

OXIDATIVE STRESS AND DEPLETION OF HEPATIC LONG-CHAIN POLYUNSATURATED FATTY ACIDS MAY CONTRIBUTE TO NONALCOHOLIC FATTY LIVER DISEASE

LUIS A. VIDELA,* RAMÓN RODRIGO,* JULIA ARAYA,[†] and JAIME PONIACHIK[‡]

* Programa de Farmacología Molecular y Clínica, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Chile;

[†] Departamento de Nutrición, Facultad de Medicina, Chile; [‡] Unidad de Gastroenterología, Departamento de Medicina, Hospital Clínico de la Universidad de Chile, Santiago-7, Chile

Abstract—Human nonalcoholic fatty liver disease (NAFLD) associated with obesity is characterized by depletion of hepatic n-3 long-chain polyunsaturated fatty acids (LCPUFA), with lower LCPUFA product/precursor ratios and higher 18:1n-9 trans levels in adipose tissue, both in patients with steatosis and in those with steatohepatitis. These changes point to modification of gene expression, with decreased fatty acid oxidation and triacylglycerol export and enhanced lipid synthesis, thereby leading to fat accumulation in the liver. Changes in oxidative stress-related parameters indicate a moderate enhancement in the pro-oxidant status of the liver in steatosis, which is further exacerbated in steatohepatitis. It is proposed that oxidative stress plays a dual role in NAFLD by contributing to steatosis due to higher peroxidation of LCPUFA, in addition to defective fatty acid desaturation and diet imbalance, and by promoting progression of steatosis to steatohepatitis, features that might involve changes in the activity of transcriptional mediators.

Keywords—Nonalcoholic fatty liver disease, Long-chain polyunsaturated fatty acids, Oxidative stress, Steatosis, Steatohepatitis, Free radicals

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a rapidly growing entity characterized by the presence of macrovesicular steatosis and hepatic necroinflammation with or without fibrosis [1,2]. This clinicopathologic syndrome is frequently associated with obesity and insulin resistance in patients with negligible alcohol consumption [2]. Although in some cases steatosis may progress to steatohepatitis, which increases the subsequent development of cirrhosis [3], the mechanisms involved in the progression of NAFLD are poorly understood.

Hepatic steatosis occurs in conditions of increased lipogenesis, defective mitochondrial/peroxisomal β -oxidation or microsomal cytochrome P4504A ω -oxidation,

and/or a diminished ability of the liver to export lipids. The above alterations may be induced by mutations of genes encoding enzymes of lipid metabolism, as shown for mice deficient in liver carnitine acyl transferase-1 [4] and triacylglycerol transferase [5] with consequent impairment of fatty acid (FA) oxidation and triacylglycerol export, respectively. Alternatively, dietary fats can accomplish regulation of hepatic lipid metabolism through modification of gene transcription [6]. This is achieved by long-chain polyunsaturated fatty acids (LCPUFA; 20:4n-6, 20:5n-3, and 22:6n-3) that are able (i) to direct fatty acids away from triacylglycerol storage, enhancing their oxidation, and (ii) to direct glucose away from FA synthesis, increasing its flux to glycogen [7–9]. This so-called fuel partitioning action of LCPUFA requires both an adequate dietary supply of the 18:2n-6 and 18:3n-3 precursor FA and an optimal activity of the Δ -5/ Δ -6 desaturases to synthesize LCPUFA in the liver [8,10]. Furthermore, the maintenance of a low oxidative stress status in the liver

Address correspondence to: Luis A. Videla, Programa de Farmacología Molecular y Clínica, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Casilla 70000, Santiago-7, Chile; Fax: +56 (2) 7372783; E-mail: lvidela@med.uchile.cl.

constitutes an additional requisite for optimum lipid homeostasis. This contention is based on the high susceptibility of LCPUFA to undergoing free radical reactions, leading to further decomposition processes with loss of unsaturated lipids [11,12]. The goal of this work was to address the hypothesis that depletion of hepatic LCPUFA was a major factor involved in the mechanism of liver steatosis found in NAFLD associated with obesity. This effect may be caused by decreased fatty acid desaturation, diet imbalance, and/or enhanced oxidative stress status in the liver, a redox imbalance that may also contribute to the progression of hepatic steatosis to steatohepatitis.

PATTERN OF LCPUFA IN THE LIVER AND ADIPOSE TISSUE IN NAFLD

Recent studies by our group [13] assessed the levels of LCPUFA in the livers of control subjects and in NAFLD patients, grouped according to the presence of macrovesicular fatty liver alone (steatosis group) or steatosis and

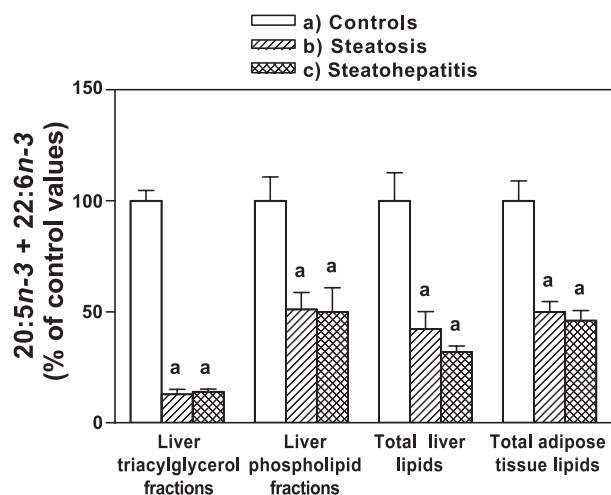


Fig. 1. Content of *n*-3 long-chain polyunsaturated fatty acids [LCPUFA; (20:5*n*-3 + 22:6*n*-3)] in the liver and adipose tissue of control subjects and patients with nonalcoholic fatty liver disease showing steatosis or steatohepatitis. Studies were performed in 19 patients with an average body mass index (BMI) of $45.6 \pm 8.3 \text{ kg/m}^2$ and an age range of 39–45 years who underwent voluntary therapeutic gastroplasty or gastrectomy with a gastrojejunal anastomosis. Eleven patients (BMI = $27.8 \pm 1.6 \text{ kg/m}^2$; age range 38–44 years) who underwent voluntary anti-reflux surgery were taken as the control group. All subjects exhibited normal values for aminotransferases in plasma and for blood tests controlling for diabetes and other chronic diseases and registered a consumption of two alcohol drinks per week to none. The content of lipid in liver and adipose tissue was assessed in methylated chloroform extracts by gas-liquid chromatography analysis using purified standards. Original data in g/100 g methyl esters are expressed as percentages of control values \pm SEM (control values of *n*-3 LCPUFA in g/100 g methyl esters: 0.86 ± 0.04 , 1.29 ± 1.4 , 5.91 ± 0.75 , and 2.18 ± 0.20 in liver triacylglycerol fractions, hepatic phospholipid fractions, total liver lipids, and total adipose tissue lipids, respectively). Significance studies: $p < .05$ compared to control values, assessed by one-way ANOVA and the Bonferroni's multiple comparison test (modified from [13]; used by permission of the publisher).

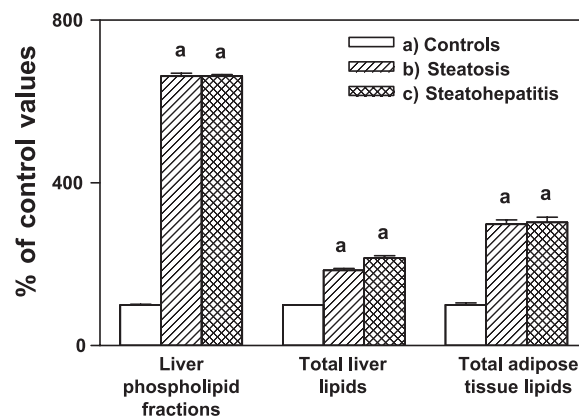


Fig. 2. Ratios of *n*-6/*n*-3 long-chain polyunsaturated fatty acids (20:4*n*-6/(20:5 + 22:6)*n*-3) in the liver and adipose tissue of control subjects and patients with nonalcoholic fatty liver disease showing steatosis or steatohepatitis. Results are expressed as percentages of control values \pm SEM [control values ($n = 11$): 0.16 ± 0.01 , 3.89 ± 0.06 , and 4.13 ± 0.69 in liver phospholipid fractions, total liver lipids, and total adipose tissue lipids, respectively]. $p < .05$ versus controls. For description of subjects, analytical methodology, and statistical analysis, see legend for Fig. 1 (modified from [13]; used by permission of the publisher).

lobular inflammation with hepatocyte ballooning (steatohepatitis group) (Fig. 1). The liver of NAFLD patients exhibited a significant increased accumulation of triacylglycerols compared to control values [mg/g liver: controls, 48 ± 4 ($n = 11$); steatosis, $256 \pm 5^*$ ($n = 10$); steatohepatitis, $250 \pm 4^*$ ($n = 9$); $*p < .05$ versus controls] [13] and decreases in LCPUFA, particularly of the *n*-3 series, in both whole liver extracts and hepatic triacylglycerol or phospholipid fractions (Fig. 1). The latter observation is independent of the presence of an inflammatory component in the liver and occurs concomitant with (i) *n*-3 LCPUFA depletion in adipose tissue (Fig. 1), (ii) higher *n*-6/*n*-3 LCPUFA ratios in total liver lipids corresponding to higher ratios in hepatic phospholipid fractions as well as in total adipose tissue lipids (Fig. 2), (iii) reduced hepatic 20:4*n*-6/18:2*n*-6 and (20:5 + 22:6)*n*-3/18:3*n*-3 ratios (Fig. 3A), and (iv) enhanced levels of 18:1*n*-9 *trans* in adipose tissue (Fig. 3B). Collectively, these data suggest that the development of fatty liver may involve different mechanisms dysregulating lipid metabolism in the liver (Fig. 4).

First, LCPUFA downregulate sterol regulatory element binding protein-1 (SREBP-1) expression and impair its processing, leading to inhibition of the transcription of lipogenic and glycolytic genes [14,15]. Decreased DNA binding of SREBP-1 may reduce that of nuclear factor- κ B and stimulatory protein-1, leading to a lower transactivation activity of the three transcription factors, thus reinforcing the downregulation of lipogenic genes [16]. On the other hand, LCPUFA upregulate the expression of genes encoding enzymes of FA oxidation, due to their function as ligand activators of the peroxisome proliferator-activated receptor- α (PPAR- α)

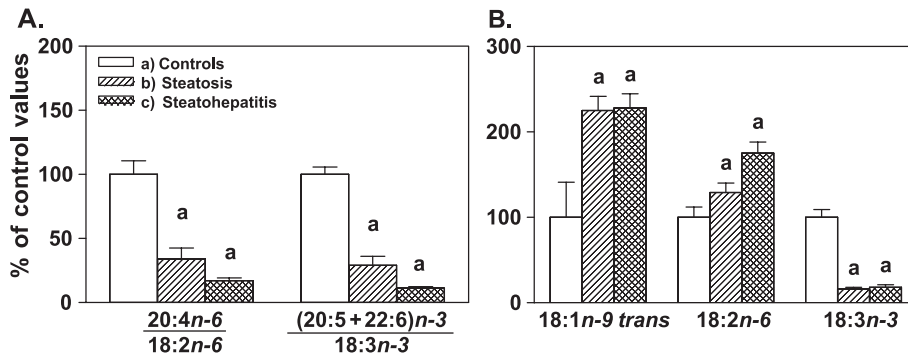


Fig. 3. (A) Liver LCPUFA product/precursor ratios and (B) adipose tissue content of 18:1n-9 trans, 18:2n-6, and 18:3n-3 in control subjects and patients with nonalcoholic fatty liver disease showing steatosis or steatohepatitis. Results are expressed as percentages of control values ± SEM [control values (n = 11): (A) liver 20:4n-6/18:2n-6 ratio, 0.47 ± 0.05; liver (20:5 + 22:6)n-3/18:3n-3 ratio, 17.3 ± 1.0; (B) adipose tissue content (in g/100 g methyl esters) of 18:1n-9 trans, 5.96 ± 2.4; 18:2n-6, 8.67 ± 1.06; 18:3n-3, 1.88 ± 0.18]. *p < .05 versus controls. For description of subjects, analytical methodology, and statistical analysis, see legend for Fig. 1 (modified from [13]; used by permission of the publisher).

[9,17,18]. Therefore, depletion of LCPUFA in the liver of NAFLD patients is expected to favor FA and triacylglycerol synthesis over FA oxidation (Fig. 4). Diminution in triacylglycerol export from the liver is also likely to occur under conditions of LCPUFA depletion, considering that PPAR-α activation by LCPUFA increases the secretion of apolipoprotein B-100 (apoB-100), a rate-limiting step in hepatic very low density lipoprotein (VLDL) formation [19]. This is in agreement with the reduced rate of apoB-100 synthesis reported in NAFLD

patients with steatohepatitis compared with BMI-matched controls without steatohepatitis and lean controls [20]. In addition, the assembly and secretion of VLDL depend on liver FA binding protein [19], a cytosolic protein that is upregulated by both LCPUFA [21] and PPAR-α activation [22]. These views are further strengthened by the substantial increase in hepatic n-6/n-3 LCPUFA ratios found in NAFLD patients (Fig. 2), considering that n-3 LCPUFA are more effective PPAR-α activators than n-6 LCPUFA [8,23].

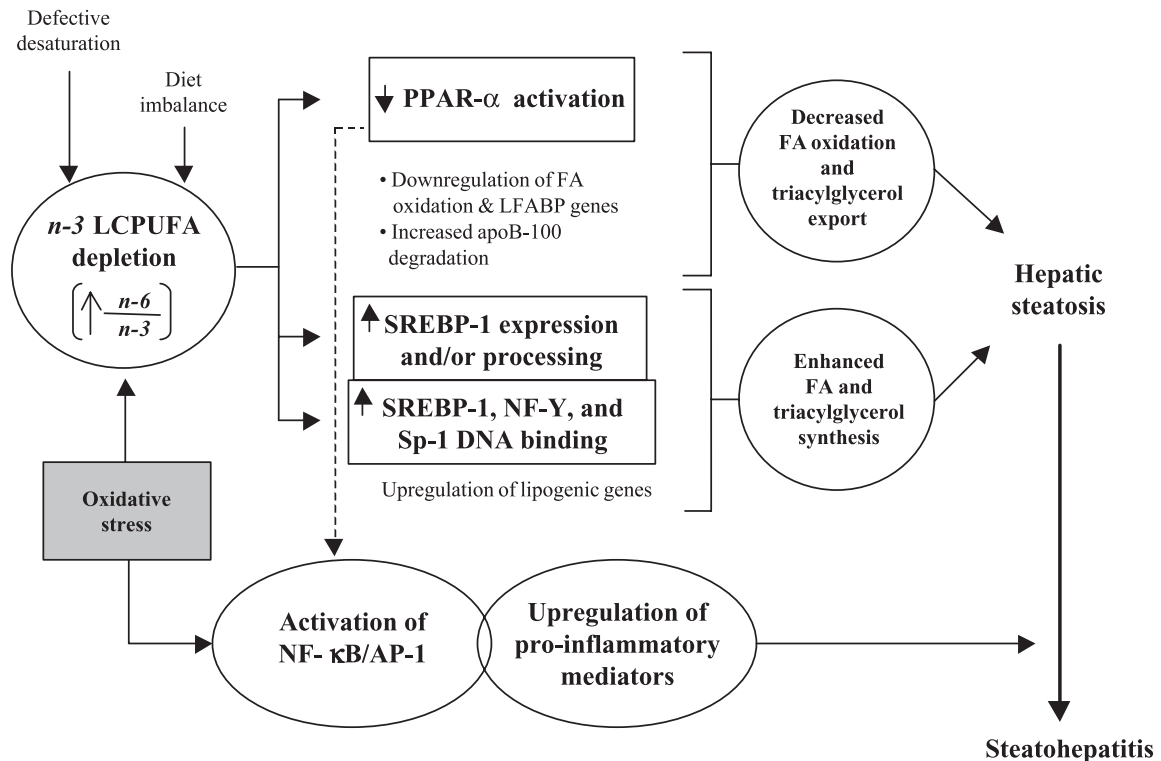


Fig. 4. Factors that influence liver LCPUFA depletion leading to hepatic steatosis and its progression to steatohepatitis in nonalcoholic fatty liver disease.

Second, pathways for desaturation and elongation of the essential FA 18:2*n*-6 and 18:3*n*-3 are required for the synthesis of LCPUFA in the liver, and the adipose tissue content of these essential FA is a suitable biomarker of dietary FA intake [24]. Although adipose tissue levels of 18:2*n*-6 FA are enhanced in NAFLD patients, suggesting an adequate intake for utilization in the liver, those of 18:3*n*-3 are drastically decreased (Fig. 3B). The latter finding points to a limiting factor in the hepatic synthesis of 18:3*n*-3-derived LCPUFA such as 22:6*n*-3 [10] and is consistent with the depletion of *n*-3 LCPUFA found in the liver of NAFLD patients (Fig. 1). Furthermore, impairment of Δ -5 and Δ -6 FA desaturases in the livers of NAFLD patients is suggested by (i) the substantial diminution in LCPUFA product/precursor ratios (Fig. 3A) and (ii) the increased content of the 18:1*n*-9 *trans* isomer in adipose tissue (Fig. 3B). The latter *trans* FA exerts a substantial inhibitory action on hepatic Δ -6 desaturase activity [10,25] and PUFA biosynthesis [10]. Thus, a decreased desaturation activity is likely to occur in the livers of NAFLD patients due to dietary imbalance (low 18:3*n*-3 intake) and *trans* FA-dependent inhibition of desaturases (high 18:3*n*-3 *trans* intake) as contributory factors leading to LCPUFA depletion (Fig. 4). However, the assessment of the activity of hepatic desaturases is needed to ensure the involvement of defective desaturation in human NAFLD. Although data concerning *n*-3 LCPUFA pattern (Fig. 1) and *n*-6/*n*-3 ratios (Fig. 2) are comparable in the liver of NAFLD patients with steatosis and steatohepatitis exhibiting normal serum transaminase levels [13], significant changes may occur with increased severity of hepatic lesions. This is in agreement with the low plasma levels of LCPUFA reported in patients with end-stage liver disease [26] or in cirrhotic patients with hepatitis B and C [27].

LIVER OXIDATIVE STRESS STATUS IN NAFLD

Imbalance in the pro-oxidant/antioxidant equilibrium in favor of pro-oxidants constitutes the major feature of the oxidative stress phenomenon [11], a condition that leads to a number of pathophysiological events in the liver [28–30]. At the cellular level, oxidative stress induces a wide spectrum of responses, depending on the cell type, the magnitude of the level of reactive oxygen species (ROS) achieved, and the duration of the exposure [31]. Under conditions of a high and/or sustained production of ROS, severe damage to PUFA, proteins, and DNA can occur, with loss of their biological functions and cell viability [11,28–31]. In addition, transient variations of low to moderate levels of ROS may represent important regulatory signals triggering defensive mechanisms to liver injury in Kupffer cells, resident macrophages in the liver [30,31], or the regulation of protein function by reversible

oxidation or nitrosation of protein sulfhydryls [32]. Therefore, dysregulation of these mechanisms can induce hepatotoxicity either through the activation of redox-sensitive transcription factors such as NF- κ B and AP-1, with the upregulation of cytotoxic, proinflammatory, and/or fibrogenic mediators [30], or by inducing irreversible nonspecific protein oxidation leading to permanent loss of function and elimination of the damaged protein [32].

Several lines of evidence suggest that chronic oxidative stress is concomitant in human NAFLD [33–35]. As can be observed in Table 1, the liver protein oxidation index is markedly enhanced in NAFLD patients with steatosis compared with controls, whereas GSH content, superoxide dismutase (SOD) activity, and the antioxidant capacity of plasma are significantly lower. Hepatic malondialdehyde (MDA) levels are increased in steatosis when expressed per milligram of protein ($p < .05$) but are not significantly different from controls when expressed per gram of liver (Table 1), a discrepancy that may be due to underestimation of the values in the latter case. Moreover, liver 3-nitrotyrosine reactivity also increases significantly in steatosis compared to control values [36]. These findings are observed in the absence of significant changes in the content of hepatic total cytochrome P450 [33], cytochrome P4502E1 (CYP2E1) (Table 1), and *p*-nitrophenol hydroxylation (*p*-NPH; as a measure of CYP2E1 activity) [33] or in the plasma levels of the lipid peroxidation end-products MDA and 4-hydroxynonenal (HNE) (Table 1). Collectively, the data reported point to a moderate enhancement in the oxidative stress status in the liver of NAFLD patients with steatosis. Because this enhanced pro-oxidant condition implies a higher free radical activity in the liver of NAFLD patients with steatosis, peroxidation of lipids containing PUFA is likely to be favored, considering that their allylic hydrogen atoms are easily removed to generate a radical site subject to O₂ addition [11,12]. These processes are followed by decomposition reactions with loss of unsaturated lipids [11,12], which are particularly relevant for LCPUFA, as the degree of lipid unsaturation increases the length of lipid radical-chain reactions [12]. Thus, oxidative stress-dependent lipid peroxidation may represent an alternate mechanism contributing to liver *n*-3 LCPUFA depletion, leading to steatosis in NAFLD patients (Fig. 4). However, the increased hepatic MDA levels found in steatosis (Table 1) suggest a higher free radical-mediated breakdown involving *n*-6 LCPUFA rather than *n*-3 LCPUFA. This observation is in agreement with the higher content of 20:6*n*-6 found in adipose tissue lipids [g/100 g FA methyl esters: controls, 0.34 \pm 0.04 ($n = 11$); steatosis, 0.69 \pm 0.06* ($n = 10$); steatohepatitis, 0.63 \pm 0.09* ($n = 9$); * $p < 0.05$ versus controls] and in liver phospholipid fractions [g/100 g FA methyl esters: controls, 2.00 \pm 1.00 ($n = 11$); steatosis, 7.00 \pm 1.1* ($n = 10$); steatohepatitis, 6.90 \pm

Table 1. Oxidative Stress-related Parameters in the Liver and Plasma of Control Subjects and Patients with Nonalcoholic Fatty Liver Disease Showing Steatosis or Steatohepatitis

Parameter	Controls (a)	Steatosis (b)	Steatohepatitis (c)	Reference
<i>Liver</i>				
Superoxide dismutase activity	100 ± 10	52 ± 5 ^a	36 ± 7 ^a	[33]
Catalase activity	100 ± 11	90 ± 14	52 ± 10 ^{a,b}	[33]
GSH content	100 ± 10	43 ± 8 ^a	73 ± 13 ^a	[33]
Cytochrome P4502E1 content	100 ± 10	142 ± 12 ^c	242 ± 22 ^{a,b}	[33]
Malondialdehyde content	100 ± 5	281 ± 9 ^a	290 ± 13 ^a	[33]
	100 ± 11	145 ± 14	188 ± 56 ^a	[34]
Protein oxidation index	100 ± 26	403 ± 103 ^{a,c}	174 ± 32 ^b	[33]
<i>Plasma</i>				
Antioxidant capacity	100 ± 3	79 ± 2 ^{a,c}	67 ± 2 ^{a,b}	[33]
Malondialdehyde levels	100	124	133 ^a	[35]
4-Hydroxynonenal levels	100	145	188 ^a	[35]

Values shown correspond to means ± SEM when available, expressed as a percentage of control values: superoxide dismutase activity, 12.6 ± 1.2 ($n = 6$) U/mg protein; catalase activity, 0.55 ± 0.06 ($n = 6$) k/mg protein; reduced glutathione (GSH) content, 23.8 ± 2.4 ($n = 5$) nmol/mg protein; cytochrome P4502E1 content, 1.0 ± 0.1 ($n = 6$) arbitrary image density; liver malondialdehyde content, 2.25 ± 0.11 ($n = 11$) nmol/mg protein [33] and 25.3 ± 2.8 ($n = 4$) nmol/g liver [34]; protein oxidation index, 0.98 ± 0.26 ($n = 5$) nmol carbonyls/mg protein; antioxidant capacity of plasma, 390 ± 11 ($n = 12$) μM iron; plasma malondialdehyde levels, 0.3 nmol/ml; plasma 4-hydroxynonenal levels, 0.3 nmol/ml. The significant differences between mean values ($p < .05$) assessed by one-way ANOVA followed by Bonferroni's multiple comparison test [33,34] or the Wilcoxon rank test [35] are shown by the letters identifying each group.

1.0* ($n = 9$); * $p < .05$ versus controls] [13], indicating a higher dietary intake and availability of the $n-6$ LCPUFA to sustain enhanced peroxidation. The direct demonstration of $n-3$ LCPUFA oxidation by assessment of aldehydic end-products such as 4-hydroxyhexenal from 22:6 $n-3$ awaits further investigation.

Depression of liver SOD activity and GSH content and enhancement of the MDA levels observed in NAFLD patients with steatosis persist in those with steatohepatitis, features that are accompanied by (i) reduction in hepatic catalase activity and CYP2E1 induction; (ii) increases in plasma MDA and HNE levels, liver inducible nitric oxide synthase [37], and hepatic nitrotyrosine reactivity [36,37]; and (iii) a further reduction in the antioxidant capacity of plasma (Table 1). In addition to the enhancement in hepatic lipid peroxidation as shown by indicators such as MDA and HNE (Table 1) [38], NAFLD patients with steatohepatitis exhibited higher 8-hydroxydeoxyguanosine content in hepatocytes and sinusoidal cells, a marker of oxidative DNA damage [38]. However, the level of hepatic oxidized proteins in steatohepatitis was comparable to that in controls (Table 1), a finding that may involve an enhanced protein degradation due to the higher susceptibility to proteolytic attack caused by ROS-induced protein carbonylation [32,39]. These studies point to a further enhancement in the oxidative stress status of the liver in steatohepatitis compared with steatosis, which is coincident with an enhanced p -NPH activity [32] related to upregulation of CYP2E1 expression (Table 1) [33,40]. The latter finding is in agreement with reports measuring liver CYP2E1 by immunohistochemistry [41] or by the pharmacokinetics

of chlorzoxazone [40,42,43]. From a pathological point of view, CYP2E1 is considered of particular interest due to (i) its poor coupling with NADPH-cytochrome P450 reductase, with significant NADPH oxidase activity generating the superoxide radical, hydrogen peroxide, and consequent lipid peroxidation response [44]; and (ii) its induction occurring primarily within the perivenular region of the liver lobule [44], a response triggered by FA and ketones [45] known to be increased in steatohepatitis [36]. The regulation of hepatic CYP2E1 expression by increased levels of FA and ketones is not exerted at the transcriptional level [45] but rather through posttranslational events prolonging its in vivo half-life [46]. In addition, insulin resistance, which is a feature of clinical nonalcoholic steatohepatitis associated with obesity, diabetes type II, or hyperlipidemia, leads to increased CYP2E1 levels by loss of the repressive effect of insulin [47]. Therefore, induction of liver microsomal CYP2E1 represents a major free-radical generating system associated with the higher oxidative stress status prevailing in human steatohepatitis, although other mechanisms cannot be ruled out. These include an increased production of ROS by induction of CYP4A [47], stimulation of the NADPH oxidase activity of polymorphonuclear cells and/or mononuclear cells infiltrating the liver [1,2], or enhanced hepatocyte mitochondrial, peroxisomal, and microsomal FA oxidation activity [9]. However, the relative contribution of the latter mechanism in ROS production remains uncertain, considering that the basal lipid oxidation rate in patients with steatohepatitis is comparable to that reported in control subjects [36] and that depletion of $n-3$ LCPUFA (Fig. 1) is likely to

diminish rather than increase hepatic FA oxidation pathways [8,9] (Fig. 4).

DUAL MECHANISTIC SIGNIFICANCE OF OXIDATIVE STRESS IN NAFLD

The data discussed in this work provide substantial evidence for a dual mechanistic significance of oxidative stress in NAFLD associated with obesity, constituting a signaling mechanism leading to hepatic steatosis and its progression to steatohepatitis (Fig. 4).

Fatty liver can result from downregulation of FA oxidation and triacylglycerol export genes and upregulation of lipogenic genes triggered by *n*-3 LCPUFA depletion induced by oxidative stress, diet imbalance, and defective FA desaturation, through decreased PPAR- α activation and/or enhanced expression and activity of SREBP-1 and related transcriptional mediators, respectively (Fig. 4). The concurrence of additional factors may contribute to hepatic steatosis, namely, (i) gene polymorphisms of enzymes of FA oxidation and lipid export that decrease lipid catabolism, (ii) mutations of the genes encoding leptin or its receptor that increase FA synthesis, (iii) excessive hepatic iron accumulation leading to exacerbation of oxidative stress and LCPUFA depletion [2,48,49], and/or (iv) enhanced peripheral lipolysis secondary to insulin resistance that increases the flux of free fatty acids and glycerol to the liver [13]. Insulin resistance and hyperinsulinemia are considered primary metabolic abnormalities in many NAFLD patients leading to fat accumulation in the liver, probably caused by excess free fatty acids, release of several peptide mediators, and/or genetic predisposition [50]. However, both the role and the relative relevance of the above mechanisms in mediating insulin resistance and the significance of excessive insulin signaling with respect to liver disease are major unresolved issues [50].

Progression of hepatic steatosis into steatohepatitis can be promoted by oxidative stress, which is substantially exacerbated in the latter stages of NAFLD (Table 1). Oxidative stress-dependent NAFLD promotion may involve stimulation of Kupffer cells and other resident cells of the hepatic sinusoid, with activation of redox-sensitive transcription factors such as NF- κ B and AP-1 and the resultant expression and release of proinflammatory cytokines, chemokines, adhesion molecules, and/or fibrogenic mediators (Fig. 4) [30,31]. This proposal may account for the increase in hepatic TNF- α mRNA and TNF receptor 1 (p55) mRNA levels reported in NAFLD patients with steatohepatitis [51] and agrees with the elevated levels of TNF- α in serum found under conditions of higher prevalence of small intestinal bacterial overgrowth but normal intestinal permeability and endotoxin levels [52]. Increased plasma levels of TNF- α in patients

with steatohepatitis coincide with higher TNF- α production by peripheral blood monocytes, with a concomitant elevation in the levels and production of interleukins 6 and 8 [53]. Interestingly, TNF- α induced by ROS in Kupffer cells further impairs electron flow in the mitochondrial respiratory chain of hepatocytes, thus predisposing to mitochondrial injury due to secondary enhancement in ROS generation [54] and thereby reinforcing the increase in the oxidative stress status of the liver. In line with this view, ultrastructural mitochondrial lesions are present in patients with steatohepatitis, with concomitant failure in ATP synthesis leading to ATP depletion [36,55,56], a critical factor determining loss of cell viability. It is important to note that the inflammatory response induced in the liver of NAFLD patients may also be related to depressed PPAR- α activity secondary to LCPUFA depletion (Fig. 4). This concept arises from observations that PPAR- α negatively interferes with NF- κ B or AP-1 transcriptional activity by forming inactive complexes with p65 or the amino-terminal domain of *c*-Jun and by inducing I κ B α [57].

PERSPECTIVES

The proposed dual etiopathogenic role of oxidative stress in NAFLD provides the basis for various directions for future research. Thus, nutritional supplementation with antioxidants might be a useful approach to prevent or reverse human NAFLD, as suggested by pilot studies showing that α -tocopherol administration improves liver test results in children [58] and adult [59] NAFLD patients. Contrary to these reports, it was found that loss of weight with or without α -tocopherol administration failed to counteract the dysregulation of proinflammatory cytokine metabolism occurring in NAFLD patients with steatohepatitis, which provided no apparent added benefit to concomitant low-fat diet and exercise [53]. In these studies, however, indexes related to oxidative stress in liver or blood were not evaluated [53,58,59] in order to adequately assess the influence of this redox imbalance in NAFLD. Moreover, modulation of the activity of transcriptional mediators may have potential therapeutic value in NAFLD, considering that (i) dietary PUFAs [5% 20:5*n*-3 plus 15% triolein (18:1*n*-9)] ameliorate hepatic steatosis through a reduction in mature SREBP-1 content with improvement in insulin resistance in leptin-deficient *ob/ob* mice [60]; (ii) the pharmacologic stimulation of PPAR- α with Wy-14,643 prevents the development of dietary steatohepatitis in mice [61], whereas activation of PPAR- γ with glitazones has a beneficial role in inflammatory processes [57] and in lipodystrophy syndromes [50]; and (iii) the inhibition of NF- κ B by means of I κ B super-repressor expression in rats prevents early alcohol-induced liver injury even in the presence of oxidative

stress [62]. Also, knowledge concerning the possible upregulation of the expression of key proteins related to steatosis (SREBP-1, lipogenic enzymes) and in NAFLD progression (cytokines, chemokines, and fibrogenic mediators) may enable the design of specific agents blunting their expression (i.e., antisense oligonucleotides). Finally, evidence has been presented that a mixture of polyenylphosphatidylcholines (PPC) containing dilinoleoylphosphatidylcholine as a potent antioxidant [63] prevents alcohol-induced oxidative stress in rat liver by restoring S-adenosylmethionine levels with replenishment of GSH stores [64]. Although PCC could be of importance in the correction of hepatic oxidative stress in NAFLD, intake of 18:2n-6 may contribute to exacerbating the increase in the n-6/n-3 (Fig. 2) ratio and the dysregulation of lipid metabolism in the liver (Fig. 4). Thus, efforts are needed to identify the etiopathogenic mechanisms underlying the different stages of NAFLD in order to define the time course effectiveness of therapies. These may include a combination of pharmacological strategies with antioxidant supplementation and weight reduction diets to interrupt the course of the disease and improve the outcome of NAFLD patients.

Acknowledgment—This work was supported by Grant 1011057 from the Fondo Nacional de Ciencia y Tecnología (FONDECYT), Santiago, Chile.

REFERENCES

- [1] Chitturi, S.; Farrell, G. C. Etiopathogenesis of nonalcoholic steatohepatitis. *Semin. Liver Dis.* **21**:27–41; 2001.
- [2] Angulo, P. Nonalcoholic fatty liver disease. *N. Engl. J. Med.* **346**:1221–1231; 2002.
- [3] Matteoni, C.; Younossi, Z. M.; McCullough, A. Nonalcoholic fatty liver disease: a spectrum of clinical pathological severity. *Gastroenterology* **116**:1413–1419; 1999.
- [4] Kuwajima, M.; Kono, N.; Horiuchi, M.; Imamura, Y.; Ono, A.; Inui, T.; Kawata, S.; Koizumi, T.; Hayakawa, J.; Saheki, T. Animal model of systemic carnitine deficiency: analysis in C3H-2 strain of mouse associated with juvenile visceral steatosis. *Biochem. Biophys. Res. Commun.* **174**:1090–1094; 1991.
- [5] Björkegren, J.; Beigneux, A.; Bergo, M. O.; Maher, J. J.; Young, S. G. Blocking the secretion of hepatic very low density lipoproteins renders the liver more susceptible to toxin-induced injury. *J. Biol. Chem.* **277**:5476–5483; 2002.
- [6] Sessler, A. M.; Ntambi, J. M. Polyunsaturated fatty acid regulation of gene expression. *J. Nutr.* **128**:923–926; 1998.
- [7] Nakamura, M. T.; Cho, H. P.; Clarke, S. D. Regulation of hepatic delta-6 desaturase expression and its role in the polyunsaturated fatty acid inhibition of fatty acid synthase gene expression in mice. *J. Nutr.* **130**:1561–1565; 2000.
- [8] Clarke, S. D. Polyunsaturated fatty acid regulation of gene transcription: a molecular mechanism to improve the metabolic syndrome. *J. Nutr.* **131**:1129–1132; 2001.
- [9] Reddy, J. K. Nonalcoholic steatosis and steatohepatitis. III. Peroxisomal β -oxidation, PPAR α , and steatohepatitis. *Am. J. Physiol.* **281**:G1333–G1339; 2001.
- [10] Mahfouz, M. M.; Smith, T. L.; Kummerow, F. A. Effect of dietary fats on desaturase activities and the biosynthesis of fatty acids in rat-liver microsomes. *Lipids* **19**:214–222; 1984.
- [11] Sies, H. Biochemistry of oxidative stress. *Angew. Chem. Int. Ed. Engl.* **25**:1058–1071; 1986.
- [12] Sevanian, A.; Hochstein, P. Mechanisms and consequences of lipid peroxidation in biological systems. *Ann. Rev. Nutr.* **5**:365–390; 1985.
- [13] Araya, J.; Rodrigo, R.; Videla, L. A.; Thielemann, L.; Orellana, M.; Pettinelli, P.; Poniachik, J. Increase in long-chain polyunsaturated fatty acids n-6/n-3 ratio in relation to hepatic steatosis in non-alcoholic fatty liver disease patients. *Clin. Sci.* **106**:635–643; 2004.
- [14] Yoshikawa, T.; Shimano, H.; Yahagi, N.; Ide, T.; Matsuzaka, T.; Nakakuki, M.; Tomita, S.; Okasaki, H.; Tamura, Y.; Iizuka, Y.; Ohashi, K.; Takahashi, A.; Sone, H.; Osuga, J.; Gotoda, T.; Ishibashi, S.; Yamada, N. Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. *J. Biol. Chem.* **277**:1705–1711; 2002.
- [15] Matsuzaka, T.; Shimano, H.; Yahagi, N.; Amemiya-Kudo, M.; Yoshikawa, T.; Hasty, A. H.; Tamura, Y.; Osuga, J.; Okasaki, H.; Iizuka, Y.; Takahashi, A.; Sone, H.; Gotoda, T.; Ishibashi, S.; Yamada, N. Dual regulation of mouse delta(5)- and delta(6)-desaturase gene expression by SREBP-1 and PPARalpha. *J. Lipid Res.* **43**:107–114; 2002.
- [16] Osborne, T. F. Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. *J. Biol. Chem.* **275**:32379–32382; 2000.
- [17] Clarke, S. D.; Thuillier, P.; Baille, R. A.; Cha, X. Peroxisome proliferator-activated receptors: a family of lipid-activated transcription factors. *Am. J. Clin. Nutr.* **70**:566–571; 1999.
- [18] Kersten, S. Effects of fatty acids on gene expression: role of peroxisome proliferator-activated receptor alpha, liver X receptor alpha, and sterol regulatory-element binding protein-1c. *Proc. Nutr. Soc.* **61**:371–374; 2002.
- [19] Lindén, D.; Lindberg, K.; Oscarsson, J.; Claesson, C.; Asp, L.; Li, M.; Gustafsson, M.; Borén, J.; Olofsson, S. O. Influence of peroxisome proliferator-activated receptor α agonists on the intracellular turnover and secretion of apolipoprotein (Apo)B-100 and ApoB-48. *J. Biol. Chem.* **277**:23044–23053; 2002.
- [20] Charlton, M.; Sreekumar, R.; Rasmussen, D.; Lindor, K.; Nair, K. S. Apolipoprotein synthesis in nonalcoholic steatohepatitis. *Hepatology* **35**:898–904; 2002.
- [21] Meunier-Durmort, C.; Poirier, H.; Niot, I.; Forest, C.; Besnard, P. Upregulation of the expression of the gene for liver fatty acid-binding protein by long-chain fatty acids. *Biochem. J.* **319**:483–487; 1996.
- [22] Carlsson, L.; Lindén, D.; Masoumeh, J.; Oscarsson, J. Effects of fatty acids and growth hormone on liver fatty acid-binding protein and PPAR α in rat liver. *Am. J. Physiol.* **281**:E772–E781; 2001.
- [23] Kersten, S.; Seydoux, J.; Peters, J. M.; González, F. J.; Desbvergne, B.; Wahli, W. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J. Clin. Invest.* **103**:1489–1498; 1999.
- [24] Baylin, A.; Kabagambe, E. K.; Siles, X.; Campos, H. Adipose tissue biomarkers of fatty acid intake. *Am. J. Clin. Nutr.* **76**:750–757; 2002.
- [25] Larqué, E.; Pérez-Llamas, F.; Puerta, V.; Girón, M. D.; Suárez, M. D.; Zamora, A.; Gil, A. Dietary trans fatty acids affect docosahexaenoic acid concentrations in plasma and liver but not brain of pregnant and fetal rats. *Pediatr. Res.* **47**:278–283; 2000.
- [26] Burke, P. A.; Ling, P. R.; Forse, R. A.; Bistrian, B. R. Conditionally essential fatty acid deficiencies in end-stage liver disease. *Nutrition* **15**:302–304; 1999.
- [27] Okita, M.; Sasagawa, T.; Tomioka, K.; Hasuda, K.; Ota, Y.; Suzuki, K.; Watanabe, A. Habitual food intake and polyunsaturated fatty acid deficiency in liver cirrhosis. *Nutrition* **18**:304–308; 2002.

- [28] Videla, L. A.; Fernández, V.; Carrión, Y.; Azzalis, L. A.; Bairy, A. C. D.; Junqueira, V. B. C. Biochemical mechanisms in hepatotoxicity: oxidative stress induced by xenobiotics and hormonal changes. *J. Braz. Assoc. Adv. Sci.* **47**:385–394; 1995.
- [29] Jaeschke, H.; Gores, G. J.; Cederbaum, A. I.; Hinson, J. A.; Pessayre, D.; Lemasters, J. J. Mechanisms of hepatotoxicity. *Toxicol. Sci.* **65**:166–176; 2002.
- [30] Tsukamoto, H. Redox regulation of cytokine expression in Kupffer cells. *Antioxid. Redox Signaling* **4**:741–748; 2002.
- [31] Martindale, J. L.; Holbrook, N. J. Cellular responses to oxidative stress: signaling for suicide and survival. *J. Cell. Physiol.* **192**:1–15; 2002.
- [32] Klatt, P.; Lamas, S. Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. *Eur. J. Biochem.* **267**:4928–4944; 2000.
- [33] Videla, L. A.; Rodrigo, R.; Orellana, M.; Fernández, V.; Tapia, G.; Quiñones, L.; Varela, N.; Contreras, J.; Lazarte, R.; Csendes, A.; Rojas, J.; Maluenda, F.; Burdiles, P.; Díaz, J. C.; Smok, G.; Thielemann, L.; Poniachik, J. Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. *Clin. Sci.* **106**:261–268; 2004.
- [34] Cortez-Pinto, H.; Filipe, P.; Camilo, M. E.; Baptista, A.; Fernandes, A.; de Moura, M. C. A peroxidação dos lípidos na esteatohepatite não alcoólica: relação com a esteatose. *J. Português Gastroenterol.* **6**:234–240; 1999.
- [35] Loguercio, C.; De Girolamo, V.; de Sio, I.; Tuccillo, C.; Ascione, F.; Baldi, F.; Budillon, G.; Cimino, L.; Di Carlo, A.; Di Marino, M. P.; Morisco, F.; Picciotto, F.; Terracciano, L.; Vecchione, R.; Verde, V.; Del Vecchio Blanco, C. Non-alcoholic fatty liver disease in an area of southern Italy: main clinical, histological, and pathophysiological aspects. *J. Hepatol.* **35**:568–574; 2001.
- [36] Sanyal, A. J.; Campbell-Sargent, C.; Mirshahi, F.; Rizzo, W. B.; Contos, M. J.; Sterling, R. K.; Luketic, V. A.; Shiffman, M. L.; Clore, J. N. Nonalcoholic steatohepatitis: association with insulin resistance and mitochondrial abnormalities. *Gastroenterology* **120**:1183–1192; 2001.
- [37] García-Monzón, C.; Martín-Pérez, E.; Lo Iacono, O.; Fernández-Bermejo, M.; Majano, P. L.; Apolinario, A.; Larrañaga, E.; Moreno-Otero, R. Characterization of pathogenic and prognostic factors of nonalcoholic steatohepatitis associated with obesity. *J. Hepatol.* **33**:716–724; 2000.
- [38] Seki, S.; Kitada, T.; Yamada, T.; Sakaguchi, H.; Nakatani, K.; Wakasa, K. In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases. *J. Hepatol.* **37**:56–62; 2002.
- [39] Stadtman, R. E. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radic. Biol. Med.* **9**:315–325; 1990.
- [40] Chalasani, N.; Gorski, J. C.; Asghar, M. S.; Asghar, A.; Foresman, B.; Hall, S. D.; Crabb, D. W. Hepatic cytochrome P450 2E1 activity in nondiabetic patients with nonalcoholic steatohepatitis. *Hepatology* **37**:544–550; 2003.
- [41] Weltman, M. D.; Farrel, G. C.; Hall, P.; Ongelman-Sundberg, M.; Liddle, C. Hepatic cytochrome P450 2E1 is increased in patients with nonalcoholic steatohepatitis. *Hepatology* **27**:128–133; 1998.
- [42] Lucas, D.; Farez, C.; Bardou, L. G.; Caisse, J.; Attali, J. R.; Valensi, P. Cytochrome P450 2E1 activity in diabetic and obese patients as assessed by chlorzoxazone hydroxylation. *Fundam. Clin. Pharmacol.* **12**:553–558; 1998.
- [43] de la Maza, M. P.; Hirsch, S.; Petermann, M.; Suazo, M.; Ugarte, G.; Binout, D. Changes in microsomal activity in alcoholism and obesity. *Alcohol. Clin. Exp. Res.* **24**:605–610; 2000.
- [44] Lieber, C. S. Cytochrome P-4502E1: its physiological and pathological role. *Physiol. Rev.* **77**:517–544; 1997.
- [45] Zangar, R. C.; Novak, R. F. Effects of fatty acids and ketone bodies on cytochromes P450 2B, 4A, and 2E1 expression in primary cultured rat hepatocytes. *Arch. Biochem. Biophys.* **337**:217–224; 1997.
- [46] Song, B. J.; Veech, R. I.; Park, S. S.; Gelboin, H. V.; Gonzalez, F. J. Induction of rat hepatic N-nitrosodimethylamine demethylase by acetone is due to protein stabilization. *J. Biol. Chem.* **264**:3568–3572; 1989.
- [47] Robertson, G.; Leclercq, I.; Farrell, G. C. Nonalcoholic steatosis and steatohepatitis. II. Cytochrome P-450 enzymes and oxidative stress. *Am. J. Physiol. Gastrointest. Liver Physiol.* **281**:G1135–G1139; 2001.
- [48] Koteish, A.; Diehl, A. M. Animal models of steatosis. *Semin. Liver Dis.* **21**:89–104; 2001.
- [49] Kaplan, L. M. Leptin, obesity, and liver disease. *Gastroenterology* **115**:997–1001; 1998.
- [50] Neuschwander-Tetri, B. A.; Caldwell, S. H. Nonalcoholic steatohepatitis: summary of an AASLD single topic conference. *Hepatology* **37**:1202–1219; 2003.
- [51] Crespo, J.; Cayon, A.; Fernández-Gil, P.; Hernández-Guerra, M.; Mayorga, M.; Domínguez-Diez, A.; Fernández-Escalante, J. C.; Pons-Romero, F. Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology* **34**:1158–1163; 2001.
- [52] Wigg, A. J.; Roberts-Thomson, I. C.; Dymock, R. B.; McCarthy, P. J.; Grose, R. H.; Cummins, A. G. The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxemia, and tumor necrosis factor α in the pathogenesis of non-alcoholic steatohepatitis. *Gut* **48**:206–211; 2001.
- [53] Kugelmas, M.; Hill, D. B.; Vivian, B.; Marsano, L.; McClain, C. J. Cytokines and NASH: a pilot study of the effects of lifestyle modification and vitamin E. *Hepatology* **38**:413–419; 2003.
- [54] Schulze-Osthoff, K.; Bakker, A. C.; Vanhaesebroeck, B.; Beyaert, R.; Jacob, W. A.; Fiers, W. Cytotoxic activity of tumor necrosis factor is mediated by early damage to mitochondrial functions. *J. Biol. Chem.* **267**:5317–5323; 1992.
- [55] Caldwell, S. H.; Swerdlow, R. H.; Khan, E. M.; Iezzoni, J. C.; Hespeneide, E. E.; Parks, J. K.; Parker Jr., W. D. Mitochondrial abnormalities in non-alcoholic steatohepatitis. *J. Hepatol.* **31**:430–434; 1999.
- [56] Cortez-Pinto, H.; Chatham, J.; Chacko, V. P.; Arnold, C.; Rashid, A.; Diehl, A. M. Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. *JAMA* **282**:1659–1664; 1999.
- [57] Delerive, P.; Fruchart, J.-C.; Staels, B. Peroxisome proliferator-activated receptors in inflammation control. *J. Endocrinol.* **169**:453–459; 2001.
- [58] Lavine, J. E. Vitamin E treatment of nonalcoholic steatohepatitis in children: a pilot study. *J. Pediatr.* **136**:734–738; 2000.
- [59] Hasegawa, T.; Yoneda, M.; Nakamura, K.; Makino, I.; Terano, A. Plasma transforming growth factor- β 1 level and efficacy of α -tocopherol in patients with non-alcoholic steatohepatitis: a pilot study. *Aliment. Pharmacol. Ther.* **15**:1667–1672; 2001.
- [60] Sekiya, M.; Yahagi, N.; Matsuzaka, T.; Najima, Y.; Nakakuki, M.; Nagai, R.; Ishibashi, S.; Osuga, J.; Yamada, N.; Shimano, H. Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology* **38**:1529–1539; 2003.
- [61] Ip, A.; Farrell, G. C.; Robertson, G. R.; Hall, D.; Kirsch, R.; Leclercq, I. Central role of PPAR- α -dependent hepatic lipid turnover in dietary steatohepatitis in mice. *Hepatology* **38**:123–132; 2003.
- [62] Uesugi, T.; Froh, M.; Arteel, G. E.; Bradford, B. U.; Gäbele, E.; Wheeler, M. D.; Thurman, R. G. Delivery of I κ B superrepressor gene with adenovirus reduces early alcohol-induced liver injury in rats. *Hepatology* **34**:1149–1157; 2001.
- [63] Aleynik, S. I.; Leo, M. A.; Takeshige, U.; Aleynik, M. K.; Lieber, C. S. Dilinoleoylphosphatidylcholine is the active antioxidant of polyenylphosphatidylcholine. *J. Invest. Med.* **47**:507–512; 1999.

[64] Aleynik, S. I.; Lieber, C. S. Polyenylphosphatidylcholine corrects the alcohol-induced hepatic oxidative stress by restoring S-adenosylmethionine. *Alcohol Alcohol.* **38**:208–212; 2003.

ABBREVIATIONS

AP-1 — activator protein-1

ApoB-100 — apolipoprotein B-100

BMI — body mass index

CYP2E1 — cytochrome P4502E1

FA — fatty acid(s)

GSH — reduced glutathione

HNE — 4-hydroxynonenal

LCPUFA — long-chain polyunsaturated fatty acid(s)

MDA — malondialdehyde

NAFLD — nonalcoholic fatty liver disease

NF- κ B — nuclear factor- κ B

PPAR- α — peroxisome proliferator-activated receptor- α

ROS — reactive oxygen species

SOD — superoxide dismutase

SREBP-1 — sterol regulatory element binding protein-1

VLDL — very low density lipoprotein