S. G., Brown, C. et al., Creatine supplementation prevents the accumulation of fat in the livers of rats fed a high-fat diet. *J. Nutr.* 2011, *141*, 1799–1804.

[44] Lombardo, Y. B., Chicco, A. G., Effects of dietary polyunsaturated fatty acids on dyslipidemia and insulin resistance in rodents and humans. A review. *J. Nutr. Biochem.* 2006, *17*, 1–13.

[45] de Roos, B., Mavrommatis, Y., Brouwer, I. A., Long-chain polyunsaturated fatty acids: new insights into mechanisms relating to inflammation and coronary heart disease. *Br. J. Pharmacol.* 2009, *158*, 413–428.

[46] Ye, D., Zhang, D., Oltman, C., Dellsperger, K. et al., Cytochrome P450 epoxygenase metabolites of docosahexanoate potently dilate coronary arterioles by activating large-conductance calcium-activated potassium channels. *J. Pharmacol. Exp. Ther.* 2002, *303*, 768–776.

[47] Hong, S., Gronert, K., Devchand, P. R., Moussignac, R. I. et al., Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids and anti-inflammation. *J. Biol. Chem.* 2003, *278*, 14677–14687.

[48] Gao, L., Wang, J., Sekkar, K. R., Yin, H. et al., Novel n-3 fatty acid oxidation products activate Nrf2 by destabilizing the association between Keap1 and Culling3. *J. Biol. Chem.* 2007, 2529–2537.

[49] Fernández, V., Tapia, G., Videla, L. A., Recent advances in liver preconditioning: thyroid hormone, *n-3* long-chain polyunsaturated fatty acids and iron. *World J. Hepatol.* 2012, *4*, 119–128.

[50] Delerive, P., Gervois, P., Fruchart, J.-C., Staels, B., Induction of IκBα expression as a mechanism contributing to the anti-inflammatory activities of PPARα activators. *J. Biol. Chem.* 2000, *275*, 36703–36707.

[51] Calder, P. C., Polyunsaturated fatty acids and inflammation. *Prostaglandins Leukot. Essent. Fatty Acids* 2006, *75*, 197–202.

[52] Spadaro, L., Magliocco, O., Spampinato, D., Piro, S. et al., Effects of polyunsaturated fatty acids in subjects with nonalcoholic fatty liver disease. *Dig. Liver Dis.* 2008, *40*, 194–199.

[53] Zhu, F. S., Liu, S., Chen, X. M., Huang, Z. G. et al., Effects of polyunsaturated fatty acids from seal oils on nonalcoholic fatty liver disease associated with hyperlipemia. *World J Gastroenterol.* 2008, *14*, 6395–6400.

[54] Hatzitolios, A., Savopoulos, C., Lazaraki, G., Sidiropoulos, I. et al., Efficacy of omega-3 fatty acids, atorvastatin, and orlistat in non-alcoholic fatty liver disease with dyslipidemia. *Indian J. Gatroenterol.* 2004, *23*, 131–134.

[55] Tanaka, N., Sano, K., Horiuchi, A., Tanaka, E. et al., Highly purified eicosapentaenoic acid treatment improves nonalcoholic steatohepatitis. *J. Clin. Gastroenterol.* 2008, *42*, 413–418.

[56] Nobili, V., Bedogni, G., Alisi, A., Pietrobattista, A. et al., Docosahexaenoic acid supplementation decreases liver fat content in children with non-alcoholic fatty liver disease: double-blind randomized controlled clinical trial. *Arch. Dis. Child* 2011, *96*, 350–353.

[57] Shapiro, H., Tehilla, M., Attal-Singer, J., Bruck, R. et al., The therapeutic potential of long-chain omega-3 fatty acids in nonalcoholic disease. *Clin. Nutr.* 2011, *30*, 6–19.

 