Biochimica et Biophysica Acta 1792 (2009) 1080–1086

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Enhancement in liver SREBP-1c/PPAR-α ratio and steatosis in obese patients: Correlations with insulin resistance and *n-3* long-chain polyunsaturated fatty acid depletion

Paulina Pettinelli [a], Talía del Pozo [b], Julia Araya [c], Ramón Rodrigo [a], A. Verónica Araya [d], Gladys Smok [e], Attila Csendes [f], Luis Gutierrez [f], Jorge Rojas [f], Owen Korn [f], Fernando Maluenda [f], Juan C. Diaz [f], Guillermo Rencoret [f], Italo Braghetto [f], Jaime Castillo [f], Jaime Poniachik [g], Luis A. Videla [a,*]

[a] *Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile*
[b] *Laboratory of Bioinformatics and Gene Expression, Institute of Nutrition and Food Technology, University of Chile, Santiago, Chile*
[c] *Department of Nutrition, Faculty of Medicine, University of Chile, Santiago, Chile*
[d] *Department of Endocrinology, University of Chile Clinical Hospital, Santiago, Chile*
[e] *Department of Pathological Anatomy, University of Chile Clinical Hospital, Santiago, Chile*
[f] *Department of Surgery, University of Chile Clinical Hospital, Santiago, Chile*
[g] *Department of Medicine, University of Chile Clinical Hospital, Santiago, Chile*

ARTICLE INFO

Article history:
Received 30 June 2009
Received in revised form 26 August 2009
Accepted 31 August 2009
Available online 4 September 2009

Keywords:
Obesity
Liver steatosis
Sterol receptor element binding protein-1c
Peroxisome proliferator activated receptor-α
Insulin resistance

ABSTRACT

Sterol receptor element-binding protein-1c (SREBP-1c) and peroxisome proliferator-activated receptor-α (PPAR-α) mRNA expression was assessed in liver as signaling mechanisms associated with steatosis in obese patients. Liver SREBP-1c and PPAR-α mRNA (RT-PCR), fatty acid synthase (FAS) and carnitine palmitoyltransferase-1a (CPT-1a) mRNA (real-time RT-PCR), and *n-3* long-chain polyunsaturated fatty acid (LCPUFA)(GLC) contents, plasma adiponectin levels (RIA), and insulin resistance (IR) evolution (HOMA) were evaluated in 11 obese patients who underwent subtotal gastrectomy with gastro-jejunal anastomosis in Roux-en-Y and 8 non-obese subjects who underwent laparoscopic cholecystectomy (controls). Liver SREBP-1c and FAS mRNA levels were 33% and 70% higher than control values ($P<0.05$), respectively, whereas those of PPAR-α and CPT-1a were 16% and 65% lower ($P<0.05$), respectively, with a significant 62% enhancement in the SREBP-1c/PPAR-α ratio. Liver *n-3* LCPUFA levels were 53% lower in obese patients who also showed IR and hipoadiponectinemia over controls ($P<0.05$). IR negatively correlated with both the hepatic content of *n-3* LCPUFA ($r=-0.55$; $P<0.01$) and the plasma levels of adiponectin ($r=-0.62$; $P<0.005$). Liver SREBP-1c/PPAR-α ratio and *n-3* LCPUFA showed a negative correlation ($r=-0.48$; $P<0.02$) and positive associations with either HOMA ($r=0.75$; $P<0.0001$) or serum insulin levels ($r=0.69$; $P<0.001$). In conclusion, liver up-regulation of SREBP-1c and down-regulation of PPAR-α occur in obese patients, with enhancement in the SREBP-1c/PPAR-α ratio associated with *n-3* LCPUFA depletion and IR, a condition that may favor lipogenesis over FA oxidation thereby leading to steatosis.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) represents the hepatic metabolic consequence of relative overnutrition and altered diet composition in the setting of reduced physical activity and sedentary behaviors [1,2]. Under these conditions, carbohydrate and lipid affluence determine significant changes in hepatic intermediary metabolism, with high blood glucose and insulin levels stimulating liver fatty acid (FA) synthesis from glucose, re-directing FAs towards triacylglycerol (TAG) formation [3]. These changes seem to play a key role in the onset of hepatic steatosis by overnutrition, which may be contributed by the development of insulin resistance (IR) promoting peripheral FA mobilization to the liver [4,5]. Although IR determines a derangement in the gluco-regulatory action of insulin, the lipogenic effects of insulin in the liver are preserved [1–3], in agreement with the higher hepatic contents of palmitic acid and TAGs observed in obese patients over control values [6].

An additional major metabolic disturbance observed in the liver of obese NAFLD patients is *n-3* long-chain polyunsaturated FA (*n-3* LCPUFA) depletion, as evidenced by the substantial diminution in eicosapentaenoic acid (20:5, *n-3*; EPA) and docosahexaenoic acid (22:6, *n-3*; DHA) levels [6,7]. This finding has been related to higher utilization of *n-3* LCPUFA due to oxidative stress [8] and defective

* Corresponding author. Programa de Farmacología Molecular y Clínica, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Independencia 1027, Sector-D, Santiago-7, Chile. Tel.: +56 2 9786256; fax: +56 2 7372783.
E-mail address: lvidela@med.uchile.cl (L.A. Videla).

0925-4439/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2009.08.015

desaturation of the essential precursor α-linolenic acid (18:3, *n-3*) [6,9], which may be compounded by dietary imbalance [6]. Under physiological conditions, the *n-3* LCPUFAs EPA and DHA and/or their oxidized derivatives constitute signaling molecules regulating hepatic lipid metabolism. This is achieved through (i) up-regulation of the expression of genes encoding proteins involved in lipid transport, FA oxidation, and thermogenesis, acting a ligand of peroxisome proliferator-activated receptor α (PPAR-α); and (ii) suppression of lipogenesis by down-regulation of sterol regulatory element binding protein 1c (SREBP-1c) [10–12]. Therefore, depletion of *n-3* LCPUFA in the liver of obese NAFLD patients might favor FA and TAG formation over FA oxidation, promoting hepatic steatosis [3], which may be compromised by derangement in TAG export from the liver [13]. In view of these considerations, changes in the expression of SREBP-1c and PPAR-α were assessed in liver samples from control subjects and obese patients with NAFLD, as potential molecular mechanisms of hepatic steatosis, in association with that of the respective target genes fatty acid synthase (FAS) and carnitine palmitoyltransferase-1a (CPT-1a). Correlations between liver SREBP-1c, PPAR-α, and the respective SREBP-1c/PPAR-α ratio with the homeostasis model assessment of IR (HOMA) and the content of hepatic *n-3* LCPUFA were established, in addition to those with the serum levels of adiponectin, a key signaling adipokine influencing IR and glucose and lipid metabolism in the liver [14].

2. Materials and methods

2.1. Patients and laboratory studies

Nineteen subjects attending the Department of Medicine of the University of Chile Clinical Hospital were studied, including eleven NAFLD patients [average body mass index (BMI) of 41.5 ± 2.1 kg/m^2, age of 33 ± 2 years], who underwent subtotal gastrectomy with a gastro-jejunal anastomosis in roux and Y as a therapy for obesity, and eight non-obese patients [BMI of 25.0 ± 0.6 kg/m^2, age of 40 ± 6 years] who underwent laparoscopic cholecystectomy (control group). The protocol was explained in detail to the subjects, who then gave their written informed consent to participate in the study before any procedure was undertaken. Exclusion criteria included positive hepatitis B or C serology, positive antibodies (antinuclear, anti-mitochondrial, and anti-smooth muscle antibodies), smoking habits or non-smokers <1-year cessation, and consumption of more than 40 g of ethanol per week. Nutritional and alcohol consumption histories with anthropometric measurements were obtained. Insulin resistance was calculated from the fasting insulin and glucose values by homeostasis model assessment (HOMA) of insulin resistance analysis (fasting insulin (μunits/mL) × fasting glucose (mmol/l)/22.5) [15]. Plasma adiponectin concentrations were measured in duplicate by radioimmunoassay with antibody against human adiponectin and dilution of recombinant adiponectin as standard, with inter- and intra-assay coefficients of variations of <7% and <5%, respectively (Linco Research, St. Charles, MO, USA). Laboratory tests included serum liver parameters, lipid profile, and iron metabolism parameters (Table 1).

Both control and obese NAFLD patients were subjected to a diet of 25 kcal/kg body weight (where 1 kcal = 4.184 kJ), with 30% of the energy given as lipids and 15% as proteins, for at least 2 days prior to surgery, and liver tissue of approximately 2 cm^3 for histological diagnoses, *n-3* LCPUFA composition, and SREBP-1c and PPAR-α mRNA determinations were taken during surgery. The samples were fixed in 10% formalin, paraffin embedded, and sections were stained with hematoxylin/eosin and Van Gieson's stains. Sections of each liver sample were observed in a blinded manner and evaluated for histological alterations by means of previously defined codes [6–8]. Liver samples for biochemical determinations were frozen at −80 °C. The Ethics Committee of the University of Chile Clinical Hospital and that of the Faculty of Medicine, University of Chile approved the study protocol that was performed in accordance with the Helsinki Declaration II criteria.

Table 1
Clinical and biochemical parameters in control subjects and in obese patients with NAFLD.

Parameter (normal range)	Controls (n = 8)	Obese NAFLD patients (n = 11)
Age (year)	40 ± 6	33 ± 2
Female/male ratio	6/2	7/4
Body weight (kg)	65 ± 2 .6	110 ± 5.1[a]
Body mass index (<25 kg/m^2)	25.0 ± 0.6	41.5 ± 2.1[a]
Waist circumference (cm)	89.7 ± 6.6	113 ± 4.2[a]
Glucose (<100 mg/dL)	89.8 ± 3.4	93.5 ± 3.6
Insulin (<20 μU/mL)	6.6 ± 0.71	25.6 ± 3.7[a]
HOMA (<2.5)	1.3 ± 0.2	6.0 ± 0.9[a]
Adiponectin (μg/mL)	14.0 ± 1.4	9.34 ± 1.00[a]
Liver parameters		
Alanine aminotransferase (9–52 IU/L)	39 ± 3.8	60 ± 10
Aspartate aminotransferase (14–36 IU/L)	25 ± 2.1	43 ± 5[a]
Alkaline phosphatase (38–126 IU/L)	96 ± 11	85 ± 8
γ-Glutamyl transpeptidase (12–43 IU/L)	42 ± 6	41 ± 7
Total bilirubin (0.2–1.3 mg/dL)	0.46 ± 0.08	0.48 ± 0.08
Lipid profile		
Total triglyceride (<150 mg/dL)	163 ± 63	170 ± 31
Total cholesterol (<200 mg/dL)	163 ± 15	190 ± 16
LDL-cholesterol (<140 mg/dL)	87 ± 11	109 ± 12
HDL-cholesterol (>40 mg/dL)	53 ± 7	40 ± 3
Iron metabolism		
Total iron (37–145 μg/dL)	64 ± 12	69 ± 9
Ferritin (ng/mL)	54 ± 23	136 ± 51
Transferrin (200–300 mg/dL)	259 ± 21	263 ± 13
Transferrin saturation (20–55%)	22 ± 4	23 ± 3

Values represent means ± S.E.M., for the number of subjects indicated.
[a] P<0.05 compared with controls, as assessed by Student's t-test for unpaired data.
Abbreviations: HOMA, homeostasis model assessment of insulin resistance [fasting insulin (μunits/mL) × fasting glucose (mmol/L)/22.5] [15]; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

2.2. Isolation of hepatic RNA and reverse transcription-polymerase chain reaction (RT-PCR) assay for SREBP-1c and PPAR-α mRNA

Total liver RNA was extracted with Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) [16] and quantified by measurement of ultraviolet absorption at 260 nm. For RT-PCR assay of mRNA, first-strand cDNA was synthesized from total RNA (5 μg) using ThermoScript RT-PCR System (Invitrogen Corp., Carlsbad, CA, USA). cDNA was amplified in a PCR reaction using Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA, USA) in the presence of primers specific for human SREBP-1c and PPAR-α. Nucleotide sequences for sense and antisense primers used were 5'-GGATTGCACTTTCGAAGACATG-3, and 5,-ACTCTGGACCTGGGTGTGCAAG-3, for SREBP-1c and 5,-AGCCCGTTATCTGAAGAGTTCC-3, and 5,-CATCCCGACAGAAAGGCACTTG-3, for PPAR-α (Invitrogen Corp., Carlsbad, CA, USA), respectively. In these conditions, a 547-bp sequence between +3 and +549 bp of human SREBP-1c and a 438 bp sequence between +61 and +498 bp of human PPAR-α were amplified. To control the relative amount of total mRNA transcribed in each reverse transcriptase reaction, an RNA 18S invariant standard [Classic II 18S Internal Standards (324 bp); Ambion, The RNA Co., Austin, TX, USA] was used. PCR conditions included denaturation, annealing, and extension at 94, 64, and 72 °C, for 30 s, 75 s, and 60 s, respectively, for 38 and 39 cycles. PCR products were electrophoresed on 1.2% agarose gels containing ethidium bromide, visualized by UV-induced fluorescence, and analyzed by densitometry using Scion Image (Scion Corp., Frederick, MD, USA).

2.3. Real-time RT-PCR assay for FAS and CPT-1a mRNA

Total RNA was extracted from homogenized liver biopsies with Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA). Quality and quantity of RNA was checked visually prior to and after DNAase digestion (TURBO DNAase-free, Ambion, The RNA Co., Austin, TX, USA) by denaturing gel electrophoresis and by photometric analysis (A_{260} and A_{280}). Synthesis of cDNA was performed with 2 μg of total RNA using SyperScrip III (Invitrogen Corp., Carlsbad, CA, USA) and random hexamers according to standard procedures. Real-time RT-PCR reactions were performed in a LightCycler system (Roche Diagnostics, Mannheim, Germany) using SYBR Green to monitor cDNA amplification. Equal amounts of cDNA corresponding to 1/15 dilution of cDNA were used in each reaction, containing 5 μL Platinum SYBR Green I SuperMix-UDG, 0.5 μL BSA 20× (Invitrogen Corp., Carlsbad, CA, USA), and 5 pmol of forward and reverse primers in a total volume of 10 μL. The standard thermal profile used was 2 min at 50 °C, 2 min at 95 °C, 50 repeats of 5 s at 95 °C, 15 s at 60 °C, and a final stage of 15 s at 72 °C. Data were analyzed using LightCycler3 analysis software (Roche Diagnostics, Mannheim, Germany). PCR efficiency was determined for each sample and gene by LinRegPCR v7.5 [17]. Two technical repeats were done for each combination of cDNA and primer pair, and the quality of the PCR reactions was checked through analysis of the dissociation and amplification curves. The products were resolved by 3% agarose gel electrophoresis to confirm the DNA fragments of expected size. Transcript levels of genes were normalized to the respective transcript level of constitutively expressed control gene, human large ribosomal protein Rp1p0 [18]. Values shown are relative transcript level (RTL)×1000. The PCR primers for amplification of FAS (NM 004104.4) were forward 5,-GGACTACAACCTCTCCCA-3, and reverse 5,-GGATGATGCTGATGATGGA-3, and of CPT-1a (NM 001876.3) were forward 5,-CGCTACTCCCTGAAAGTG-3, and reverse 5,-CTTGACCATACCCATCCAG-3, (Invitrogen Corp., Carlsbad, CA, USA).

2.4. Determination of liver n-3 LCPUFA

Liver samples frozen in liquid nitrogen were homogenized (50 mg/mL) in a 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). To determine the changes in the levels of EPA (20:5,*n-3*) and DHA (22:6, *n-3*), lipids were extracted and derivatized to FA methyl esters (FAME) [19]. Briefly, liver homogenates containing the internal standard were extracted with a methanol/chloroform/H_2O mixture (2/1/1.8 by vol) and FAME in all samples was analyzed by GLC, as described previously [6]. The individual FAME peaks were identified by comparison of the retention times of the individual FAs from an authentic standard mixture and converted to concentration using the 17:0 internal standard. Results are expressed as g of *n-3* LCPUFA/100 g FAME.

2.5. Statistical analyses

Data showing Gaussian distribution using the Kolmogorov–Smirnov test are expressed as means ± S.E.M. for the number of patients indicated. Statistical analysis of the differences between mean values from control subjects and obese NAFLD patients was assessed by Student's *t*-test for unpaired data. The differences were considered statistically significant at $P<0.05$. To analyze the association between different variables, the Spearman rank order correlation coefficient was used. All statistical analyses were computed using GraphPad Prism™ version 2.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Clinical and biochemical characteristics of obese patients and control subjects

Table 1 shows the general characteristics of the patients studied. Subjects were predominantly female and exhibited comparable ages. Control patients ($n=8$) had normal liver histology, whereas those in the obese group ($n=11$) presented either simple macrovesicular steatosis ($n=7$) or steatosis and lobular inflammation with hepatocyte ballooning (steatohepatitis)($n=4$). NAFLD patients were significantly more obese than controls, as evidenced by their body weight, body mass index, and waist circumference being 69%, 66%, and 26% higher than controls, respectively. Fasting blood glucose levels in control and NAFLD patients were comparable, whereas fasting insulin levels in obese patients were 284% higher than controls ($P<0.05$), resulting in 359% enhancement ($P<0.05$) in the HOMA index of IR. Fasting levels of plasma adiponectin in obese patients were 33% lower ($P<0.05$) than control values. The majority of patients were asymptomatic, with plasma lipid levels (total cholesterol, HDL-cholesterol, LDL-cholesterol, and triacylglycerols), parameters related to iron metabolism (total iron, ferritin, transferrin, and transferrin saturation), and alkaline phosphatase and γ-glutamyl transpeptidase activity in serum and total serum bilirubin being within normal ranges in the studied groups. Control patients exhibited normal serum ALT and AST activity, whereas six (55%) obese NAFLD patients exhibited serum ALT and AST levels higher than the normal range. Considering that liver TAG content and the changes in the hepatic composition of *n-6* and *n-3* PUFA are comparable in obese NAFLD patients with steatosis or steatohepatitis [6,7], all obese patients studied were joined in a single group.

3.2. Obesity is associated with liver n-3 LCPUFA depletion

The content of EPA in the liver of obese NAFLD patients was comparable to that in control subjects (Table 2). However, the content of DHA was 57% lower ($P<0.05$) in the liver of obese patients than control values, with a net 53% diminution being observed in the total *n-3* LCPUFA (EPA + DHA) content (Table 2).

3.3. Obesity is related to liver SREBP-1c and FAS up-regulation and PPAR-α and CPT-1a down-regulation

The upper panels of Fig. 1A and B show representative agarose gel electrophoresis of the RT-PCR products for SREBP-1c mRNA and PPAR-α mRNA, respectively, with those of 18S rRNA to compare lane-lane equivalency in total RNA content in liver samples from control patient 1, NAFLD patient 2 with steatosis, and NAFLD patient 3 with steatohepatitis. Densitometric quantification of RT-PCR products of the mRNA of SREBP-1c in the liver of obese NAFLD patients, expressed as SREBP-1c mRNA/18S rRNA, revealed values 33% higher than those in the control group ($P<0.05$) (Fig. 1A, lower panel), whereas hepatic PPAR-α mRNA/18S rRNA were 16% lower ($P<0.05$) in obese patients over controls (Fig. 1B, lower panel). Accordingly,

Table 2
Content of EPA and DHA in liver total lipids from control subjects and obese patients with NAFLD.

Fatty acid	Controls ($n=8$)	Obese NAFLD patients ($n=11$)
EPA (g/100 g FAME)	1.30 ± 0.37	1.36 ± 0.42
DHA (g/100 g FAME)	18.5 ± 1.7	7.90 ± 1.30[a]
EPA + DHA (g/100 g FAME)	19.8 ± 1.8	9.30 ± 1.60[a]

Values represent means ± S.E.M., for the number of subjects indicated.
[a] $P<0.05$ compared with controls, as assessed by Student's *t*-test for unpaired data.
Abbreviations: DHA, docosahexaenoic acid (22:6, *n-3*); EPA, eicosapentaenoic acid (20:5, *n-3*); FAME, fatty acid methyl esters.

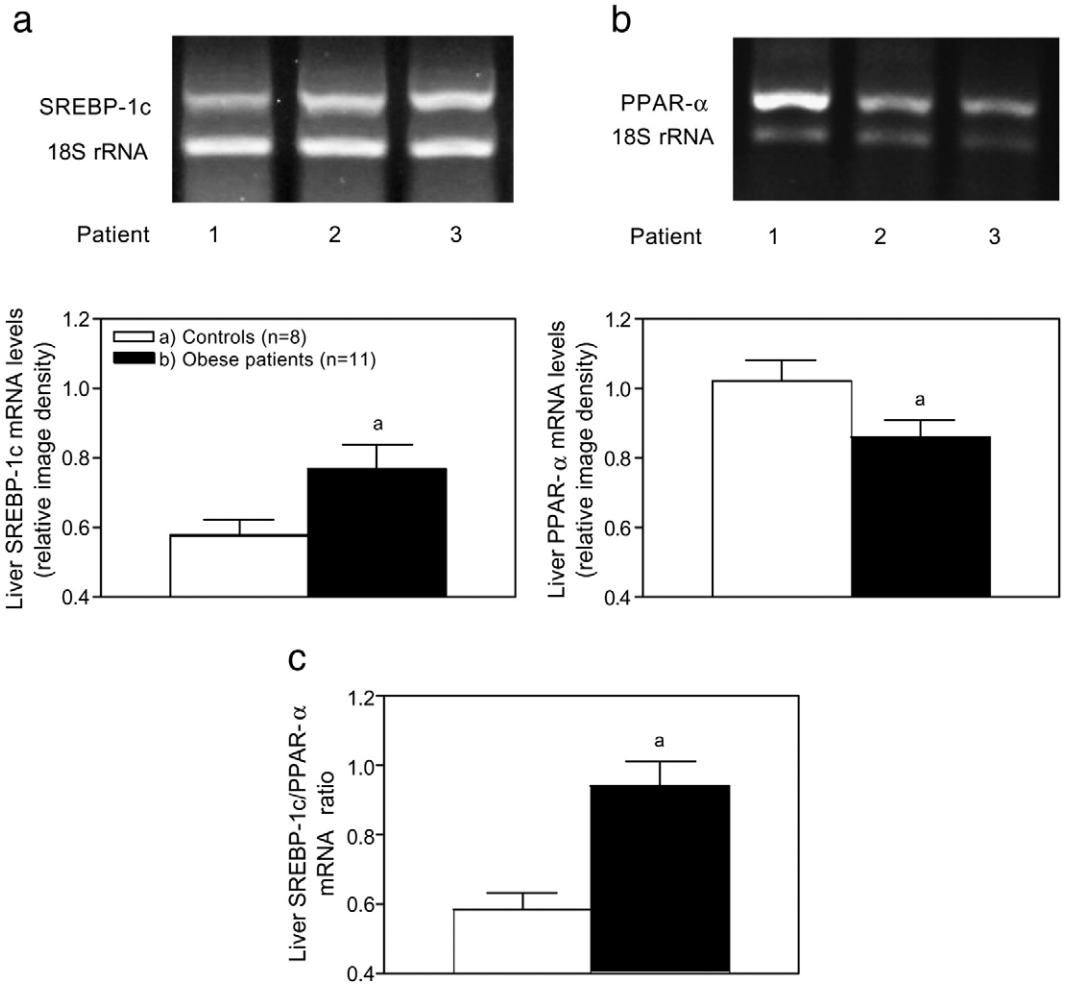

Fig. 1. Expression of SREBP-1c mRNA (A), PPAR-α mRNA (B), and SREBP-1c mRNA/PPAR-α mRNA ratio (C) in the liver of control subjects and obese patients with NAFLD. The upper panels represent agarose gel electrophoresis of the RT-PCR products for SREBP-1c mRNA (547 bp) and 18S rRNA (324 bp)(A) and for PPAR-α mRNA (438 bp) and 18S rRNA (324 bp) (B) in total liver RNA samples (5 μg) from patient 1 (control), patient 2 (steatosis), and patients 3 (steatohepatitis). Bar graphs in the lower panels corresponding to densitometric quantification of relative SREBP-1c mRNA (SREBP-1c mRNA/18S rRNA)(A) and PPAR-α mRNA (PPAR-α mRNA/18S rRNA)(B) levels represent means ± S.E.M. for the number of patients indicated in parentheses. [a]$P<0.05$ compared to controls, as assessed by Student's *t*-test for unpaired data.

the liver of obese patients exhibited respective calculated SREBP-1c mRNA/PPAR-α mRNA ratios 62% higher ($P<0.05$) than control subjects (Fig. 1C). Values for the SREBP-1c mRNA/PPAR-α mRNA ratios were comparable in obese patients with esteatosis and steatohepatitis (data not shown). Up-regulation of SREBP-1c in the liver of obese patients (Fig. 1A) coincided with a 70% enhancement in the mRNA expression of hepatic FAS over controls (Fig. 2), parameters that were significantly correlated in the studied patients ($r=0.88$; $P<0.0001$). Furthermore, down-regulation of hepatic PPAR-α (Fig. 1B) was associated with a significant 65% diminution in the levels of liver CPT-1a mRNA (Fig. 2) ($r=0.86$; $P<0.0001$).

3.4. Liver SREBP-1c/PPAR-α ratios are positively correlated with serum insulin levels or with HOMA and negatively associated with liver n-3 LCPUFA levels

In the studied patients, IR assessed by the HOMA index negatively correlated with both the hepatic content of *n-3*-LCPUFA ($r=-0.55$; $P<0.01$) and the plasma levels of adiponectin ($r=-0.62$; $P<0.005$). Liver SREBP-1c mRNA expression showed a positive correlation with HOMA ($r=0.43$; $P<0.05$), without significant association with the plasma levels of adiponectin ($r=-0.09$; not significant). Hepatic PPAR-α mRNA expression negatively correlated with HOMA ($r=-0.44$; $P<0.05$) and showed a positive association with the plasma adiponectin levels ($r=0.54$; $P<0.01$). Furthermore, the SREBP-1c/PPAR-α mRNA ratio and *n-3* LCPUFA showed a negative significant correlation ($r=-0.48$; $P<0.02$) (Fig. 3A) and significant positive associations with either HOMA ($r=0.75$;

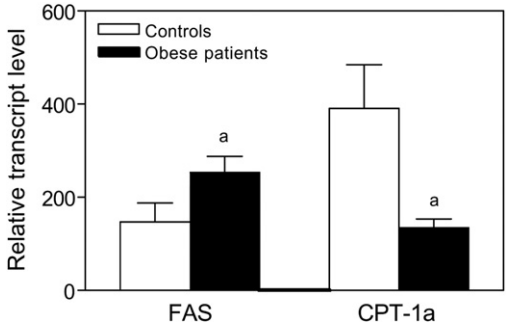

Fig. 2. Expression of fatty acid synthase (FAS) mRNA and carnitine palmitoyltransferase-1a (CPT-1a) mRNA in the liver of controls subjects ($n=8$) and obese patients ($n=11$) with NAFLD. Measurements were carried out by real-time RT-PCR and values shown were normalized to the respective transcript level of the constitutively expressed control gene Rp1p0, expressed as means ± S.E.M. for the number of patients indicated in parentheses. [a]$P<0.05$ compared to controls, as assessed by Student's *t*-test for unpaired data.

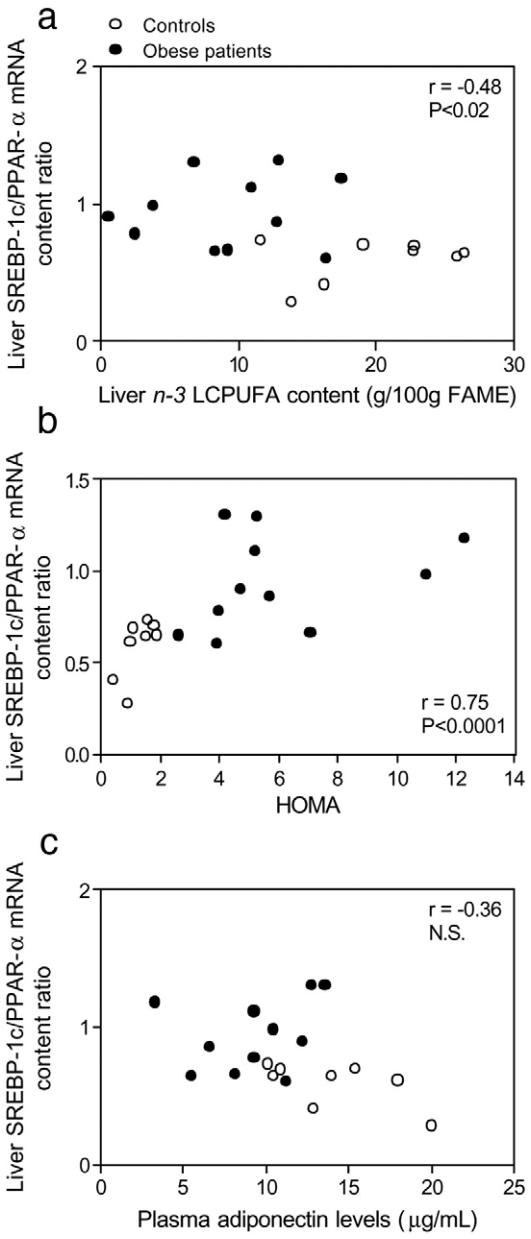

Fig. 3. Correlations between liver SREBP-1c/PPAR-α mRNA ratio and the hepatic content of *n-3* LCPUFA content (A), the homeostasis model assessment of insulin resistance (HOMA)(B), and the plasma levels of adiponectin (C) in control subjects and obese NAFLD patients. The Spearman rank order correlation coefficient was used to analyze the association between different variables.

$P<0.0001$) (Fig. 3B) or serum insulin levels ($r=0.69$; $P<0.001$), whereas that with plasma adiponectin levels was not significant ($r=-0.36$; $P>0.05$) (Fig. 3C).

4. Discussion

Saturated FAs are the major metabolic fuel in the liver under most circumstances in healthy human subjects, whereas the contribution of hepatic *de novo* lipogenesis (DNL) to TAG content is very small (1.6–4.7%) [20,21]. However, in insulin-resistant states such that occurring in obese hyperinsulinemic NAFLD patients, fasting DNL is substantially increased, a response that is likely to involve up-regulation of the enzymes of hepatic DNL [20,21]. Data presented in this study show that the liver of obese NAFLD patients exhibit levels of the pro-lipogenic transcription factor SREBP-1c that are 33% higher than lean controls. Up-regulation of liver SREBP-1c is in agreement with the enhanced DNL observed in obesity [20–22], considering that SREBP-1c transcriptionally activates most genes required for hepatic lipogenesis [23], as shown by the 70% increase in FAS expression. Insulin is known to activate the hepatic expression of SREBP-1c under normal conditions [23], an action that persists in IR states underlying hyperinsulinemia such as that observed in the current study (Fig. 4). This contention is supported by the significant correlation found between liver SREBP-1c mRNA levels and HOMA established. In addition to this mechanism, increased liver DNL in obese NAFLD patients may be contributed by the hepatic *n-3* LCPUFA depletion observed, favoring the proteolytic release of membrane-bound SREBP-1c and its nuclear abundance and/or altering membrane lipid composition associated with IR (Fig. 4) [10–12]. In addition to the above mechanisms triggering SREBP-1c transcription and processing, interference with SREBP-1c ubiquitination and/or sumoylation by specific enzymes may also play a role in enhancing SREBP-1c transcriptional activity [24]. However, this contention underlying diminished degradation and/or inactivation of SREBP-1c, respectively, remains to be studied in the liver of obese patients.

Up-regulation of liver SREBP-1c in obesity was found concomitantly with down-regulation of hepatic PPAR-α, as evidenced by the significant diminution in PPAR-α mRNA levels over control values, in association with a 65% decrease in PPAR-α-dependent CPT-1a expression. Data reported in human liver indicate that PPAR-α expression (i) is lower than in rodent liver [25]; (ii) represents a functional and conserved process capable of trans-activating response elements in genes responsible for FA oxidation and lipid transport [26,27]; and (iii) is regulated by genetic and/or environmental factors [28]. According to the latter view, obesity-associated liver down-regulation of PPAR-α expression in NAFLD patients may be related to the expression of a PPAR-α variant transcript [25,28], leading to production of a truncated form of PPAR-α that upon nuclear translocation exerts a potent dominant negative activity [28]. Furthermore, down-regulation of liver PPAR-α mRNA expression in obese NAFLD subjects could be achieved through a tumor necrosis factor-α (TNF-α)-dependent mechanism as shown for PPAR-γ [29], considering the pro-inflammatory cytokine signaling established in NAFLD [1–3,30]. However, further studies in obese NAFLD patients are needed to support the involvement of both PPAR-α splice variant and TNF-α in liver PPAR-α mRNA down-regulation. In addition to obesity-induced changes in liver PPAR-α transcription, transcriptional activation of PPAR-α target genes should also be considered, as this complex phenomenon requires the binding of specific ligands and the orchestrated recruitment and assembly of several cofactors into multi-subunit protein co-activator complexes on promoters [27]. Considering that human PPAR-α is activated through direct binding of LCPUFA to its ligand-binding domain [31], liver PPAR-α function may be compromised in obesity by deficient PPAR-α activation due to diminished binding of *n-3* LCPUFA secondary to depletion of these naturally occurring ligands (Fig. 4) reported in this work (Table 2) and elsewhere [6,7,32]. Alternatively, availability of co-activators of PPAR-α function may be reduced, as shown for CREB-binding protein (CBP) in subjects expressing truncated PPAR-α [28]. Finally, in the studied patients, liver PPAR-α expression positively correlated with plasma adiponectin levels, whereas it showed a negative association with HOMA. These correlations suggest that hypoadiponectinemia in IR-obese NAFLD patients may impair liver PPAR-α function, as hypothesized from data obtained in experimental obese animals [33]. However, this aspect and availability of PPAR-α co-activators in PPAR-α function require further investigation in human obesity. Down-regulation of liver PPAR-α expression without significant alteration in that of SREBP-1 has been reported in NAFLD [34], changes that are difficult to interpret due to lack of assessment of clinical and biochemical parameters including body mass index, IR, and liver function tests. Recently, NAFLD patients with BMI≥25

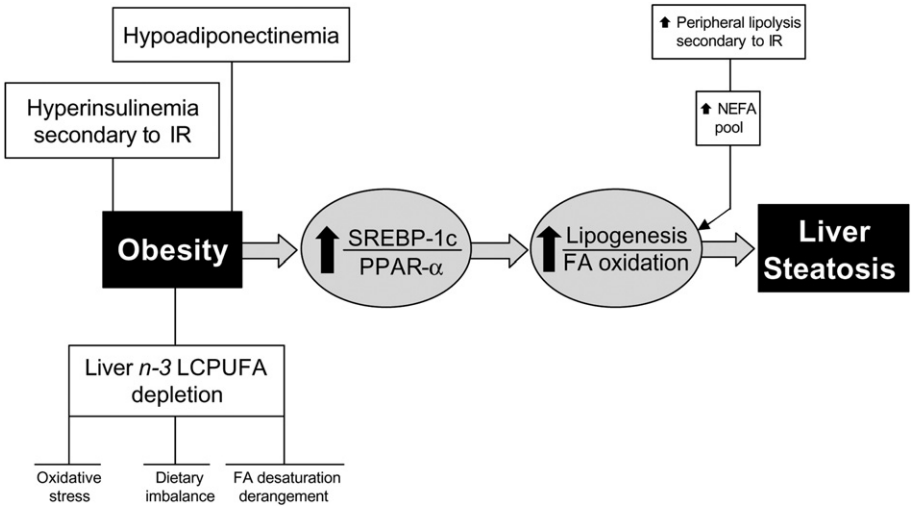

Fig. 4. Dysregulation of liver lipid metabolism leading to hepatic steatosis in obese NAFLD patients. FA loading underlying overnutrition can increase mitochondrial production of reactive oxygen species (ROS) leading to (i) depletion of *n-3* LCPUFA due to enhanced lipid peroxidation, with the involvement of dietary imbalance and decreased FA desaturation; and (ii) the onset of IR due to ROS- and FA-dependent activation of serine-threonine kinases that phosphorylate the insulin receptor and related insulin-receptor substrates thus decreasing insulin signaling [3,36]. Hyperinsulinemia secondary to IR and liver *n-3* LCPUFA depletion up-regulate the expression and processing of hepatic SREBP-1c, respectively, which occur concomitantly with liver PPAR-α deactivation coupled to *n-3* LCPUFA depletion. The substantial increase in the SREBP-1c/PPAR-α mRNA content ratio is associated with an enhanced lipogenic potential relative to FA oxidation and export, a change that is favored by increased FA flux into the plasma nonesterified FA (NEFA) pool due to IR-dependent peripheral lipolysis thus determining liver steatosis.

exhibited significantly lower liver PPAR-α mRNA levels over those with BMI<25, in agreement with our results, however, liver SREBP-1c expression was comparable in the studied groups [35]. The discrepancy observed in liver SREBP-1c expression could be due to the fact that obese patients in our study exhibited morbid obesity, as evidenced by BMI>40 (Table 1).

A major finding in the current study is the substantial enhancement in the hepatic SREBP-1c/PPAR-α mRNA content ratio found in obesity, implying a metabolic imbalance between DNL and FA oxidation in favor of the former as a central issue determining liver steatosis in NAFLD (Fig. 4). Furthermore, liver SREBP-1c/PPAR-α ratio is significantly correlated with both HOMA and serum insulin levels, pro-lipogenic factors conditioning the increases in peripheral lipolysis and in the nonesterified FA plasma pool reaching the liver (Fig. 4). Of particular interest is the negative ($P<0.02$) correlation established between liver SREBP-1c/PPAR-α ratio and *n-3* LCPUFA content in control and obese NAFLD patients, which support the crucial role of the depletion of *n-3* LCPUFA as a fuel partitioning factor directing FAs away from oxidation and toward TAG storage [36]. In agreement with this view, obese NAFLD patients supplemented with *n-3* LCPUFA for 12 months ameliorated hepatic steatosis compared with similar patients refusing the treatment, as measured by ultrasonography and echo-Doppler, although cellular *n-3* LCPUFA status and IR were not assessed [37]. Thus, considering that steatosis in the obese NAFLD patient is the result of multiple metabolic abnormalities taking place in the setting of dietary imbalance and involving the onset of oxidative stress and IR (Fig. 4), future therapeutic trials may require different treatment targets [38].

In conclusion, the expression of transcription factors controlling lipid metabolism is markedly altered in the liver of obese NAFLD patients. This is evidenced by the increased mRNA expression of SREBP-1c inducing lipogenic genes such as FAS, with the parallel diminution in that of PPAR-α controlling FA oxidation (CPT-1a) and secretion. Consequently, the liver SREBP-1c/PPAR-α ratio is substantially elevated, a change that may determine higher rates of hepatic lipogenesis over those of FA oxidation, with development of steatosis. Although hepatic DNL is mainly controlled by SREBP-1c signaling amplified by nuclear factor Y and stimulatory protein 1 [12,24,36], induction of SREBP-1c by liver X receptor-α and/or activation of carbohydrate response element binding protein could also play a role [12,39], however, further studies are required to verify this contention in human morbid obesity. Down-regulation of hepatic PPAR-α in NAFLD reported in this work may have a pro-inflammatory connotation, considering the antagonizing effect of PPAR-α on nuclear factor-κB and activating protein-1 action [2], in agreement with the enhancement in the activity of the latter pro-inflammatory mediators recently reported in the liver of obese NAFLD patients with steatohepatitis [40].

Acknowledgments

This work was supported by grants 1060105 and 1090020 from Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) Chile.

References

[1] P. Angulo, Nonalcoholic fatty liver disease, New Engl. J. Med. 346 (2002) 1221–1231.
[2] J. George, C. Liddle, Nonalcoholic fatty liver disease: pathogenesis and potential for nuclear receptors as therapeutic targets, Mol. Pharm. 5 (2008) 49–59.
[3] L.A. Videla, R. Rodrigo, J. Araya, J. Poniachik, Insulin resistance and oxidative stress interdependency in non-alcoholic fatty liver disease, Trends Mol. Med. 12 (2006) 555–558.
[4] S. Nielsen, Z. Guo, C.M. Johnson, D.D. Hensrud, M.D. Jensen, Splanchnic lipolysis in human obesity, J. Clin. Invest. 113 (2004) 1582–1588.
[5] E. Fabbrini, B.S. Mohammed, F. Magkos, K.M. Korenblat, B.W. Patterson, S. Klein, Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease, Gastroenterology 134 (2008) 424–431.
[6] J. Araya, R. Rodrigo, L.A. Videla, L. Thielemann, M. Orellana, P. Pettinelli, J. Poniachik, 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. 106 (2004) 635–643.
[7] A. Elizondo, J. Araya, R. Rodrigo, J. Poniachik, A. Csendes, F. Maluenda, J.C. Díaz, C. Signorini, C. Sgherri, M. Comporti, L.A. Videla, Polyunsaturated fatty acid pattern in liver and erythrocyte phospholipids from obese patients, Obesity 15 (2007) 24–31.
[8] L.A. Videla, R. Rodrigo, M. Orellana, V. Fernández, G. Tapia, L. Quiñones, N. Varela, J. Contreras, R. Lazarte, A. Csendes, J. Rojas, F. Maluenda, P. Burdiles, J.C. Díaz, G. Smok, L. Thielemann, J. Poniachik, Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients, Clin. Sci. 106 (2004) 261–268.
[9] M. Borkman, L.H. Storlien, D.A. Pan, A.B. Jenkins, D.J. Chisholm, L.V. Campbell, The relationship between insulin sensitivity and the fatty acid composition of phospholipids of skeletal muscle, N. Engl. J. Med. 328 (1993) 238–244.
[10] Y.B. Lombardo, A.G. Chicco, Effects of dietary polyunsaturated n-3 fatty acids on dyslipidemia and insulin resistance in rodents and humans. A review, J. Nutr. Biochem. 17 (2006) 1–13.

[11] D.B. Jump, D. Botolin, Y. Wang, J. Xu, B. Christian, O. Demeure, Fatty acid regulation of hepatic gene transcription, J. Nutr. 135 (2005) 2503–2506.
[12] H. Sampath, J.M. Ntambi, Polyunsaturated fatty acid regulation of genes of lipid metabolism, Annu. Rev. Nutr. 25 (2005) 317–340.
[13] D. Lindén, K. Lindberg, J. Oscarsson, C. Claesson, L. Asp, L. Li, M. Gustafsson, J. Borén, S.O. Olofsson, Influence of peroxisome proliferator-activated receptor α agonists on the intracellular turnover and secretion of apolipoprotein (apo) B-100 and apoB48, J. Biol. Chem. 277 (2002) 23044–23053.
[14] T. Kadowaki, T. Yamauchi, Adiponectin and adiponectin receptors, Endocr. Rev. 26 (2005) 439–451.
[15] D.R. Matthews, J.P. Hosker, A.S. Rudenski, B.A. Naylor, D.F. Tearcher, R.C. Turner, Homeostasis model assessment: insulin resistance and β-cell function from fasting glucose and insulin concentration in man, Diabetologia 28 (1985) 412–419.
[16] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[17] C. Ramakers, J.M. Ruijter, R.H. Deprez, A.F. Moorman, Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data, Neurosci. Lett. 339 (2003) 62–66.
[18] K. Dheda, J.F. Huggett, S.A. Bustin, M.A. Johnson, G. Rook, A. Zumla, Validation of housekeeping genes for normalizing RNA expression in real-time PCR, Biotechniques 37 (2004) 112–119.
[19] E.C. Bligh, W.J. Dyer, A rapid method of total lipid extraction, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[20] J.-M. Schwarz, P. Linfoot, D. Dare, K. Aghajanian, Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets, Am. J. Clin. Nutr. 77 (2003) 43–50.
[21] M.T. Timlin, E.J. Parks, Temporal pattern of de novo lipogenesis in the postprandial state in health men, Am. J. Clin. Nutr. 81 (2005) 35–42.
[22] K.L. Donnelly, C.O. Smith, S.J. Schwarzenberg, J. Jessurun, M.D. Bold, E.J. Parks, Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease, J. Clin. Invest. 115 (2005) 1343–1351.
[23] S. Tamura, I. Shimomura, Contribution of adipose tissue and de novo lipogenesis to nonalcoholic fatty liver disease, J. Clin. Invest. 115 (2005) 1139–1142.
[24] R. Sato, SREBPs: protein interaction and SREBPs, FEBS J. 276 (2009) 622–627.
[25] C.N.A. Palmer, M.-H. Hsu, K.J. Griffin, J.L. Raucy, E.F. Johnson, Peroxisome proliferator activated receptor-α expression in human liver, Mol. Pharmacol. 53 (1998) 14–22.
[26] R. Mukherjee, L. Jow, D. Noonan, D.P. McDonnell, Human and rat peroxisome proliferator activated receptors (PPARs) demonstrate similar tissue distribution but different responsiveness to PPAR activators, J. Steroid Biochem. Mol. Biol. 51 (1994) 157–166.
[27] J.K. Reddy, Peroxisome proliferators and peroxisome proliferator-activated receptor α. Biotic and xenobiotic sensing, Am. J. Pathol. 164 (2004) 2305–2321.
[28] P. Gervois, I.P. Torra, G. Chinetti, T. Grotzinger, G. Dubois, J.C. Fruchart, J. Fruchart-Najib, E. Leitersdorf, B. Staels, A truncated human peroxisome proliferator-activated receptor α splice variant with dominant negative activity, Mol. Endocrinol. 13 (1999) 1535–1549.
[29] J. Ye, Regulation of PPARγ function by TNF-α, Biochem. Biophys. Res. Commun. 374 (2008) 405–408.
[30] J. Poniachik, A. Csendes, J.C. Díaz, J. Rojas, P. Burdiles, F. Maluenda, G. Smok, R. Rodrigo, L.A. Videla, Increased production of IL-1α and TNF-α in lipopolysaccharide-stimulated blood from obese patients with non-alcoholic fatty liver disease, Cytokine 33 (2006) 252–257.
[31] K. Murakami, T. Ide, M. Suzuki, T. Mochizuki, T. Kadowaki, Evidence for direct binding of fatty acids and eicosanoids to human peroxisome proliferators-activated receptor alpha, Biochem. Biophys. Res. Commun. 260 (1999) 609–613.
[32] A. Elizondo, J. Araya, R. Rodrigo, C. Signorini, C. Sgherri, M. Comporti, J. Poniachik, L.A. Videla, Effects of weight loss on liver and erythrocyte polyunsaturated fatty acid pattern and oxidative stress status in obese patients with non-alcoholic fatty liver disease, Biol. Res. 41 (2008) 59–68.
[33] T. Yamauchi, J. Kamon, H. Waki, Y. Terauchi, N. Kubota, K. Hara, Y. Mori, T. Ide, K. Murakami, N. Tsuboyama-Kasaoka, O. Ezaki, Y. Akanuma, O. Gavrilova, C. Vinson, M.L. Reitman, H. Kagechika, K. Shudo, M. Yoda, Y. Nakano, K. Tobe, R. Nagai, S. Kimura, M. Tomita, P. Froguel, T. Kadowaki, The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity, Nat. Med. 7 (2001) 941–946.
[34] M. Kohjima, M. Enjoji, N. Higuchi, M. Kato, K. Kotoh, T. Yoshimoto, T. Fujino, M. Yada, R. Yada, N. Harada, R. Takayanagi, M. Nakamuta, Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease, Int. J. Mol. Med. 20 (2007) 351–358.
[35] M. Nakamuta, M. Kohjima, N. Higuchi, M. Kato, K. Kotoh, T. Yoshimoto, M. Yada, R. Yada, R. Takemoto, K. Fukuizumi, N. Harada, A. Taketomi, Y. Maehara, M. Nakashima, M. Enjoji, The significance of differences in fatty acid metabolism between obese and non-obese patients with non-alcoholic fatty liver disease, Int. J. Mol. Med. 22 (2008) 663–667.
[36] S.D. Clarke, Polyunsaturated fatty acid regulation of gene transcription: a molecular mechanism to improve the metabolic syndrome, J. Nutr. 131 (2001) 1129–1132.
[37] M. Capanni, F. Calella, M.R. Biagini, S. Genise, L. Raimondi, G. Bedogni, G. Svegliati-Baroni, F. Sofi, S. Milani, R. Abbate, C. Surrenti, A. Casini, Prolonged n-3 polyunsaturated fatty acid supplementation ameliorates hepatic steatosis in patients with non-alcoholic fatty liver disease: a pilot study, Aliment. Pharmacol. Ther. 23 (2006) 1143–1151.
[38] L.A. Videla, Oxidative stress and insulin resistance as interdependent pathogenic mechanisms in non-alcoholic fatty liver disease associated with obesity, in: S. Alvarez, P. Evelson (Eds.), Free Radical Pathophysiology, Transworld Research Network, Kerala, India, 2008, pp. 369–385.
[39] P. Ferré, F. Foufelle, SREBP-1c transcription factor and lipid homeostasis: clinical perspective, Horm. Res. 68 (2007) 72–82.
[40] L.A. Videla, G. Tapia, R. Rodrigo, P. Pettinelli, D. Haim, C. Santibañez, A.V. Araya, G. Smok, A. Csendes, L. Gutierrrez, J. Rojas, J. Castillo, O. Korn, F. Maluenda, J.C. Díaz, G. Rencoret, J. Poniachik, Liver NF-κB and AP-1 DNA binding in obese patients, Obesity 17 (2009) 973–979.