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Review Article

Free radical biology for medicine: learning from nonalcoholic fatty liver disease

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ABSTRACT

Reactive oxygen species, when released under controlled conditions and limited amounts, contribute to cellular proliferation, senescence, and survival by acting as signaling intermediates. In past decades there has been an epidemic diffusion of nonalcoholic fatty liver disease (NAFLD) that represents the result of the impairment of lipid metabolism, redox imbalance, and insulin resistance in the liver. To date, most studies and reviews have been focused on the molecular mechanisms by which fatty liver progresses to steatohepatitis, but the processes leading toward the development of hepatic steatosis in NAFLD are not fully understood yet. Several nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs) α/γ/δ, PPARγ coactivators 1α and 1β, sterol-regulatory element-binding proteins, AMP-activated protein kinase, liver-X-receptors, and farnesoid-X-receptor, play key roles in the regulation of lipid homeostasis during the pathogenesis of NAFLD. These nuclear receptors may act as redox sensors and may modulate various metabolic pathways in response to specific molecules that act as ligands. It is conceivable that a redox-dependent modulation of lipid metabolism, nuclear receptor-mediated, could cause the development of hepatic steatosis and insulin resistance. Thus, this network may represent a potential therapeutic target for the treatment and prevention of hepatic steatosis and its progression to steatohepatitis. This review summarizes the redox-dependent factors that contribute to metabolism alterations in fatty liver with a focus on the redox control of nuclear receptors in normal liver as well as in NAFLD.

Contents

Abbreviations: ROS, reactive oxygen species; NR, nuclear receptor; FA, fatty acid; TAG, triacylglycerol; MCD, malonyl-CoA decarboxylase; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD1, stearoyl-CoA desaturase-1; G-3-P, glycerol 3-phosphate; CPT-1, carnitine palmitoyl transferase 1; TCA, tricarboxylic acid; HMG-CoAR, 3-hydroxy-3-methylglutaryl-CoA reductase; ER, endoplasmic reticulum; 4-HNE, 4-hydroxynonenal; NASH, nonalcoholic steatohepatitis; UCP-2, uncoupling protein 2; PPARα/γ/δ, peroxisome proliferator-activated receptors α/γ/δ; PGC-1α/β, PPARγ coactivators 1α and 1β; SREBP, sterol-regulatory element-binding protein; AMPK, AMP-activated protein kinase; LXR, liver-X-receptor; FXR, farnesoid-X-receptor; ACOX, acyl-CoA oxidase; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; FAT/CD36, FA translocase; NRF, nuclear respiratory factor; mtTFA, mitochondrial transcription factor A; NAFLD, nonalcoholic fatty liver disease; FOX, forkhead box class; MAPK, mitogen-activated protein kinase; CREB, cAMP-responsive element-binding protein; IRS, insulin receptor substrate; HC, hydroxycholesterol; NFE2L2, nuclear factor (erythroid-derived 2)-like 2; UPR, unfolded protein response; GPX, glutathione peroxidase; GST, glutathione *S*-transferase; GRx, glutathione reductase; ALA, α-lipoic acid

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Introduction

Oxidative stress, which accounts for the dysfunction or death of hepatocytes and other liver cells, contributes to the pathogenesis of acute and chronic liver diseases [1,2]. Even though reactive oxygen (ROS)[1] and nitrogen species are normally produced by the metabolism of normal cells, in hepatic diseases an overproduction of free radicals that overcomes the antioxidant defenses occurs, inducing liver injury [3]. At high concentrations, free radicals are dangerous for several cellular constituents. However, at low or moderate concentrations, they may act as regulatory mediators in signaling processes. Various sources of free radicals are implicated and can be classified as mitochondrial, principally from Complexes I and III, and extramitochondrial, such as cytochrome P450, xanthine oxidase, nitric oxide synthase, and NADPH oxidase [2]. Neutrophils and Kupffer cells are the primary producers of free radicals in the liver, whereas the major sites of ROS release in hepatocytes are the cytochrome P450 system and mitochondria [4]. In addition, iron may act synergistically with other free radical sources to promote liver lipid peroxidation through the Fenton reaction [5].

Nonalcoholic fatty liver disease (NAFLD), the most frequent hepatic pathology [6], is characterized by the development of oxidative stress and changes in redox balance [7]. The pathogenesis of NAFLD is multifactorial and includes lipid metabolism alterations, mitochondrial dysfunction, inflammation, and oxidative stress [8–12]; moreover, hepatic iron deposits in some cases may contribute to NAFLD, even though their role is still controversial [13]. Excessive accumulation of lipids is strongly associated with insulin resistance [14], and it is widely accepted that NAFLD represents the hepatic manifestation of a systemic impairment of the insulin network [15]. However, it is still unclear whether insulin resistance causes lipid storage in liver or whether the increase in lipids itself or their metabolite intermediates may play a causal role in the development of hepatic or systemic insulin resistance [16]. The homeostasis of metabolic pathways is finely modulated through a network of programs, which involves transcription factors, kinases, and phosphatases, as well as nuclear receptors (NRs). The result is a fine balancing of the intermediary metabolism to meet metabolic demands.

Free radicals play a role in the activation or inhibition of signaling pathways that can modulate cellular lipid metabolism. An example of how oxidative stress may dysregulate redox signaling leading to hepatic steatosis is provided by alcoholic liver disease (ALD) [17]. In fact, the oxidation of ethanol determines a more reduced cellular state and activates the microsomal induction with consequent impaired utilization of oxygen and free radical-induced toxicity [18], which in turn inhibit fatty acid oxidation and promote lipogenesis through the modulation of several NRs [19,20]. Even though NAFLD is histologically identical to ALD, it is not associated with alcohol consumption and presents a different natural history [21]. The dysregulation of redox biology in NAFLD has already been extensively reviewed, particularly pointing out its role in the progression of steatohepatitis and in the involvement of adipokines and immune system [22–26]. The definition of redox-dependent molecular alterations responsible for the development of steatosis provides new insights into the role of ROS as controllers of liver lipid metabolism under physiological and pathological conditions. Moreover, as several findings suggest that increased ROS levels induce various signaling pathways that may trigger insulin resistance in numerous settings [27], a redox control may be implicated in the early development of fatty liver.

Taking into account the pivotal role played by several NRs and transcription factors in the development of NAFLD [28], this review outlines the recent knowledge of the role played by free radicals in the regulation of the transcriptional network that modulates lipid metabolism in NAFLD, suggesting a redox-centered pathogenic theory. Finally, updated evidence of the impact of NAFLD on hepatic antioxidant defense and on the role of antioxidant targeting therapy is also discussed.

Redox regulation of key enzyme activity in lipid metabolism in NAFLD

The liver plays a central role in all the steps of lipid metabolism (schematized in Fig. 1):

- lipogenesis by conversion of excess carbohydrates;
- fatty acid (FA) oxidation to produce energy;
- cholesterol and phospholipid metabolism.

Lipid metabolism is controlled by:

(1) the activity of key enzymes triggered by the binding of an activator or inhibitor;
(2) post-translational modifications, which may shift the equilibrium between an inactive and an active enzyme; and
(3) transcriptional regulation, which affects the level of expression of key enzymes and is effective over a longer time scale.

Cellular redox state may affect the activity of several enzymes involved in lipid metabolism, cause post-translational modifications directly (glutathionylation, carbonylation), or by the modulation of phosphatases/kinases, act as second messengers or induce conformational changes to NRs and/or act as NR ligands [29]. The intracellular redox status is established by several redox pairs, such as NADH/NAD^+, NADPH/$NADP^+$, and reduced glutathione/oxidized glutathione [30,31]. Thus, these ratios serve as an index of the availability of reducing equivalents required for lipogenesis. When an excess of reducing equivalents occurs in rat liver mitochondria, β-oxidation may be partially suppressed [32,33]. Some of the enzymes involved in lipid metabolism whose activity is modulated by redox status are shown in Table 1.

NAFLD is a condition in which hepatocytes, which normally hold only small amounts of storage lipid, contain supraphysiological amounts of fat, caused by an imbalance between lipid uptake and synthesis that exceeds oxidation and removal. Patients affected by NAFLD show an increase in both uptake and synthesis

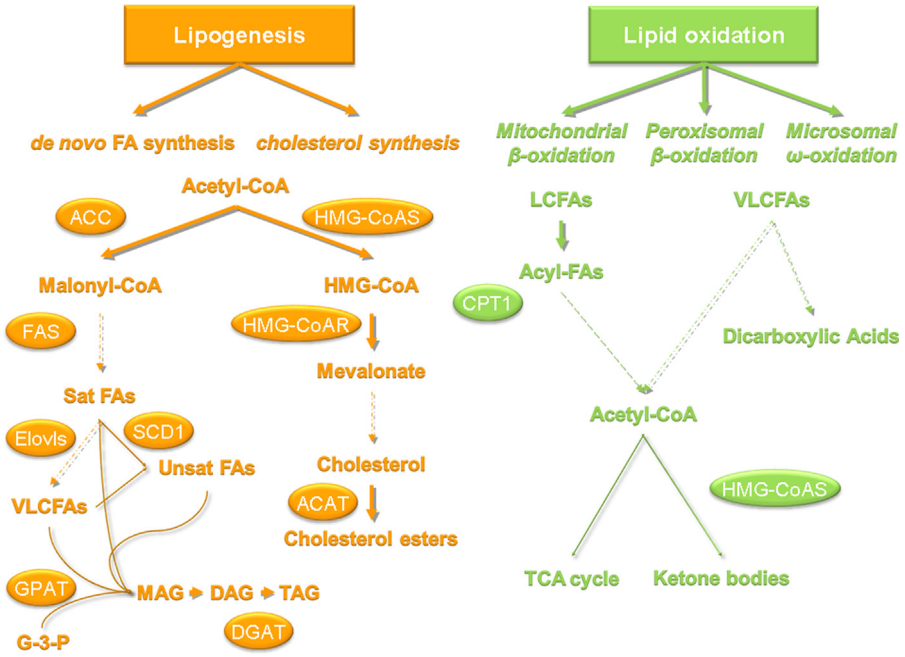

Fig. 1. Liver lipid metabolism. Hepatic lipogenesis includes *de novo* synthesis of fatty acids (FAs) from acetyl-CoA or malonyl-CoA and further processing to triglycerides (TAGs) as energy stores. *De novo* synthesis of FA is started by acetyl-CoA carboxylase (ACC; the rate-limiting step), which converts acetyl-CoA into malonyl-CoA, and fatty acid synthase (FAS), which catalyzes all of the reaction steps in the conversion of malonyl-CoA to saturated FA. The conversion of saturated to monounsaturated FAs is catalyzed by the endoplasmic reticulum enzyme stearoyl-CoA desaturase-1 (SCD1). The formation of very long-chain FAs (VLCFAs) is mediated by elongases (Elovls), a family of microsomal enzymes. TAG synthesis begins with the acylation of glycerol 3-phosphate (G-3-P) with a fatty acyl-CoA, producing lysophosphatidic acid, followed by further acylation and dephosphorylation to yield diacylglycerol (DAG). The acylation of G-3-P represents the first and committed step in glycerolipid biosynthesis; the reaction is catalyzed by acyl-CoA:G-3-P acyltransferase (GPAT), located both in microsomes and in mitochondria. The final step in TAG synthesis involves diacylglycerol acyltransferase (DGAT), which converts DAG to TAG. The liver is also the major site of cholesterol synthesis, which depends on the cellular level of cholesterol induced by changes in the amount and activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoAR), which catalyzes the formation of mevalonate, the limiting step in cholesterol biosynthesis. Cholesterol is synthesized in a 19-step process involving the activity of nine different enzymes. Cholesterol is converted to cholesterol esters primarily by the enzyme acyl-CoA acyltransferase (ACAT), which is allosterically activated by cholesterol itself. Oxidation of FAs occurs within mitochondria and peroxisomes (β-oxidation), or endoplasmic reticulum (ω-oxidation), and facilitates degradation of activated FAs to acetyl-CoA. Short- and medium-chain FAs freely enter mitochondria; in contrast, long-chain FAs (LCFAs) are activated to acyl-CoA esters targeted to esterification or to mitochondrial β-oxidation. Mitochondria are the gateway to the regulation of β-oxidation. Transfer across the mitochondrial membrane is dependent on the activity of carnitine palmitoyl transferase 1 (CPT1), which may be inhibited by malonyl-CoA, produced in the first step of FA synthesis. Peroxisomes are preferentially involved in β-oxidation chain shortening of VLCFAs. Nevertheless, the peroxisomal pathway is quantitatively lesser: FAs are chain-shortened only to shorter chain FAs followed by the transfer of these to mitochondria for full oxidation. VLCFAs are also metabolized by the cytochrome P4504A ω-oxidation system to dicarboxylic acids. These acids are the preferred substrates for the peroxisomal β-oxidation pathway and are further shortened by mitochondria. The final product of FA oxidation, acetyl-CoA, is further processed through the tricarboxylic acid (TCA) cycle or, in the case of FA abundance, is converted into ketone bodies, a process directly controlled by mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoAS).

[34,35], whereas experimental reduction of lipogenesis, consisting in hepatic expression of malonyl-CoA decarboxylase (MCD) or inhibition of acetyl-CoA carboxylase (ACC), reverses hepatic steatosis [36,37]. Liver steatosis increases both lipid oxidation and the tricarboxylic acid (TCA) cycle, whereas ketogenesis is not modified, suggesting that hepatocytes try to counteract lipid excess by enhancing oxidation [38]. β-Oxidation involves four individual reactions that generate reducing equivalents (NADH or FADH2). The energy produced is stored in the form of ATP by the oxidative phosphorylation system coupled with the transfer of electrons along the respiratory chain. The increased formation of reducing equivalents by lipid oxidation causes an overflow of electrons through the mitochondrial respiratory chain, resulting in higher free radical generation (Fig. 2) [10,12]. Peroxisomal β-oxidation generates hydrogen peroxide and is not coupled with phosphorylating systems [39]. Microsomal oxidation also participates in the adaptive response induced by lipid accumulation and in the disorder of redox balance; in particular, cytochromes P4502E1 and P4504A are the major microsomal sources of oxidative stress in NAFLD [40]. Thus, increased oxidation of fat by microsomes and peroxisomes occurs in NAFLD and contributes to oxidative stress (Fig. 2) [41–43].

Liver steatosis induces changes in the redox potential of hepatocytes in both cytoplasm and mitochondria toward a more reduced state, as demonstrated by alterations in the $NADH/NAD^+$ ratio calculated for both cellular compartments from the β-hydroxybutyrate dehydrogenase and lactate dehydrogenase reactions [44], regulating some metabolic pathways occurring in these compartments. The increased formation of reducing equivalents could impair FA oxidation and the TCA cycle [45], but it could also enhance the formation of glycerol-3-phosphate (G-3-P) and thus lipogenesis [46]. In phosphoenolpyruvate carboxykinase-null mice, a genetic model of liver steatosis, high mitochondrial reduction–oxidation state and increased TCA cycle intermediate concentration, as well as reduced oxygen consumption, occur. However, both TCA and pyruvate cycles are dramatically reduced [47].

Saturation of lipids may also modify cellular redox status. Among free FAs, monounsaturated fatty acids, such as oleic acids, are less toxic than palmitate, a saturated acid, because the latter increases the $NADH/NAD^+$ ratio and promotes uncoupling between glycolysis and TCA cycle fluxes, leading to increased ROS production [48]. The hepatic accumulation of saturated FAs can promote redox imbalance and the formation of reactive oxygen intermediates, mainly inducing endoplasmic reticulum (ER) stress and apoptosis [49]. In fact, polyunsaturated FA supplementation significantly improves redox balance and reduces hepatic steatosis [50]. Because saturated FAs are the first product of *de novo* lipogenesis and exert lipotoxic effects by promoting liver fat accumulation, stearoyl-CoA desaturase-1 (SCD1) is able to prevent this accumulation and its toxic effects [51]. On the other

Table 1
Enzymes involved in hepatic lipid metabolism regulated by redox status.

Pathway	Enzyme	Redox regulation
Fatty acid synthesis	Fatty acid synthase	Inhibited by a reduction in NADPH/$NADP^+$ or by a reduction in GSH/GSSG
Fatty acid synthesis	Glycerol-3-phosphate acyltransferase	Activated by an increase in NADH/NAD^+
Cholesterol synthesis	3-Hydroxy-3-methylglutaryl-CoA reductase	Inhibited by a reduction in GSH/GSSG; activated by an increase in NADH/NAD^+
Fatty acid oxidation	Acyl-CoA dehydrogenase	Inhibited by an increase in NADH/NAD^+
Fatty acid oxidation	β-Hydroxyacyl-CoA dehydrogenase	Inhibited by an increase in NADH/NAD^+
Tricarboxylic acid cycle	Citrate synthase	Inhibited by an increase in NADH/NAD^+
Tricarboxylic acid cycle	Isocitrate dehydrogenase	Inhibited by an increase in NADH/NAD^+
Tricarboxylic acid cycle	α-Ketoglutarate dehydrogenase	Inhibited by an increase in NADH/NAD^+

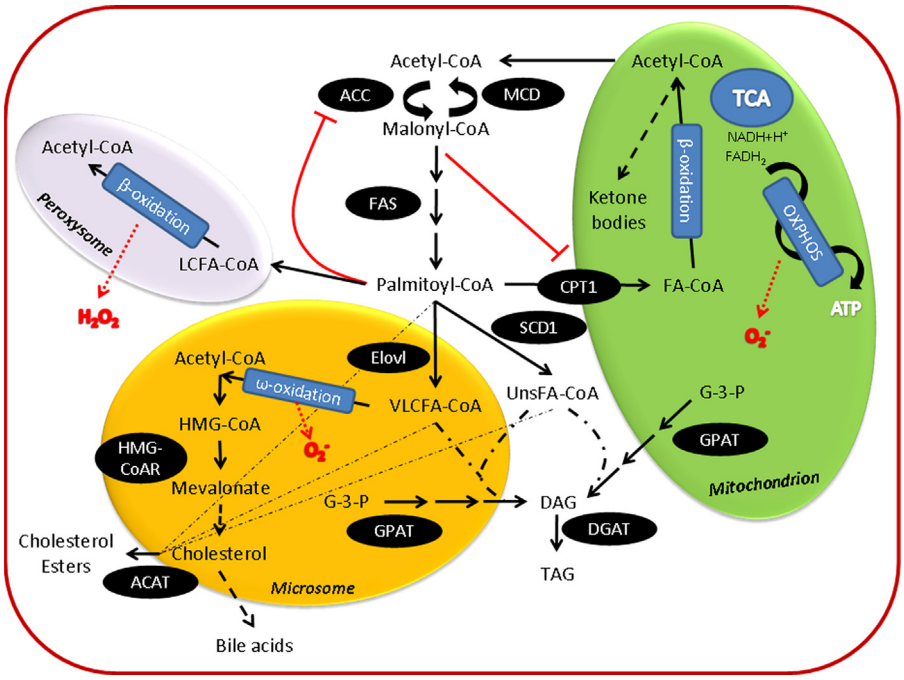

Fig. 2. Sources of free radicals in fatty liver during lipid metabolism. Liver steatosis increases lipid oxidation and activity of the TCA cycle, whereas ketogenesis is not modified. The increased formation of reducing equivalents by lipid oxidation causes an overflow of electrons through the mitochondrial respiratory chain (OXPHOS), resulting in higher free radical generation. Excess of long-chain fatty acids (LCFAs) enhances peroxisomal β-oxidation, with consequent generation of hydrogen peroxide. Accumulation of very long chain fatty acids (VLCFAs) induces microsomal oxidation with consequent production of free radicals particularly by cytochromes P4502E1 and P4504A. Other abbreviations used: ACC, acetyl-CoA carboxylase; MCD, malonyl-CoA desaturase; FAS, fatty acid synthase; CPT1, carnitine palmitoyl transferase 1; SCD1, stearoyl-CoA desaturase 1; Elovl, elongase; HMG-CoAR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; GPAT, acyl-CoA:G-3-P acyltransferase; ACAT, acyl-CoA acyltransferase; DGAT, diacylglycerol acyltransferase; DAG, diacylglycerol; TAG, triacylglycerol.

hand, SCD1 is strongly downregulated in a dietary model of NAFLD, resulting in enhanced delivery of FAs to mitochondria and oxidation that in turn increases mitochondrial ROS production and release [52], and its causes are the object of active investigation.

The role of cholesterol accumulation in hepatic steatosis is an emerging topic, because it can further contribute to the alteration of cellular redox status. Several studies reported that free cholesterol (but not cholesteryl esters) accumulates in human NAFLD and strongly associates with the progression and the severity of liver damage [53,54]. This accumulation arises from high cholesterol synthesis rather than elevated flux from intestinal absorption [51,55]. The accumulation of free cholesterol in liver mitochondria leads to specific mitochondrial glutathione depletion, sensitizes the hepatocytes to tumor necrosis factor and Fas-mediated apoptosis and induces steatohepatitis [56]. Very interestingly the catalytic activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR), the rate-limiting step in cholesterol synthesis, may be regulated by its thiol redox status [57]. Accordingly, cholesterol accumulation depletes mitochondrial glutathione and this redox change milieu may inhibit hepatic HMG-CoAR activity, as suggested in [58].

Redox-dependent post-translational protein modifications in NAFLD

Redox-dependent post-translational protein modifications arise either from direct oxidation of amino acid residues or through the formation of reactive intermediates by the oxidation of other cellular components. Furthermore, a significant portion of ROS-induced post-translational modifications result in the addition of reactive carbonyl functional groups on proteins, generically termed "protein carbonylation," with the most reactive and common of these carbonyl groups being in the form of aldehydes [59]. Protein carbonylation can result directly from a variety of reactions or from an indirect mechanism involving the hydroxyl radical-mediated oxidation of lipids. Recent studies suggested that protein carbonylation formed from lipid-derived aldehydes is more prevalent than that formed via direct amino acid side-chain oxidation [60]. The most reactive aldehydes generated from polyunsaturated fatty acid oxidation are α,β-unsaturated aldehydes, including 4-hydroxynonenal (4-HNE) [61]. Posttranslational protein oxidation frequently leads to enzyme inactivation or targeted degradation, but it can also lead to a functional gain for certain metabolic signaling pathways [59].

Accumulation of oxidatively modified proteins and lipid peroxides acids was described in NAFLD and is assumed to induce liver disease progression [45,62,63]. Protein glutathionylation also increases in livers with nonalcoholic steatohepatitis (NASH) [64]. However, selectively oxidized target proteins are poorly characterized and merit further investigation.

Some proteins involved in FA metabolism may be post-translationally modified. In particular, specific adducts between 4-HNE and CPT-1 were observed in a model of NAFLD, with consequent decreased expression and activity of the protein [65]. Similarly, adducts between 4-HNE and the uncoupling protein 2 (UCP-2) were also reported both in rats and in patients with NASH, but the 4-HNE–UCP-2 adduct was associated with increased uncoupling [12].

Redox balance and insulin control of lipid metabolism in NAFLD

Postprandial insulin secretion induces hepatic glucose uptake by stimulating the translocation of glucose transporter 2 from intracellular vesicles to plasma membrane; moreover, high postprandial insulin inhibits gluconeogenesis and stimulates glycogen synthesis [66]. When not redirected to glycogen pools, excess glucose is used in *de novo* lipogenesis. Insulin triggers lipogenesis, suppresses peripheral lipolysis by inhibiting hormone-sensitive lipase, and indirectly antagonizes mitochondrial FA oxidation by increasing malonyl-CoA concentration [67,68]. Millimolar concentrations of H_2O_2 may activate insulin signaling and/or induce metabolic actions of insulin by the tyrosine phosphorylation of the insulin receptor β-chain [69,70]. The insulin receptor kinase activity itself may be oxidatively regulated, because ATP binding, required for the receptor autophosphorylation process, is modulated by H_2O_2 [71]. However, the main underlying mechanism for the insulinomimetic effect of H_2O_2 is the inhibition of the catalytic activity of various protein and lipid phosphatases, which act as negative regulators and off-mechanisms of insulin signaling [72–75]. Free radicals may strengthen the insulin signaling by acting as second messengers in adipocytes and muscle cells [76,77], but evidence for this in hepatocytes is still lacking. The redox environment may also regulate the insulin-degrading enzyme and thus insulin half-life and action [78].

Hepatic lipid accumulation is strongly dependent on insulin sensitivity; however, the causal relationship has not yet been completely defined, because it is not clear whether hepatic steatosis increases insulin resistance or, conversely, NAFLD is the final effect of insulin resistance. It is conceivable that a common underlying molecular pathophysiology may account for the two effects [79]. Very interestingly, it has been demonstrated that oxidation of palmitate but not oleate induces insulin resistance through excess mitochondrial electron flux and consequent ROS production by inhibiting signal transduction through c-Jun NH_2-terminal kinase (JNK) [80]. Moreover, it was observed that CPT-1 reduction and superoxide dismutase activity, together with impaired FA oxidation and glutathione oxidation, occur before insulin resistance [81].

Different lipid classes may also have different effects on insulin sensitivity, according to their oxidative properties. Dietary supplementation of *n*-3 long-chain-polyunsaturated FAs reduced hepatic lipid content, with concurrent antioxidant and anti-inflammatory responses and improvement of insulin sensitivity [50,82]. Hepatic cholesterol accumulation *per se* does not induce insulin resistance but increases hepatic steatosis [83,84]. Several experimental models clarified that both high fat and cholesterol are needed to produce hepatic steatosis and insulin resistance [83,85]. Because free cholesterol accumulation in mitochondria leads to glutathione depletion and lipid peroxidation [56], and because cholesterol accumulation also leads to oxysterol formation [86], it is conceivable that cholesterol accumulation impairs redox status and induces insulin-dependent signaling. Taken together, this evidence suggests that lipids accumulating in liver steatosis function as bioactive substances that interfere with the ability of hepatocytes to respond to changes in insulin level. However, to date, studies failed to identify a specific lipid necessary and sufficient to determine hepatic insulin resistance [79]. Thus, excess bioactive lipids do not act alone as contributing factors but rather as part of a signal in which radical molecules generate reactive intermediates and exert signaling functions.

Redox control of lipid metabolism by nuclear receptors in normal liver and NAFLD

Control of lipid metabolism is mediated by a network of NRs that respond to several ligands to regulate the expression of enzymes that participate in hepatic lipogenesis or lipoxidation in a thin form of coordination [87]. Several endogenous and exogenous lipids such as cholesterol or FAs act as physiological NR ligands, and NRs may be viewed as “lipostats” because their activation frequently promotes metabolism/catabolism of their respective ligands and/or provides a negative feedback for self-termination of synthesis [88]. Very interestingly, free radical-derived molecules may act as NR ligands or alter their DNA binding or nuclear import [29]. Among the various NRs taking part in the physiological regulation of lipid metabolism and pathological modulation by free radicals during hepatic steatosis (Table 2), the role of peroxisome proliferator-activated receptors α/γ/δ (PPARα/γ/δ), PPARγ coactivators 1α and 1β (PGC-1α/β), sterol-regulatory element binding proteins (SREBPs), AMP-activated protein kinase (AMPK), liver-X-receptors (LXRs), and farnesoid-X-receptor (FXR) are specifically addressed here (Fig. 3).

PPAR family

PPARs are activated by peroxisome proliferators—a miscellaneous group of rodent hepatocarcinogens that include hypolipidemic drugs, plasticizers, and herbicides; there are four PPAR isoforms within vertebrates termed α, γ, and β/δ [89].

PPARα induces genes involved in mitochondrial as well as peroxisomal and microsomal FA oxidation [90–92]. Both polyunsaturated FAs eicosapentaenoic acid (C20:5*n*-3) and docosahexaenoic acid (C22:6*n*-3), and long chain-FA-CoA, bind and activate PPARα and increase FA oxidation, gluconeogenesis, and ketogenesis [93]. PPARα binds to the PPAR response element as a heterodimer with the retinoid-X-receptor (RXR) [94]. The expression of PPARα, by activation of acyl-CoA oxidase (ACOX)—a key enzyme initiating FA β-oxidation—was significantly reduced in a rodent model of NAFLD; on the other hand, PPARα agonists improved steatosis [95,96]. Mice deficient in PPARα and those deficient in both PPARα and ACOX exhibit severe hepatic steatosis when subjected to fasting, indicating that a defect in PPARα-inducible FA oxidation accounts for severe FA overload in liver, in contrast to the wild-type mice, which respond by enhancing FA oxidation [92,97,98]. The expression of PPARα is also reduced in patients with NAFLD, and PPARα gene polymorphism distribution may influence the pathogenesis of human hepatic steatosis [99,100]. Human liver cells exposed to H_2O_2 show a downregulation of PPARα expression, as well as its target genes ACOX and CPT-1, which control FA oxidation, indicating that the alteration of lipid homeostasis might be dependent on a free radical-mediated signaling (Fig. 4) [101]. On the other hand, PPARα expression is associated with that of Cu^{2+}, Zn^{2+}-superoxide dismutase (SOD)

Table 2
Modifications in the expression of nuclear receptors that modulate hepatic lipid metabolism during NAFLD.

Nuclear receptor	Target proteins	Modification in NAFLD
Peroxisome proliferator-activated receptor α	Acyl-CoA oxidase	Reduced expression in humans [99] and animal models [95]
Peroxisome proliferator-activated receptor γ		Increased [99,110] or decreased [109] expression in humans
Peroxisome proliferator-activated receptor γ coactivator α	Mitochondrial transcription factor A	Reduced expression in humans [137]
Sterol-regulatory element-binding protein 1c	Acetyl-CoA carboxylase, fatty acid synthase	Increased expression in humans [99,150]
Sterol-regulatory element-binding protein 2	Hydroxymethyl glutaryl-CoA reductase	Increased expression in humans [154] and rodents [153]
AMP-activated protein kinase		No modifications in humans [150,173]
Liver-X-receptor	Sterol-regulatory element-binding protein 1c	Increased expression in humans [173]
Farnesoid-X-receptor		Decreased expression in humans [207]

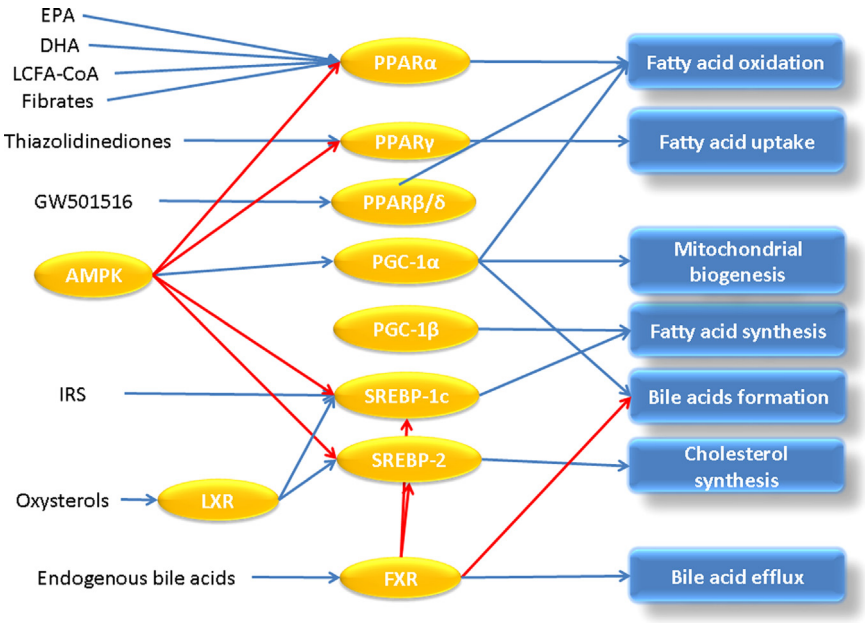

Fig. 3. Nuclear receptor interplay involved in the regulation of lipid metabolism. Fatty acid oxidation is favored by peroxisome proliferator-activated receptors α and β/δ (PPARα and β/δ), respectively activated by polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), long-chain fatty acid-CoA (LCFA-CoA), fibrates, and the synthetic ligand GW501516. In contrast, PPARγ, activated by thiazolidinediones, triggers fatty acid uptake. PPARγ coactivator 1α (PGC-1α) orchestrates a complex program of metabolic changes that occur during the transition of a fed to a fasted liver, including mitochondriogenesis, fatty acid oxidation, and bile acid homeostasis; PGC-1β is induced by dietary intake of fats and leads to hyperlipidemia by activating hepatic lipogenesis. Sterol-regulatory element-binding protein 1c (SREBP-1c), activated by insulin receptor substrate proteins (IRSs), controls hepatic de novo lipogenesis by regulating the expression of key genes involved in fatty acid synthesis and glucose metabolism, whereas SREBP-2 predominantly regulates cholesterol synthesis. AMP-activated protein kinase (AMPK) negatively regulates PPARα and PPARγ, as well as SREBP-1c and -2, but activates PGC-1α to turn on ATP-producing processes, such as fatty acid oxidation, and turn off ATP-consuming processes such as fatty acid synthesis. Liver-X-receptors (LXRs), activated by physiological concentrations of sterols, are crucial for the control of lipid homeostasis by enhancing gene transcription involved in regulation of fatty acid and cholesterol metabolism through SREBP induction. Activation of farnesoid-X-receptor (FXR) by endogenous bile acids inhibits bile acid synthesis from cholesterol and also protects against the toxic accumulation of bile acids through increased conjugation in the liver and secretion into bile canaliculi.

expression in liver [102], and a peroxisome proliferator-response element has been identified in the catalase (CAT) gene [103], suggesting that PPARs may also modulate antioxidant response (Fig. 4). PPARα also protects hepatocytes from potential oxidative damage developed during fasting, because fasted PPARα-null mice exhibit marked hepatic steatosis associated with elevated levels of lipid peroxidation, nitric oxide synthase activity, and hydrogen peroxide accumulation, as well as reduced levels of total glutathione (GSH), mitochondrial GSH, and activities of major antioxidant enzymes [104].

PPARγ plays a crucial role in adipogenesis and insulin sensitization; the main role of PPARγ in the liver is related to the regulation of glucose and lipid metabolism; however, its hepatic expression level is 9–12% that of adipose tissue in humans [105,106]. High expression levels are associated with induction of PPARγ-responsive genes related to lipid metabolism. These include: (1) lipoprotein lipase; (2) proteins involved in FA uptake, binding, and transport, such as FA translocase (FAT/CD36), FA transport proteins 2 and 5, and FA-binding proteins 1 and 5; and (3) LXR favoring both PPARγ and FAT/CD36 expression [107,108]. Assessment of hepatic PPARγ expression in humans with hepatic steatosis has produced conflicting results, showing comparable, decreased, or increased levels [99,109,110]. A comparison between these studies suggests that the expression of PPARγ is enhanced in association with increased body mass index and insulin resistance, which may represent additional mechanisms upregulating genes that encode lipogenic proteins leading to hepatic steatosis [110]. The use of PPARγ ligands such rosiglitazone or pioglitazone for treatment of NAFLD patients has demonstrated an improvement in glucose and lipid metabolism, as well as hepatic inflammation and fat storage [111–113]. However, the treatment response of PPARγ ligands is extremely variable and difficult to predict, because of single-nucleotide polymorphisms in several target genes [114]. The mechanisms underlying the responsiveness or nonresponsiveness

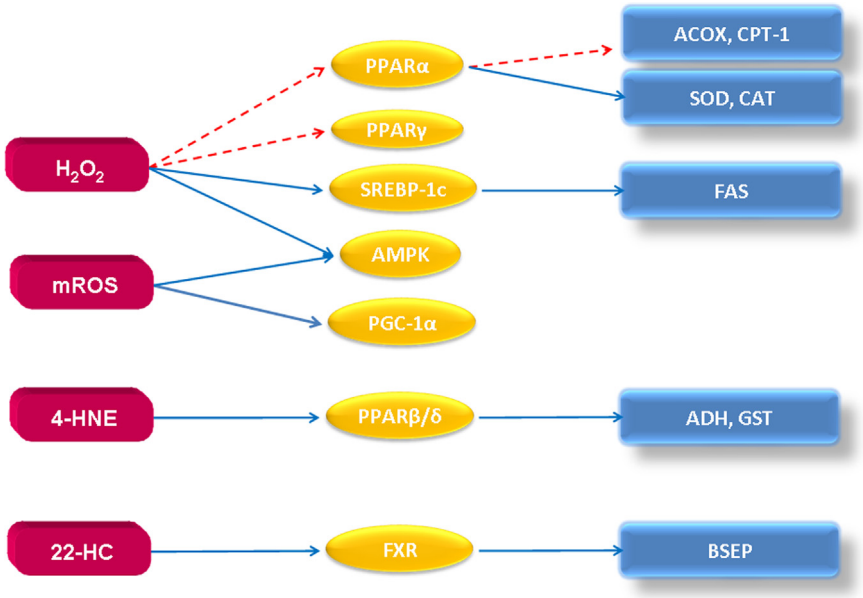

Fig. 4. Interplay between nuclear receptors and free radicals during fatty liver progression. Hydrogen peroxide may downregulate peroxisome proliferator-activated receptors α and γ, with consequent repression of acyl-CoA oxidase (ACOX) and carnitine palmitoyl transferase-1 (CPT-1) and a possible induction of Cu^{2+}, Zn^{2+}-superoxide dismutase (SOD) and catalase (CAT). On the other hand, hydrogen peroxide activates sterol-regulatory element-binding protein 1c (SREBP-1c) and lipogenesis through fatty acid synthase (FAS) induction, as well as AMP-activated protein kinase (AMPK). The latter nuclear receptor can also be activated by reactive oxygen species specifically produced by mitochondria (mROS), probably to preserve the redox environment. Mitochondrial ROS may also play a key role in the control of PPARγ coactivator 1α (PGC-1α) expression, with improvement in insulin sensitivity as final effect. The activation of PPARβ/δ by the lipid peroxidation product 4-hydroxynonenal (4-HNE) upregulates alcohol dehydrogenase (ADH) and glutathione *S*-transferases (GSTs), suggesting a feedback mechanism pointing at a reduction of liver toxicity. The oxysterol 22-hydroxycholesterol (22-HC) activates farnesoid-X-receptor (FXR), inducing the expression of the bile salt export pump (BSEP), which mediates the secretion of bile acids from the liver.

of this treatment are largely unknown [112,113]. Very interestingly, it has been demonstrated that during adipocyte differentiation there is an mTORC1-dependent increase in mitochondrial metabolism and biogenesis with consequent increased production of mitochondrial Complex III ROS resulting in the induction of PPARγ transcriptional machinery required to initiate adipocyte differentiation [115]. However, exposure of endothelial cells to H_2O_2 leads to inhibition of PPARγ gene expression, but does not affect protein level or activity (Fig. 4); these effects are preserved by catalase and are not reproduced by other oxidant molecules [116]. The lipid peroxidation product 4-HNE is effective in the modulation of PPARγ activity through the pathways modulating leukemic cell growth and differentiation [117]. However, there is no evidence relating to the free radical biology of PPARγ in hepatocytes, and this field merits further investigation.

PPARβ/δ is expressed in most metabolically active tissues, but is the only subtype that is not a target of current drugs. Early data have suggested that adipose differentiation-related protein, a lipid droplet coating protein, acts as a lipid sensor, being a PPARβ/δ target gene [118]. Further studies revealed that PPARβ/δ controls an array of metabolic genes involved in glucose homeostasis and fatty acid synthesis/storage, mobilization, and catabolism in a tissue-specific manner [119–121]. Several synthetic ligands have also been developed, to improve symptoms of metabolic disorders [122,123]. Experimental data support that the combined hepatic and muscular effects of PPARβ/δ constitute a "fatty acid futile cycle," resulting in improved glucose and lipid metabolism [124]. The effect of the selective PPARβ/δ ligand GW501516 in a mouse model of hepatic steatosis consisted in reduced hepatic TAG and in the number of fatty liver droplets [125]. A study using adenovirus-mediated liver-restricted PPARβ/δ activation demonstrated the control of hepatic energy substrate homeostasis by coordinated regulation of glucose and fatty acid metabolism, through transcriptional mechanisms partly mediated by its coactivator, PGC-1β, and by AMPK [126].

The extent of liver toxicity by treatment with the xenobiotics azoxymethane, arsenic, or carbon tetrachloride in PPARβ/δ-null mice is more severe than in wild-type mice, suggesting that the presence of this receptor is important in ameliorating the effects of hepatotoxicants [127]. One possible explanation for the increased susceptibility of PPARβ/$\delta^{-/-}$ mice to hepatotoxicity is that oxidative damage increases the production of an endogenous ligand for PPARβ/δ, which would in turn stimulate lipid metabolism and degradation of lipid peroxidation intermediates. Accordingly, it was reported that 4-HNE is able to activate PPARβ/δ receptor in transient transfection reporter assays [128]. Moreover, the activation of PPARβ/δ by 4-HNE increased the expression of alcohol dehydrogenase and glutathione *S*-transferases, providing evidence of a feedback regulation of gene expression that reduces liver toxicity (Fig. 4) and reveals a cross talk between PPARβ/δ and energy homeostasis in cellular events during inflammation [128].

PGC-1 family

Transcriptional coactivators are recruited to liganded NRs and function in part by stimulating gene expression, covalently modifying histones, and mediating interactions between transcription factors [129]. PGC-1 family members are multifunctional transcriptional coregulators that act as "molecular switches" in many metabolic pathways. PGC-1α and PGC-1β regulate adaptive thermogenesis, mitochondrial biogenesis, and glucose/FA metabolism by interacting with various transcription factors in a tissue-specific manner [130].

PGC-1α is abundantly expressed in tissues with high energy demand and strongly stimulates the program of nuclear- and mitochondrial-encoded mitochondrial genes as well as organelle

biogenesis through its coactivation of nuclear respiratory factors 1 and 2 (NRF1 and NRF2) and the estrogen-related receptor α [131–133]. The induction of NRF1 and NRF2 leads in turn to the increased expression of mitochondrial transcription factor A (mtTFA) as well as other mitochondrial subunits of the electron transport chain complex such as ATP synthase, cytochrome *c*, and cytochrome oxidase [131,134]. mtTFA translocates to the mitochondrial matrix, where it stimulates mitochondrial DNA replication and mitochondrial gene expression [135]. PGC-1α orchestrates a complex program of metabolic changes that occur during the transition of a fed to a fasted liver, including gluconeogenesis, FA oxidation, ketogenesis, and bile acid homeostasis. These effects are achieved by coactivating key hepatic transcription factors, such as hepatic nuclear factor 4α, PPARα, forkhead box class O1 (FOXO1), FXR, and LXRs [136]. Transcriptional activity of PGC-1α is tightly related to peripheral insulin sensitivity in NAFLD patients through epigenetic modifications [137].

There is significant evidence as regards the role of PGC-1α as a redox sensor. When primary vascular endothelial cells are treated with NO donors, a short-term downregulation of PGC-1α mediated by protein kinase G occurs after short-term treatment, whereas a long-term treatment upregulates PGC-1α, suggesting that the primary effect of NO is the suppression of PGC-1α expression, and the induction phase must be a secondary process [138]. These changes lead to variations in the expression of antioxidant genes, suggesting that NO can elicit both pro-oxidant and antioxidant effects through the regulation of PGC-1α expression, and the analysis of several tissues from endothelial NO synthase-deficient mice suggests that NO regulation of the mitochondrial detoxification system through PGC-1α takes place *in vivo* and is not confined to the vascular endothelium only [138]. ROS generation and the resultant alteration of intracellular redox status activate the PGC-1α signaling pathway through p38 mitogen-activated protein kinase (MAPK) and cAMP-responsive element-binding protein (CREB) phosphorylation in skeletal muscle contraction [139]. Moreover, the use of allopurinol (an inhibitor of xanthine oxidase) before exercise severely attenuated exercise activation of the PGC-1α signaling pathway, thus providing strong evidence to suggest that mitochondrial biogenesis in skeletal muscle is controlled at least in part by nonmitochondrial ROS [139]. Very interestingly, ROS induced the activation of CREB and PGC-1α in a line of human liver cells (Fig. 4), and the overexpression of hepatic SOD1 in genetically diabetic mice improved insulin sensitivity by downregulating this signaling and attenuating FOXO1 [140]. These findings suggest that mitochondria ROS may play a key role in the control of PGC-1α expression, with improvement in insulin sensitivity, pointing out this signaling pathway as a potentially suitable therapeutic target for the prevention or treatment of insulin resistance [140].

PGC-1β expression is increased in response to dietary intake of fats and leads to hyperlipidemia by activating hepatic lipogenesis and very low density lipoprotein secretion [136]. Several factors are involved in mediating the effects of PGC-1β on lipid metabolism, including SREBPs, LXRs, and FOXA2 [136,141].

SREBP family

De novo lipogenesis is modulated by multiple mechanisms, including increased expression of lipogenic enzymes under the control of several specific transcription factors; this is particularly true for members of the SREBP family. SREBP molecules are transcribed and translated into inactive precursors embedded in the membrane of the endoplasmic reticulum; the release of the transcriptionally active domain by a sequential two-step proteolytic machinery is controlled by complex metabolic regulation. The mature forms of SREBPs translocate into the nucleus and facilitate the expression of target genes [142,143]. SREBP-1c controls hepatic *de novo* lipogenesis primarily by regulating the expression of key genes involved in FA homeostasis and glucose metabolism, whereas SREBP-2 predominantly regulates cholesterol synthesis [16,144,145]. Insulin stimulates lipogenesis by hepatic expression of SREBP-1c through activation of insulin receptor substrate (IRS) proteins [146,147]. In addition, IRS also suppresses FA oxidation through the inhibition of FOXA2 [148].

In NAFLD patients, IRS-1 expression is enhanced and positively correlated with SREBP-1c expression, leading to the increased synthesis of FA in hepatocytes; moreover, negative feedback via AMPK does not occur and the activation of FOXA upregulates FA oxidation [99,149,150]. Liver-specific expression of transcriptionally active SREBP-1c is associated with hepatic steatosis [151]. The prevention of SREBP-1a phosphorylation by MAPKs protects against liver steatosis development in mice [152]. Hepatic cholesterol accumulation is sustained by SREBP-2 activation in obese and diabetic mice [153]. SREBP-1c, together with its target gene FAS, is not induced in NAFLD patients; in contrast, an increase in SREBP-2 and its target genes HMG-CoA reductase and the mitochondrial cholesterol-transporting polypeptide steroidogenic acute regulatory protein was observed [54]. The recent demonstration of an enhanced SREBP-2 maturation in nonobese patients with liver steatosis associated with increased synthesis of cholesterol increases the role played by abnormal cholesterol metabolism in NAFLD pathogenesis [154].

In HepG2 cells hypoxia and H_2O_2 induced activation of Akt and hypoxia inducible factor 1, together with upregulation of SREBP-1, with a final increase of FAS (Fig. 4). The authors suggested that the increased activity of FAS enhances lipogenesis, with more NADPH consumed, and that this rebalances the redox state, which permits the cells to compensate for the shortfall in oxygen [155]. Indeed, H_2O_2 directly induces SREBP-1c transcriptional activity in the same cells, leading to lipid accumulation [156]. Because SREBP-1c activation is under the regulation of insulin signaling, H_2O_2 activates this pathway via the insulin-signaling cascade; alternatively, endoplasmic reticulum stress evoked by the accumulation of misfolding proteins under the stimulation of ROS and other stressors may activate JNK [157] and then SREBPs [156,158,159]. It is worth noting that some natural antioxidant compounds reduce fatty liver by downregulation of SREBP-1c and consequently FAS, ACC, and SCD1 [160]. Because the antioxidants tested are not able to bind SREBP-1c directly, it is conceivable that they act as modulators of the cellular redox environment.

AMP-activated protein kinase

AMPK is a heterotrimeric kinase that contains two regulatory subunits, β and γ, and one of the isoforms of the α-catalytic subunit, α1 or α2. AMP, the classical activator of AMPK, binds the γ-subunit and induces a conformational change allowing phosphorylation at the Thr^{172} residue of the α-subunit or reducing the ability of phosphatases to remove the phosphate [161]. AMPK is activated by hypoxia, ischemia, hyperosmolality, ROS, hypoglycemia, and stimulation of signaling pathways [162–164]. Regardless of the stimulus, once activated, AMPK turns on ATP-producing processes, such as FA oxidation and glycolysis, and turns off ATP-consuming processes such as FA synthesis [165]. When intracellular FA levels increase, AMPK inhibits hepatic FA and cholesterol synthesis by SREBP-1c downregulation [166,167]. The SREBP-1c gene is induced by insulin through increased activity of LXRs [168]; on the other hand, AMPK—activated by glucagon [169]—directly inhibits ligand-induced LXR activity [170]. Moreover, AMPK activation leads to PPARα and PPARγ inhibition and also to phosphorylation and consequent activation of PGC-1α [171,172].

AMPK levels in NAFLD patients are similar to those in healthy people [150,173]. Because of its favorable global metabolic effects, AMPK is considered a possible therapeutic target in the prevention and treatment of hepatic steatosis [166]. Sauchinone—an AMPK-activating ligand—inhibits LXRα-mediated SREBP-1c induction and SREBP-1c-dependent hepatic steatosis, thereby protecting hepatocytes from oxidative stress induced by fat accumulation in an animal model [174]. The activation of AMPK by synthetic polyphenols protects against liver steatosis by repression of lipogenesis through direct inhibition of SREBP-1c and SREBP-2 [175].

AMPK is transiently and concentration-dependently activated by H_2O_2 in NIH-3T3 cells (Fig. 4), and this activation is significantly blocked by the pretreatment of 0.5% dimethyl sulfoxide, a potent hydroxyl radical scavenger, indicating that the AMPK cascade is highly sensitive to oxidative stress [176]. In vascular endothelial cells mitochondria generate ROS for AMPK activation by a mechanism independent of AMP/ATP concentration, indicating a role in cellular defense because it leads to cardioprotection and the inactivation of caspase-3 [177]. Indeed, it was shown that mitochondrial ROS production (and not an increase in the AMP/ATP ratio) is required for hypoxic AMPK activation; in particular, ROS generated by Complex III of mitochondria are the stimulus for the activation of AMPK signaling, as demonstrated by a failure of hypoxia to activate AMPK in cells that are deficient in cytochrome *b*, a subunit of the mitochondrial Complex III [178]. Exposure of recombinant AMPKαβγ complex or HEK 293 cells to H_2O_2 is associated with increased kinase activity and also results in S-glutathionylation of the AMPKα and AMPKβ subunits [44]. Moreover, activation and S-glutathionylation of the AMPKα subunit are present in the lungs of acatalasemic mice or mice treated with the catalase inhibitor aminotriazole, i.e., conditions under which intracellular H_2O_2 steady-state levels are increased [44].

The activation of AMPK by oxidative stress could be justified by its ability to activate reducing metabolic pathways in tumor cells to preserve the redox environment; in fact, the inhibition of ACC by AMPK maintains NADPH levels by decreasing NADPH consumption in FA synthesis and increasing NADPH generation by FA oxidation [179]. Though the effects of a redox-dependent regulation of AMPK in NAFLD still need to be clarified, nevertheless when palmitate-charged HepG2 cells are treated with the flavonoid luteolin, the phosphorylation of AMPK is enhanced, together with upregulation of CPT-1 and ACC and downregulation of SREBP-1c and FAS, with a final lipid-lowering effect [180].

Liver-X-receptor family and farnesoid-X-receptor

LXRs α (NR1H3) and β (NR1H2) are critical for the control of lipid homeostasis; they respond to physiological concentrations of sterols [181]. Upon ligand-induced activation, both isoforms constitute compulsory heterodimers with the RXR and regulate gene expression through binding to LXR-response elements in the promoter regions of the target genes [182]. In the absence of ligands, LXR recruits complexes of corepressors that are exchanged with coactivators on receptor activation [183].

LXRs are crucial for the control of lipid homeostasis and not only for enhancing gene transcription involved in regulation of cholesterol metabolism [184]. As well as inducing SREBP-1c, LXRα also induces carbohydrate-response element-binding protein A, a glucose-sensitive transcription factor that promotes the hepatic conversion of excess carbohydrate to lipid, activating lipogenic gene expression [185], and directly enhances the expression of lipogenic genes through LXRα-response elements in their promoters [186]. In NAFLD, LXR expression is four times higher than in healthy subjects and is significantly correlated to SREBP-1c [173]. Elevated cholesterol production may induce an increase in oxysterol levels, LXR and SREBP-1c activation, and fatty acid synthesis [187].

Oxysterols are mostly oxygenated forms of cholesterol [188,189], formed enzymatically in the first steps of cholesterol metabolism or directly from cholesterol by ROS, and act as active molecules and ligands for LXR in the regulation of lipid metabolism, but also in several cell signaling pathways [190,191]. Circulating levels of oxysterols are increased in NAFLD patients, suggesting an important role for these ligands in the pathogenesis of this disease [86]. LXR-linking oxysterols are intermediate compounds of cholesterol synthesis (24,25-hepoxycholesterol) or steroid hormone synthesis (22-hydroxycholesterol and 20-hydroxycholesterol) or products of cholesterol hydroxylase (24-hydroxycholesterol, 24-HC; 25-hydroxycholesterol, 25-HC; 27-hydroxycholesterol, 27-HC) [192]. Among them, 24-HC and 27-HC are the main circulating oxysterols in humans. 27-HC is the product of hepatic mitochondrial 25-hydroxylase, involved in the mitochondrial transport of cholesterol [193].

Because oxysterols are natural ligands of LXR it has been suggested that LXR may act as a cellular sensor of oxidative stress.

Some evidence suggests that redox status may modulate the activation of LXRs. The expression of LXRs and PPARs is impaired by a strong peroxidation induced by iron ascorbate in macrophages, and this leads to downregulation of ATP-binding cassette A1, a crucial unidirectional cholesterol exporter, thus impairing cholesterol efflux; this effect is reversed by the use of powerful antioxidants, such as Trolox and butylated hydroxytoluene, suggesting that the redox environment is crucial in the regulation of LXR-mediated cholesterol handling [194]. Vitamin C supplementation selectively activates LXRα-dependent signaling pathways in mononucleate cells, which in turn downregulates its effector gene matrix metalloproteinase-9, thus reducing the atherogenic process [195]. It is also interesting to note that deletion of the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2), which activates the antioxidative cellular response, leads to severe liver steatosis in animals fed a high-fat and atherogenic diet by downregulating LXRs and the other NRs that regulate fatty acid metabolism in hepatocytes [196]. Indeed, a deficiency in NFE2L2 enhances the ability of the LXRα agonist to promote hepatic steatosis, as mediated by lipogenic gene induction, whereas NFE2L2 overexpression in hepatocytes represses gene transactivation by LXR-binding site activation [197]. Thus, given the role of NFE2L2 biology in the modulation of cellular redox status [198], it is conceivable that the changes in the redox environment that occur in NAFLD could contribute to the direct activation of LXRs.

FXR regulates, directly or through the orphan nuclear receptor small heterodimer partner, a wide variety of target genes critically involved in the control of bile acid, lipid, and glucose homeostasis and in the regulation of immune responses [199]. Like LXRs, FXR may also form a heterodimer with RXR to modulate the expression of target genes [200]. Activation of FXR inhibits bile acid synthesis from cholesterol and also protects against the toxic accumulation of bile acids through increased conjugation in the liver and secretion into bile canaliculi [199]. Thus, FXR signaling is functionally related to LXR signaling [201]. FXR is a biosensor for endogenous bile acids: chenodeoxycholic acid (a primary bile acid) is the most potent natural FXR agonist, and lithocholic acid and deoxycholic acid (secondary bile acids) also activate FXR, but to a lesser extent [202,203].

FXR-deficient mice display elevated serum levels of TAG and cholesterol, demonstrating the crucial role of FXR in lipid metabolism [204]. In human cells, FXR activation induces expression of PPARα and its target genes [205]. Moreover, FXR is induced by PGC-1α both directly and indirectly via PPARγ [206]. Thus, FXR reduces lipogenesis and promotes increased uptake, catabolism, and oxidation of TAGs and FAs. In NAFLD patients, the decreased

expression of hepatic FXR is associated with an increased expression of LXRs, SREBP-1c, and hepatic TAG synthesis [207]. Consistent with this, activation of FXR by bile acids or synthetic FXR agonists lowers plasma TAGs by repressing hepatic SREBP-1c expression and also by increasing hepatic FA oxidation [208,209].

To date, there is no evidence that changes in cellular redox status or radical compounds may directly modulate the FXR signaling pathway. However, it was reported that the oxysterol 22-HC activates FXR (and not LXRs), inducing the expression of the bile salt export pump, accounting for the secretion of bile acids from the liver (Fig. 4) [210]. This finding suggests that oxysterols may act as dual ligands that regulate both LXR and FXR target genes in NAFLD, in a complementary pathway aimed at removing excess cholesterol. A support for the cross talk is provided by the demonstration that NRF2 activation improves experimental steatosis by both LXR inhibition and FXR activation and that in human NAFLD the transcript levels of LXRα and SREBP-1 are inversely correlated with those of NFE2L2 and FXR [197].

Hepatic lipotoxicity: from low to high redox imbalance

The progression of simple steatosis to NASH may be the result of hepatic lipotoxicity caused by an excess of FAs, which may induce excessive ROS production and consequent cellular dysfunction and death through apoptosis and/or necrosis [211,212]. In fact, hepatocytes overwhelmed by FAs (in particular saturated) activate a variety of intracellular responses resulting in lipotoxic stress in both mitochondria and endoplasmic reticulum [213].

Mitochondrial FA oxidation is still functional in NAFLD patients presenting with simple steatosis or steatohepatitis [214]. We observed an increased rate of mitochondrial substrate oxidation during the early setting of liver injury in a NASH model, followed by an impairment of mitochondrial function and increased oxidative stress, despite the adaptive uncoupling mechanism, as the disease progresses [10,12]. Moreover, the enhanced mitochondrial oxidative metabolism in humans with high intrahepatic triglycerides provides a potential link with oxidative stress and liver damage [38]. Studies performed on liver cells tried to identify the mechanisms of mitochondria-mediated lipotoxicity and demonstrated that saturated FA excess enhanced TCA cycle fluxes with consequent increase in mitochondrial ROS production and apoptosis [48], whereas exposure to a lipid emulsion triggered excess of mitochondrial ROS leading to necrosis but not apoptosis [215]. The role of cholesterol accumulation in the induction of mitochondrial oxidative stress through GSH depletion sensitizing hepatocytes to proinflammatory cytokines has been previously cited [56].

An excess of saturated FAs in hepatic cell lines impairs TAG synthesis in the ER, probably because of the formation of lipid intermediates that induce ER stress with accumulation of unfolded or misfolded proteins [216]. This causes the so-called unfolded protein response (UPR), activating an intracellular signaling cascade that leads to an increased transcription of ER-resident chaperones and a decreased overall protein synthesis [212]. A human study demonstrated that the UPR is activated to varying degrees in NAFLD liver [217], and its signaling pathway was linked to lipid and membrane biosynthesis, insulin action, inflammation, and apoptosis [218]. Under normal conditions, the redox environment in the ER lumen is different from that of other cell organelles and favors disulfide formation for protein folding and assembly, indicating that ROS formation and oxidative stress are integral components of the UPR [219]. Oxidative stress during ER disruption may be the result of an enzymatic mechanism or of GSH depletion [220]. Moreover, accumulation of unfolded proteins in the ER may generate mitochondrial ROS production through Ca^{2+} release and depolarization of the inner membrane [221]. Thus, the ER-stress-induced oxidative stress and UPR triggered by altered lipid metabolism are closely related in the progression of NAFLD and may represent another interesting therapeutic target.

Antioxidant defense mechanisms and lipid metabolism in NAFLD

The liver is richly endowed with antioxidant defense mechanisms, which include chemicals such as GSH, vitamins C and E, and enzymes such as SOD, CAT, glutathione peroxidase (GPX), glutathione *S*-transferase (GST), glutathione reductase (GRx), peroxiredoxins, and thioredoxins [222].

Several lines of evidence indicate that the antioxidant defense mechanisms are altered in NAFLD even though the results are controversial. The hepatic glutathione content and SOD activity were decreased in patients presenting with liver steatosis and exacerbated in those with steatohepatitis [223]. An animal study on fa/fa mice fed a high-fat diet confirmed the reduction of hepatic glutathione as well as GRx, GPX, SOD, and CAT activity [224]. In contrast, the activity of Cu^{2+}/Zn^{2+}-SOD, CAT, and GPX was markedly elevated in the liver of NAFLD patients, as well as SOD and CAT gene expression [99,225]. Another study showed that the expression of NAD(P)Q:quinone oxidoreductase 1 and GST was increased and related to the progression of NAFLD [226]. This report also suggested that the antioxidant response to NAFLD progression was induced by NFE2L2 activation [226].

A persistent condition of increased ROS induces antioxidant gene expression through the activation of the antioxidant-response element, primarily regulated by NFE2L2 [227,228]. Very interestingly, NFE2L2 plays an important role in energy regulation, because its activation induces a larger cluster of genes associated with lipid metabolism [229]. This was also confirmed by gene disruption studies, which evidenced altered expression of genes involved in metabolic pathways as well as exacerbation of NAFLD in NFE2L2-knockout mice [230,231]. In contrast, enhanced expression of NFE2L2 attenuated liver steatosis in MCD-fed mice, probably increasing hepatic antioxidant and detoxification capacity, but also by inhibition of lipid deposition and expression of CD36 and PPARα [232]. Chemically activated NFE2L2 decreased obesity induced by a high-fat diet, but also lipid accumulation and synthesis (through inhibition of FAS and ACC gene expression) in liver [233]. However, even though it is conceivable that NFE2L2 negatively regulates lipid metabolism [234], targeted knockout of NFE2L2 in adipose tissue resulted in reduction of adipogenesis by inhibition of PPARγ [235], suggesting that the modulatory role of this nuclear factor on lipid metabolism could be tissue specific. Very interestingly, genetically obese mice with constitutive activation of NFE2L2 showed impaired glucose tolerance and insulin resistance, together with reduced expression of PPARγ and SREBP-1c in skeletal muscle and decreased lipid accumulation in white adipose tissue, but induced NAFLD [236]. Taken together, all these results indicate a close connection between antioxidant signaling and lipid metabolism, even though several aspects of this network need to be further investigated.

Antioxidant therapy in NAFLD

Because several antioxidants may favorably influence the cellular redox environment and consequently the molecular mechanisms implicated in NAFLD, the use of such compounds may be beneficial in the treatment of hepatic steatosis, as the redox imbalance plays a major role in its early pathogenesis, preceding insulin resistance and lipid metabolism alterations [237].

This hypothesis is supported by considerable *in vitro* and *in vivo* evidence. Experimental data demonstrate the effects of some antioxidants in the beneficial modulation of both redox status and lipid metabolism in liver through the involvement of specific NRs. Carnitine and carnitine–lipoic acid prevent lipotoxicity by increasing mitochondrial β-oxidation and reducing intracellular oxidative stress through PPARγ and CPT1 upregulation [238]. The antioxidant effect of L-carnitine on human hepatocytes has also been associated with an involvement of PPARα [101]. α-Lipoic acid (ALA) activates both sirtuin 1—a longevity-associated protein that regulates energy metabolism and life span in response to nutrient deprivation—and AMPK, leading to lipid-lowering effects [239]. Moreover, ALA reduces hepatic steatosis by downregulating SREBP-1c and ACC [240]. The polyphenolic compound silybin, widely used as a hepatoprotectant, is effective in the prevention of mitochondrial dysfunction and oxidative stress in an animal model of NAFLD [241]. Moreover, it exerts antisteatotic effects because of changes in liver expression of key enzymes involved in lipid homeostasis [242]. Geniposide shows protective effects against hepatic steatosis in rats fed a high-fat diet owing to its antioxidant actions and as a regulator of PPARα expression [243]. Resveratrol decreases NAFLD severity in rats through its antioxidant activities but also AMPK activation [244,245]. Green tea extracts protect against liver damage in ob/ob mice but with different effects on adipogenesis and hepatic antioxidant defenses [246,247]. Oxysterols are also considered to be potential candidates for the treatment of NAFLD. Even though they induce proinflammatory mechanisms *in vitro* [248], 27-HC administration can reduce hepatic inflammation and modulate intracellular cholesterol distribution in a rodent model through LXR modulation [249]. It was demonstrated that 5-cholesten-3β-25-diol-3-sulfate, which is synthesized by the cytosolic sulfotransferase SULT2B1b through sulfation of 25-HC, decreases serum and hepatic lipid levels in a mouse model, possibly by inhibiting the LXRα/SREBP-1c signaling pathway and by counteracting the action of its precursor 25-HC. All this suggests that this molecule is a viable candidate for NAFLD treatment [250]. FXR agonists may also prove clinically useful for treating hepatic steatosis [251]. Treatment with obeticholic acid—a first-in-class selective FXR agonist—protects against hepatic fat deposition together with decreased expression of genes involved in lipogenesis [252]. However, to date there are no reports on a possible antioxidant effect of FXR activation in liver.

Despite this large number of experimental studies encouraging the use of antioxidant therapy in the treatment of NAFLD, there are few clinical trials that support the efficacy of these compounds. Several studies evaluated the potential of vitamin E in the treatment of NAFLD. Early pilot studies provided conflicting results: vitamin E treatment caused a reduction in serum aminotransferase activity in children [253] and improved liver pathology in adults affected with NAFLD [254], but other studies have not demonstrated any benefit either in children or in adults [255,256]. In recent times, a study on pediatric NASH patients demonstrated that vitamin E treatment improved ballooning and NASH activity score, but the treatment had no effect on liver fibrosis or portal and lobular inflammation [257]. Another recent study on adults has shown that vitamin E improves serum transaminase levels and reduces hepatic steatosis and lobular inflammation, but is unable to improve liver fibrosis [258]. Overall, the data on the use of vitamin E alone in the treatment of NAFLD indicates that, at least in the early stages of the disease, vitamin E may improve serum biochemical tests and some histological features of NASH. However, there is no definitive evidence that vitamin E improves hepatic histology in humans, such as portal inflammation and fibrosis. Thus, it seems that vitamin E alone is insufficient for full treatment of NAFLD [259]. The combination of vitamins E and C improved liver fibrosis in a small trial [260], but this finding was not subsequently confirmed in a study on NAFLD children [261]. The complex formed by silybin–phospholipid–vitamin E has been tested in NAFLD patients showing an improvement in liver enzyme levels, hyperinsulinemia, and indexes of liver fibrosis in treated individuals [262]. Some hepatoprotective drugs such pentoxyfilline, ursodesoxycholic acid, and betaine show beneficial effects improving antioxidant status, aminotransferase level, serum fibrosis markers, and metabolic parameters in NASH patients [263–265]. Finally, a recent meta-analysis concluded that there are insufficient data to either support or refute the use of antioxidant supplements for patients with NAFLD [266].

There could be various reasons extensive efforts in evaluating the benefits and harmful effects of antioxidant supplements in NAFLD resulted in neutral or detrimental results. First of all, it is likely that oxidative stress is not the only pathogenetic mechanism contributing to liver damage in NAFLD; thus antioxidant efficacy should be investigated as a cotreatment with other drugs. On one side there is a need for large, well-designed, randomized, double-blind, controlled trials, with well-defined endpoints, various dosages, and proven biomarkers that correlate well with clinical outcome and ideally also provide insights into the mechanism of their action. Moreover, the available antioxidative compounds present with nonselective effects [267]; this encourages carrying out tests for targeted compounds, such as selective molecule and nanocarrier technology. Finally, several scientists agree with the small commitment of the pharmaceutical industry in funding these studies, probably because antioxidants are not expensive and modestly effective after a short-term use. Hence, we need more extensive prospective randomized clinical trials on this topic, mainly supported by public funding agencies.

Concluding remarks

The onset of NAFLD is characterized by changes in the hepatocellular redox status that lead to impaired regulation of lipid metabolism. On the other hand, alterations in lipid metabolism modify the redox state of the liver, which in turn may lead to several modifications such as mitochondrial dysfunction, peripheral insulin resistance, and fat accumulation, perpetuating the liver injury. The molecular mechanisms accounting for these alterations are not completely understood, but involve redox modification of enzyme activity and expression, post-translational modulation, and activation of NRs with consequent modification of the metabolic network. This evidence suggests that the pathogenesis of NAFLD is redox-centered. Thus, the identification of redox-dysregulated NRs as targets for the treatment of NAFLD is a promising approach. The impact of modulation of several NRs by use of direct agonists/antagonists might be far from beneficial, because of the lack of a full understanding of the molecular mechanisms of these receptors as well as lack of tissue specificity. Because antioxidant molecules favorably modulating the cellular redox environment may also regulate NRs known to play a role in lipid metabolism, fine-tuning of the redox status might be effective in the restoration of the signaling pathways that lead to the development and progression of fatty liver.

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