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COMPREHENSIVE INVITED REVIEW

Redox Balance in the Pathogenesis of Nonalcoholic Fatty Liver Disease: Mechanisms and Therapeutic Opportunities

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is currently the most common liver disease in the world. It encompasses a histological spectrum, ranging from simple, nonprogressive steatosis to nonalcoholic steatohepatitis (NASH), which may progress to cirrhosis and hepatocellular carcinoma. While liver-related complications are confined to NASH, emerging evidence suggests both simple steatosis and NASH predispose to type 2 diabetes and cardiovascular disease. The pathogenesis of NAFLD is currently unknown, but accumulating data suggest that oxidative stress and altered redox balance play a crucial role in the pathogenesis of steatosis, steatohepatitis, and fibrosis. We will examine intracellular mechanisms, including mitochondrial dysfunction and impaired oxidative free fatty acid metabolism, leading to reactive oxygen species generation; additionally, the potential pathogenetic role of extracellular sources of reactive oxygen species in NAFLD, including increased myeloperoxidase activity and oxidized low density lipoprotein accumulation, will be reviewed. We will discuss how these mechanisms converge to determine the whole pathophysiological spectrum of NAFLD, including hepatocyte triglyceride accumulation, hepatocyte apoptosis, hepatic inflammation, hepatic stellate cell activation, and fibrogenesis. Finally, available animal and human data on treatment opportunities with older and newer antioxidant will be presented. *Antioxid. Redox Signal.* 15, 1325–1365.

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I. Introduction: Importance of Nonalcoholic Fatty Liver Disease and of Altered Redox Balance for the Pathogenesis of Nonalcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is characterized by liver fat accumulation exceeding 5% of hepatocytes, in the absence of significant alcohol consumption, viral infection, or any other known cause of liver disease. NAFLD is currently the most common chronic liver disease, affecting 30% of the general adult population in the Western world (181). NAFLD encompasses a histological spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), the latter characterized by the presence of hepatocyte necrosis and inflammatory infiltration, along with different degrees of fibrosis. While simple steatosis is believe to remain stable over time, NASH may develop cirrhosis and related complications (portal hypertension, liver failure, and hepatocellular carcinoma) in up to 15%–25% of cases over 10 years (181). Beside liver-related complications, NAFLD is increasingly recognized as a risk factor for the development of metabolic disorders, including diabetes and metabolic syndrome, and for cardiovascular disease, independently of traditional risk factors. Thus, the clinical implications of NAFLD derive from its common occurrence and its potential liver-related and cardio-metabolic complications (181).

The pathogenesis of NAFLD, and particularly the mechanisms responsible for liver injury and NAFLD progression, remain poorly understood but are of significant biomedical importance, as identification of these processes may help to identify novel therapeutic targets to treat this disease.

Reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^•$), and reactive nitrogen species (RNS), including nitric oxide (NO) and peroxynitrite ($ONOO^-$), are physiologically generated in normal hepatocytes and at low concentrations are critical for normal physiologic processes, including oxidative respiration, growth, regeneration, apoptosis, and microsomal defense (139).

ROS toxicity is due to an unbalanced equilibrium between the rates of production of ROS and the rate of their removal: when the redox balance between reactive species generation and antioxidant defense mechanisms is disrupted and the levels of oxidation products exceed the capacity of normal antioxidant systems, ROS damage cellular macromolecules and oxidative stress occurs (58).

In the latest years, altered oxidative metabolism of triglyceride at both hepatic and extrahepatic level, an imbalance in pro/antioxidant systems, and an increased oxidative stress are increasingly recognized as a crucial pathogenic moment for the development of hepatic steatosis, necroinflammation, and fibrosis that characterize NASH. Many of these alterations can be found in NAFLD-associated metabolic disorders, including obesity and diabetes, and in the inflammatory process at the basis of atherosclerosis, thus suggesting common molecular mechanisms act at both hepatic and extrahepatic levels to produce liver and cardio-metabolic disease. Initial efforts to clarify the pathogenesis of NAFLD and NASH led to the formulation of the two-hit hypothesis (50): this model considered hepatic steatosis as the first hit, increasing the susceptibility of the liver to the second hit, responsible for the development of necroinflammation and fibrosis. In the latest years, it has become increasingly clear that the same noxae can determine both steatosis and necroinflammation and that initial and subsequent mechanisms can mutually influence each other. Altered redox balance and increased oxidative stress are among the strongest candidates for the pathogenesis of NAFLD and have been the focus of extensive research in the latest years (57). This review will focus on the main pro- and antioxidant mechanisms potentially involved in the pathogenesis of NAFLD.

Many cellular systems are important sources of ROS, including the mitochondrial respiratory chain (39), the cytochrome P450s (202), oxidative enzymes (xanthine oxidase, aldehyde oxidase, cyclo-oxygenase, monoamine oxidase, and the NADPH oxidase complex) (346), and several heme proteins (ferrohemoglobin and myoglobin) and biochemicals (catecholamines, quinones, and tetrahydrobiopterins) that are susceptible to autoxidation.

In addition to these cellular sources of ROS, environmental sources of ROS include radiation, UV light, smoke, and certain drugs such as antibiotics (*e.g.*, nitrofurantoin), antineoplastic agents (*e.g.*, bleomycin), anthracyclines (*e.g.*, Adriamycin), and Methotrexate, which can alter redox cycle.

ROS are toxic to cells because they can react with many cellular macromolecules, inactivating enzymes or denaturing proteins and causing DNA damage such as strand breaks, base removal, or base modifications. Lipid peroxidation can lead to destruction of biological membranes and to production of reactive aldehydic products such as malondialdehyde (MDA) or 4-hydroxynonenal (4-HNE) (174).

Several enzymatic and nonenzymatic mechanisms protect cells against ROS-induced damage: superoxide dismutases neutralize O_2^-; catalase and glutathione (GSH) peroxidase systems neutralize H_2O_2; glutathione transferases are able to neutralize reactive intermediates and lipid aldehydes; metallothioneins, heme oxygenase, and thioredoxin remove various ROS species. Ceruloplasmin and ferritin combine with pro-oxidative metals, such as iron, and neutralize them though complex formation. Nonenzymatic mechanisms include low-molecular-weight antioxidant such as GSH, vitamin E, ascorbate (vitamin C), vitamin A, ubiquinone (coenzyme Q10), uric acid, and bilirubin (93). Other sources of ROS are macrophages and neutrophils whose NADPH oxidase produces ROS (9) and the enzyme myeloperoxidase, which produces hypochlorite upon catalyzing a reaction between H_2O_2 and chloride.

A. Summary

NAFLD encompasses a histological spectrum ranging from simple steatosis to steatosis plus necroinflammation (NASH), with or without fibrosis. NAFLD and its progressive form NASH are common conditions in the general population. Oxidative stress is considered to have a pathogenic role in both hepatic fat accumulation and in necroinflammation and fibrogenesis.

II. Free Fatty Acid Oxidation as a Source of ROS

A. Overview of hepatic fatty acid oxidative pathways and their altered homeostasis in NAFLD

In the liver fatty acid (FA) is metabolized through three distinct pathways: β-oxidation, which occurs mainly in mitochondria, but also in peroxisomes, and microsomial ω-oxidation in the endoplasmic reticulum (ER) by members of the cytochrome P450 4A family. The extramitochondrial FA oxidative pathways become increasingly important as FA availability increases in the liver, as is the case of NAFLD (11, 12). These three FA oxidative pathways, whose key enzymes are regulated by PPAR-α, will be briefly described (234).

B. Mitochondrial FA oxidation: β-oxidation

Mitochondrial β-oxidation is primarily involved in the oxidation of short-chain ($<C_8$), medium-chain (C_8–C_{12}), and long-chain (C_{12}–C_{20}) FAs. Mitochondrial β-oxidation progressively shortens FAs into acetyl-CoA subunits, which either condense into ketone bodies, that serve as oxidizable energy substrates for extrahepatic tissues, or enter the tricarboxylic acid cycle for further oxidation to water and carbon dioxide.

Several lines of evidence suggest that mitochondrial dysfunction is a central abnormality responsible for the progression to steatohepatitis from simple steatosis in NAFLD (221). Mitochondrial dysfunction is characterized by mitochondrial outer-membrane permeabilization. Mitochondrial outer-membrane permeabilization causes the release of multiple proteins from the mitochondrial intermembrane space into the cytosol. This leads to caspase activation in the cytosol, disruption of the mitochondrial respiratory chain, loss of mitochondrial transmembrane potential ($\Delta\psi_m$), free-radical production, and loss of mitochondrial structural integrity. We will highlight the relationships between structural changes, decreased mitochondrial function, and disease progression in

NAFLD and the subcellular and molecular mechanisms resulting in mitochondrial dysfunction in NAFLD.

Mitochondrial β-oxidation is regulated by carnitine palmitoyltransferase 1 (CPT-I), carnitine concentration, and malonyl-CoA. CPT-I is an outer membrane enzyme playing a key-role for the entry of long-chain free FAs (FFAs) into the mitochondria and whose activity is inhibited by malonyl-CoA. Malonyl-CoA is formed by acetyl-CoA carboxylase (ACC) and is the first step in the synthesis of FAs from acetyl-CoA (175). α-β-dehydrogenation of the acyl-CoA ester is due to a family of four chain length-specific straight-chain acyl-CoA dehydrogenases. Medium-chain acyl-CoA dehydrogenase deficiency is the most common inherited disorder of mitochondrial FA oxidation in humans. Mice with disrupted medium-chain and very-long-chain acyl-CoA dehydrogenase genes manifest defects in FA oxidation, and these animals develop micro- and macrovascular hepatic steatosis (295).

Short-chain and medium-chain FFAs freely enter the mitochondria without requiring CPT-I and other protein involved in the carnitine shuttle (71).

The second, third, and fourth steps in the mitochondrial β-oxidation pathway are performed by 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. A single mitochondrial trifunctional protein (MTPr) performs all of these three enzyme activities. This heterotrimeric protein consists of four α-subunits and four β-subunits and catalyzes long-chain FA oxidation. Homozygous null mice for the α-subunit of MTPr (MTPr-$\alpha^{-/-}$ mice) develop hepatic steatosis immediately after birth and die 6–36 h after birth; MTPr defects in humans are recessively inherited, and children with defects of any of the three enzymatic activities exhibit microvesicular hepatic steatosis (236). Besides genetic disorders affecting mitochondrial FA function, several drugs and toxins, including alcohol, can inhibit mitochondrial β-oxidation enzymes, leading to hepatic steatosis (236).

The cycle previously described is repeated to split the FAs into acetyl-CoA subunits. In the presence of high glucose and energy levels, acetyl-CoA is converted to citrate, which can leak out of the mitochondrial matrix into the cytosol through the tricarboxylate carrier. In the cytosol, citrate regenerates acetyl-CoA, which is converted to malonyl-CoA by ACC. Malonyl-CoA regulates both hepatic FA oxidation and lipid synthesis. High malonyl-CoA levels inhibit CPT-I enzyme activity and consequently the rate of FFA entering the mitochondria. Then, during period of caloric overconsumption and excessive energy supply, increased malonyl-CoA levels promotes hepatic FA synthesis and suppress FA oxidation. On the other side, in fasting conditions, low hepatic malonyl-CoA levels allow import of long-chain FFAs and β-oxidation.

FFA oxidation in mitochondria is associated with conversion of oxidized cofactors NAD^+ and FAD into their reduced form NADH and $FADH_2$. The reduced cofactors are re-oxidized by the mitochondrial respiratory chain in the mitochondrial inner membrane (71). During oxidation of NADH and $FADH_2$, electrons are transferred to the first complexes of the respiratory chain. The migration of electrons along the respiratory chain causes protons to be extruded from the mitochondrial matrix into the mitochondrial intermembrane space and a large electrochemical potential across the inner membrane to be used for adenosine triphosphate (ATP) synthesis. When ATP synthesis is required, protons flow back into the matrix through the F0 portion of ATP synthase moving the molecular rotor of ATP synthase into the F1 portion and allowing the conversion of ADP into ATP. The adenine nucleotide translocator in exchange for cytosolic ADP extrudes ATP from mitochondria (214). The entire biochemical process is referred to as oxidative phosphorylation.

Most of the electrons flowing along the respiratory chain reach cytochrome c oxidase to be coupled with oxygen and protons to produce water (175) despite minor electron leakages at upstream sites of the respiratory chain. Some of these electrons can directly react with oxygen to form the superoxide anion radical that is dismuted by mitochondrial manganese superoxide dismutase (MnSOD) into hydrogen peroxide (H_2O_2) and detoxified into water by mitochondrial glutathione peroxidase and catalase, which may have a role in protection against endogenous or exogenous H_2O_2 in hepatocyte mitochondria (250). Thus, the respiratory chain even in healthy mitochondria generates ROS. Physiologically, most mitochondrial ROS are detoxified into water and only a small amount of ROS persists. Larger amounts of ROS leak out from impaired mitochondria and can damage other cellular components.

As previously described, hepatic mitochondria of patients with NASH show both morphological and functional changes compared to healthy subjects: their megamitochondria have ultrastructural lesions with paracrystalline inclusions (250) and have a decreased ATP re-synthesis rate after a fructose challenge, leading to a transient hepatic ATP depletion (213). In patients with NASH, there is a compensatory removal of FFA through an enhanced mitochondrial β-oxidation and ketogenesis, aiming at offsetting the higher hepatic FFA availability (251). Several mechanisms have been proposed to explain the increased hepatic FFA removal: a compensatory enlargement of the pool size of FFAs, an enhanced CPT-I activity, an increased expression of PPAR-α and of the uncoupling protein-2 (UCP-2), and an enlargement of hepatic peroxisomial compartment.

The augmented pool of FFAs could induce hepatic PPAR-α activation. An increased hepatic PPAR-α expression has been found in several genetic models of obesity-associated steatosis, including ob/ob and db/db mice (176). The pool of FAs synthesized *de novo* from carbohydrates activates PPAR-α more potently than those FAs taken up from the circulation (86); however, in NASH other mechanisms, including tumor necrosis factor-α (TNF-α), nuclear factor (kappa)B (NF-κB), and ROS (86), can counteract the FFA-induced PPAR-α upregulation. As a result, the increased PPAR-α expression induced by *de novo* FA synthesis is still insufficient to remove the excess of lipids from the liver (37).

In rodent models of diabetes-associated NASH, NASH CPT-I and CPT-II expression levels have been found to be increased, thereby enhancing the entry of long-chain FA into mitochondria (210). Upregulation of CPT is due not only to PPAR-α activation and to enhanced availability of long-chain FAs (161), but also to a decreased affinity of CPT-I for its physiological inhibitor, malonyl-CoA (45). The loss of CPT-inhibition by malonyl-CoA may explain why β-oxidation is increased in type 2 diabetes despite high insulin and malonyl-CoA levels.

An increased hepatic expression of UCP-2 has been demonstrated in genetically obese ob/ob mice (45). UCPs are proteins located in the mitochondrial inner membrane, where they mediate proton leak to uncouple substrate oxidation from ATP synthesis. FFAs can induce an increase in the proton

leak in the respiratory chain through PPAR-α-induced UCP-2 overactivation (40, 47). FFAs are weak acids that exist in equilibrium between the ionized ($RCOO^-$) and the protonated form (RCOOH). In the intermenbrane space of mitochondria, the equilibrium is displaced toward the protonated form because the pH is acidic. Therefore, FFAs are soluble into phospholipids and they can enter the mitochondrial matrix through the inner mitochondrial membrane. In the alkaline mitochondrial matrix, FFAs dissociate into the ionized form $RCOO^-$, which moves back through the UCP (118). The shuffling of one molecule of FFA lets one proton to reenter the matrix at each cycle. The energy of respiration produces heat instead of ATP since ATP synthase is by-passed.

UCP-2 may also enhance the re-oxidation of NADH into NAD^+, which is required for both β-oxidation and the tricarboxylic acid cycle (69). Under this point of view, the increased UCP-2 oxidation may be considered an attempt to prevent steatosis by increasing hepatic FA oxidation (105). A further beneficial effect of UCP-2 might be the loss of electrons in the respiratory chain before the over-reduction of respiratory complexes occurs, thereby reducing mitochondrial ROS formation (119).

In NASH, adipocytes, Kupffer cells, and hepatocytes secrete TNF-α, which acts on its receptors on hepatocytes to trigger a cascade reaction involving caspase-8 activation, Bid truncation, and Bax translocation to mitochondria, where the latter associates with Bak, eventually leading to permeabilization of the outer mitochondrial membrane (213, 296). Complexes I and III of the respiratory chain have been consistently involved in ROS generation (354). Cytochrome C is released from mitochondria, thereby preventing electron flow from complex III to cytochrome c and subsequently cytochrome c oxidase. The accumulation of electrons along the respiratory chain is further promoted by a simultaneously enhanced mitochondrial β-oxidation rate, which increases both the formation of NADH and $FADH_2$, and the flow of electrons to the respiratory chain (213). Electrons can freely react with oxygen to form superoxide anion radical ($O_2^{\bullet -}$) and other ROS (342). Thus, mitochondrial ROS formation is enhanced (213). Further, inducible nitric oxide synthase (iNOS) transforms $O_2^{\bullet -}$ into peroxynitrite, which can damage DNA, and in the presence of iron more powerful oxidants, including $OH^{\bullet}$, and ferryl species, are produced. Consequently, reactive species damage mtDNA, leading to a decreased synthesis of mtDNA-encoded respiratory chain polypeptides and to a further block of the electron flow through the respiratory chain. MtDNA is very sensitive to oxidative injuries since it lacks protective histones and several DNA repair mechanisms. Mitochondrial cardiolipin may also be damaged by ROS and the released reactive lipid peroxidation products can boost a vicious cycle, eventually leading to an enhanced ROS production. Increased ROS generation in the liver can injure hepatocytes through other mediators, such as 4-HNE, and trigger the synthesis of several cytokines, including TNF-α, transforming growth factor-β (TGF-β), interleukin-8 (IL-8), and Fas ligand (213).

C. Peroxisomal β-oxidation

The peroxisomal β-oxidation system is composed of two pathways, one of which is inducible by peroxisome proliferators. The peroxisome proliferator-inducible β-oxidation pathway metabolizes straight-chain saturated fatty acyl-CoAs, whereas the second noninducible pathway metabolizes 2-methyl-branched fatty acyl-CoAs (38). Peroxisomal β-oxidation metabolizes less abundant, relatively more toxic and biologically active molecules, including very-long-chain FAs ($>C_{20}$), which are subsequently oxidized in mitochondria once their chain has been shortened, 2-methyl-branched FAs, dicarboxylic acids, prostanoids, and C_{27} bile acid intermediates (11). An effective peroxisomal β-oxidation system is needed to minimize the deleterious effects and to prevent hepatic FA accumulation and steatosis. The peroxisomal β-oxidation system consists of four steps involving two distinct enzymes. Straight-chain acyl-CoA oxidase is responsible for the initial oxidation of very-long-chain fatty acyl-CoAs to their corresponding *trans*-2-enoyl-CoAs in the PPAR-α-regulated system, whereas branched-chain acyl-CoA oxidase metabolizes branched-chain fatty acyl-CoAs in the noninducible system. A single enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase [L-bi/multifunctional enzyme (L-PBE/MFP1), catalyzes the second and third reactions in the classical β-oxidation system, hydration, and dehydrogenation of enoyl-CoA esters to 3-ketoacyl-CoA. Ketoacyl-CoAs generated by L-PBE/MFP1 are converted to acetyl-CoA, and an acyl-CoA that is two carbon atoms shorter than the original molecule. The shortened acyl-CoA re-enters the β-oxidation cycle, and this process is repeated for about five cycles, resulting in the removal of about 10 carbon atoms. The appropriately chain-shortened acyl-CoAs are then further oxidized in the mitochondria (234). The second and third steps in the noninducible β-oxidation pathway are performed by D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase (D-bi/multifunctional enzyme 2), and the resulting 3-ketoacyl-CoAs are cleaved by the third enzyme of this system, known as sterol carrier protein X, which possesses thiolase activity.

Targeted deletion of the straight-chain acyl-CoA oxidase gene in the mouse induces high levels of very-long-chain FAs ($>C_{22}$) in serum, growth retardation, severe hepatic steatosis and steatohepatitis, and hepatocellular carcinoma (67, 95).

D. Microsomal ω-oxidation of FAs: role of cytochrome P450 enzymes in the pathogenesis of NASH

Microsomal ω-oxidation by cytochrome P450 4A (CYP4A) enzymes oxidizes saturated and unsaturated FAs. FAs are first ω-hydroxylated in the ER, and the resulting ω-hydroxy FA is then dehydrogenated to a dicarboxylic acid in the cytosol. Dicarboxylic acids are converted to dicarboxylyl-CoAs for oxidation by the classical β-oxidation pathway. Although ω-oxidation is a minor pathway of FA metabolism, significant amounts of dicarboxylic acids can be formed under conditions of FA overload in the liver (*e.g.*, obesity and diabetes) and in situations where the mitochondrial oxidation system is inadequate to metabolize FAs. Dicarboxylic acids, substrates for the peroxisomal β-oxidation system, are also PPAR-α ligands; therefore, they can induce the all three FA oxidation systems, including the CYP4A pathway, in the liver (164, 234, 236).

Several lines of evidence suggest that mitochondrial function is impaired in human NASH: in subjects with NASH hepatic mitochondria show ultra-structural abnormalities, decreased mitochondrial DNA levels, decreased protein expression of several mitochondrial DNA-encoded polypeptides,

and a lower activity of complexes I, II, III, IV, and V (ATP synthase). These alterations are either spontaneous or induced by the mitochondrial respiratory chain inhibitory drug diethylaminoethoxyhexestrol (33, 96, 212, 213). Patients with NASH have decreased respiratory chain complex activity and an impaired ability to resynthesize ATP after a fructose challenge, which transiently depleted hepatic ATP stores (213). As previously mentioned, mitochondrial respiratory chain dysfunction can directly lead to the production of ROS: if electron flow is interrupted at any point in the respiratory chain, the preceding respiratory intermediates can transfer electrons to molecular oxygen to produce super oxide anions and hydrogen peroxide (20, 76).

As the oxidative capacity of the mitochondria becomes impaired, FAs accumulate in the cytosol. In the initial step of peroxisomal β-oxidation, hydrogen peroxide is formed through the action of acyl-CoA oxidase, which donates electrons directly to molecular oxygen (167). Microsomal ω-oxidation of FAs, catalyzed primarily by cytochrome P450 enzymes 2E1, 4A10, and 4A14, forms ROS through flavoprotein-mediated donation of electrons to molecular oxygen. Additionally, dicarboxylic acids, other products of microsomal FA ω-oxidation, impair mitochondrial function by uncoupling oxidative phosphorylation. Protonated dicarboxylic acids cycle from the inner to the outer mitochondrial membrane, resulting in dissipation of the mitochondrial proton gradient without concomitant ATP production (102). Increased availability of intracellular FAs, as well as PPAR-α activation, induces microsomal cytochrome P450 enzymes 2E1 (CYP2E1) and CYP4A1 activity, leading to generation of ROS and dicarboxylic acids. The net result of extra-mitochondrial FA oxidation is thus a further increase in oxidative stress and mitochondrial impairment. The biological activity of CYP2E1 will be discussed in detail below, as elevated hepatic expression of CYP2E1 has been observed in patients with NASH (140, 322) (Fig. 1) and in other NAFLD-associated disorders, including obesity and diabetes (329).

CYP2E1 is a member of the P450 family, which can metabolize a large number of small, hydrophobic substrates and drugs (23, 26, 153, 289). Acetone and FAs, such as linoleic and arachidonic acid, but also molecular oxygen constitute its physiological substrates (137). CYP2E1 is the most powerful P450 enzyme in the initiation of NADPH-dependent lipid peroxidation (62). It shows higher NADPH oxidase activity than other P450 enzymes, and it is poorly coupled with NADPH-cytochrome (61). CYP2E1 is predominantly found in hepatocytes but significant amounts are also found in the Kupffer cells (134) and in many organs, including the brain (95). Within cells, CYP2E1 is mainly located in the membrane of the ER from where is transported out to the Golgi apparatus

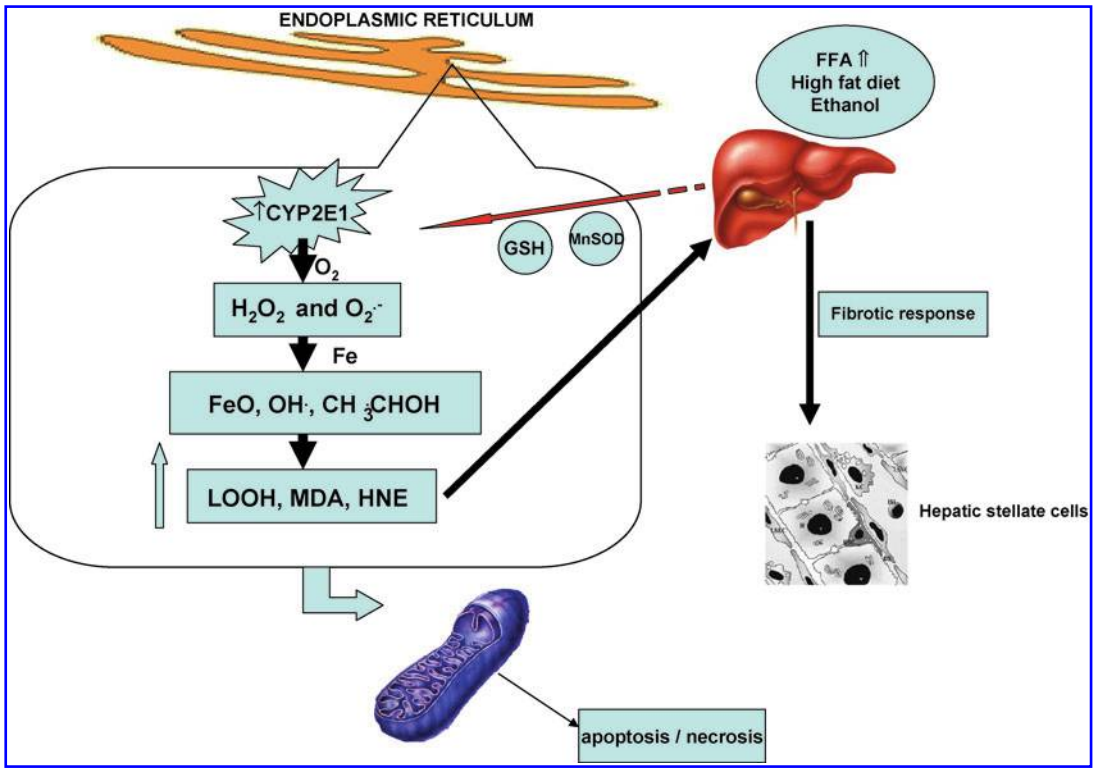

FIG. 1. A model of CYP2E1-dependent oxidative stress and toxicity. Upon activation, CYP2E1 produces ROS, including O_2^{-}. Ethanol treatment makes iron to be available, and more potent oxidants, such as OH^{-}, ferryl species, and 1-hydroxyethyl radical are generated. Initially, the hepatocytes can buffer the CYP2E1-dependent oxidative stress through the induction of several antioxidant enzymes. When these defensive mechanisms are defeated, the hepatocytes are damaged by the CYP2E1-related oxidants. Oxidant products oxidize protein and DNA, impair enzyme activity, and enhance cell membrane lipid peroxidation whose breakdown products include malonylaldehyde and 4-hydroxynonenal. Mitochondria membranes are the most damaged. So, increased membrane permeability, due to a decreased $\Delta\psi m$, causes pro-apoptotic factors to leak out of the mitochondria triggering apoptosis. CYP2E1, cytochrome P450 enzymes 2E1; HNE, hydroxynonenal; MDA, malonylaldehyde; ROS, reactive oxygen species. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

and subsequently to the plasma membrane (192). CYP2E1 is also located in the microsomes and mitochondria (191). Two isoforms of CYP2E1 are present in mitochondria, a highly phosphorylated form and a shortened 40-kDa amino-terminal-truncated form, which can be further NH_2-terminally truncated to produce a mature mitochondrial form of CYP2E1. The phosphorylation and amino-terminal truncation are supposed to cause conformational changes, altered interactions with molecular chaperons, signal recognition particles, and direct the CYP2E1 to the mitochondria. The mitochondrial CYP2E1 requires adrenodoxin and adrenodoxin reductase (and NADPH) as electron donors (191, 193). Small amount of the $O_2^{\cdot -}$ can be produced from decay of the oxygenated P450 complex, whereas H_2O_2 can stem from both dismutation of $O_2^{\cdot -}$ and decay of the peroxy-P450 complex (64, 160).

CYP2E1 can be induced under a variety of metabolic or nutritional conditions. CYP2E1 is induced in diabetes and obesity. CYP2E1 induction in diabetes may be associated with elevated production of ketone bodies (96) or with resistance to insulin, which was able to downregulate CYP2E1 expression at the posttranscriptional level in a cultured rat hepatocytes (52). The carbohydrate content of the diet influences CYP2E1 levels, as a low-carbohydrate diet increased the extent of induction of MEOS by ethanol (291) and high-fat/low-carbohydrate diets resulted in the highest levels of CYP2E1 induced by ethanol (352). As a matter of fact, chronically obese, overfed rats and rats fed a high-fat diet as well as those under fasting or prolonged starvation displayed increased CYP2E1 levels (123, 233). In NASH CYP2E1 is inducible by ketones and by a high-fat diet, especially when coupled with low dietary carbohydrate intake (22).

In addition to insulin, other hormones can affect CYP2E1 levels. Hypophysectomy and triiodothyronine increase CYP2E1 protein and mRNA levels, in contrast to insulin, which lowers them (211).

An accurate and feasible method to assess CYP2E1 activity *in vivo* is the measurement of plasma metabolites of chlorzoxazone by HPLC. Chlorzoxazone is an analgesic drug, which, upon oral administration, is specifically metabolized by hepatic CYP2E1. Chlorzoxazone clearance was found to be greater in the NASH patients than in control subjects, and also lymphocyte-derived CYP2E1 mRNA levels were higher in the NASH patients (38). Recently, Varela *et al.* (307) assessed CYP2E1 polymorphisms in nondiabetic obese women with steatosis or NASH. They found that CYP2E1 activity was significantly higher in patients with liver injury in NASH compared to simple steatosis or healthy controls, and that this correlated with Rsa1/Pst1 CYP2E1 polymorphisms: the rare c1 allele was present in 37% of patients with simple steatosis and 44% of subjects with NASH, as compared to 27% of healthy controls (307). Thus, CYP2E1 might be an important candidate in the pathogenesis of human NASH (38) since in obese patients with nonalcoholic liver disease, increased CYP2E1 protein content and activity correlated with the development of liver injury (202). In addition, animal models, such as the rat methionine-choline-deficient model of NASH, displayed elevated CYP2E1 in the liver (321). CYP2E1 induction in o*b*/*ob* mice, an experimental model of obesity, was associated with substantial induction of histological liver injury and, coupled with higher TNF-α activity, and oxidative and nitrosative stress (56). Increased oxidative species were MDA, protein carbonyls, 4-HNE-protein adducts, and iNOS, whereas increased nitrative stress was due to the formation of 3-nitrotyrosine protein adducts in the liver (56). Superoxide radical shows high affinity toward NO originated from NOS activity. Superoxide radical reacts with $NO^{\cdot}$ to originate peroxynitrite (14). Peroxynitrite nitrates free and protein-associated tyrosine residues and produces nitrotyrosine (56). Oxidative and nitrative species can trigger the intrinsic cytotoxic pathway of the apoptosis pathway. Reactive nitrogen species (RNS) and ROS can affect c-Jun N-terminal kinase and p38 mitogen-activated protein (MAP) fueling cellular apoptosis (241).

Figure 1 depicts a model of CYP2E1-dependent oxidative stress and toxicity. Upon activation, CYP2E1 produces ROS and O_2^{-}. Ethanol treatment makes iron to be available, and more potent oxidants, such as OH^{-}, ferryl species, and 1-hydroxyethyl radical are generated. Initially, the hepatocytes can buffer the CYP2E1-dependent oxidative stress through the induction of several antioxidant enzymes. When these defensive mechanisms are overcome, hepatocytes are damaged by the CYP2E1-related oxidants. Oxidant products oxidize protein and DNA, and impair enzyme activity, and they enhance cell membrane lipid peroxidation whose breakdown products include MDA and 4-HNE. Mitochondria membranes are the most damaged. So, increased membrane permeability, due to a decreased $\Delta\psi m$, causes pro-apoptotic factors to leak out of the mitochondria triggering apoptosis.

E. Summary

FFA are oxidized by mitochondrial β-oxidation, peroxisomal β-oxidation, or microsomal ω-oxidation in hepatocytes. NASH is associated with mitochondrial dysfunction and lower activity of ATP synthase. As a consequence, there is a shift toward peroxisomal and microsomal FFA oxidation, with increased generation of ROS.

III. Lysosomal-Mitochondrial Axis as a Mediator of FFA Hepatic Lipotoxicity in NASH

Recent experimental evidence suggests that lysosomes play an important part in mediating FFA-induced generation of ROS and mitochondrial dysfunction in NASH.

Li *et al.* (150) incubated primary mouse hepatocytes with various concentrations of long-chain FFA with different degrees of saturation, including palmitate (saturated), oleate (1 double bond), and linoleic (2 double bonds) for up to 24 h. Hepatocyte mitochondrial function was monitored by real-time imaging and cytochrome c redistribution, and ROS production was assessed by the nonfluorescent cell-permeant compound 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which upon oxidation becomes deesterified and fluorescent (dichlorofluorescin, DCF). The temporal relationship of lysosomal and mitochondrial permeabilization was established. Activity of the lysosomal protease cathepsin B was suppressed by genetic deletion or and pharmacological inhibition by CA074 or E-64. In a separate experiment, mitochondrial function and liver damage were assessed in cathepsin B-knockout mice and wild-type animals placed on a high-carbohydrate diet for 16 weeks. Exposure of hepatocytes to saturated FFA resulted in mitochondrial depolarization, cytochrome c release, and increased ROS production. Lysosomal permeabilization and cathepsin B redistribution into the cytoplasm occurred several hours before mitochondrial

dysfunction. Either pharmacological or genetic inhibition of cathepsin B preserved mitochondrial function. Finally, *in vivo,* cathepsin B inactivation protected mitochondria, decreased oxidative stress, and attenuated hepatic injury in mice. These data further confirm that the excessive accumulation of FFAs in hepatocyte induces mitochondrial dysfunction, oxidative stress, and liver injury in a dose- and saturation-dependent manner, and it discloses a novel mechanisms underlying this association, that is, lysosomal disruption and activation of cathepsin B. Molecular mechanisms connecting FFA to cathepsin B release involve intracellular translocation of Bax to the lysosome and subsequent lysosomal permeabilization leading to release of cathepsin B. Besides mitochondrial dysfunction, capthesin B in the cytosol may contribute to hepatocyte death through NF-κB translocation into the nucleus with increased production and release of TNF-α (86) (Fig. 2).

This study is the first to provide evidence that in both human and murine hepatocytes, saturated FFAs induce mitochondrial dysfunction and increased ROS production. In the study by Li *et al.* (150), these events are downstream of lysosomal permeabilization, and genetic or pharmacological inhibition of cathepsin B protects against FFA-induced mitochondrial dysfunction and oxidative stress both *in vitro* and *in vivo*, pointing at lysosomal integrity preservation as an attractive tool for the treatment of NASH. The safety and efficacy of an approach targeting cathepsin B to prevent FFA mitochondrial permeabilization and hepatocyte lipotoxicity in NASH warrant further *in vivo* evaluation.

A. Summary

Saturated FAs are more toxic for mitochondria than unsaturated FAs. Lysosomal permeabilization is a crucial step mediating saturated FA toxicity to the mitochondria, and preventing cathepsin B liberation from the lysosome may have therapeutic applications in NASH.

IV. Modulation of Mitochondrial Function and Potential Therapeutic Implications for NAFLD

Currently available strategies to enhance mitochondrial β-oxidation of FFA include

1. Inhibition of ACC system: antisense oligonucleotides (ASOs), ACC antagonists, and AMP-activated protein kinase (AMPK) agonists (metformin, AICAR).
2. PPAR-α agonists: fibrates.
3. PPAR-β/δ agonists: GW501516, PPAR-γ coactivator 1α (PGC-1α) modulation, and PPAR-δ agonists (L-165041).

A. Inhibition of ACC system

ACCs are important regulatory enzymes of both lipogenesis and mitochondrial β-oxidation. They catalyze the carboxylation of acetyl-CoA into malonyl-CoA, which is an intermediate in the synthesis of FAs and a natural inhibitor of mitochondrial β-oxidation (Fig. 3). Two isoforms of ACC (ACC1 and ACC2), encoded by separate genes, have been identified. ACC1 is a 265-kDa protein that is mainly expressed in liver and adipose tissue (84, 222, 259).

ACC2 is a 280-kDa protein that, compared to ACC1, contains 117 additional amino acids at the N-terminus to anchor the protein to the outer mitochondrial membrane. ACC2 is mainly expressed in muscle, but its expression in the liver is also of biological relevance (1).

Studies with knockout mouse models and with functional gene suppression by ASOs demonstrated that these two isoenzymes deliver malonyl-CoA into 2 functionally separate pools: a cytosolic pool used for lipogenesis and a subcytosolic pool next to the mitochondrial outer membrane to inhibit CPT-I (44, 254).

Genetically, ACC2-deleted mice (ACC2$^{-/-}$ mice) showed enhanced mitochondrial FA β-oxidation in heart, muscle, and liver, decreased fat storage, and improved insulin sensitivity,

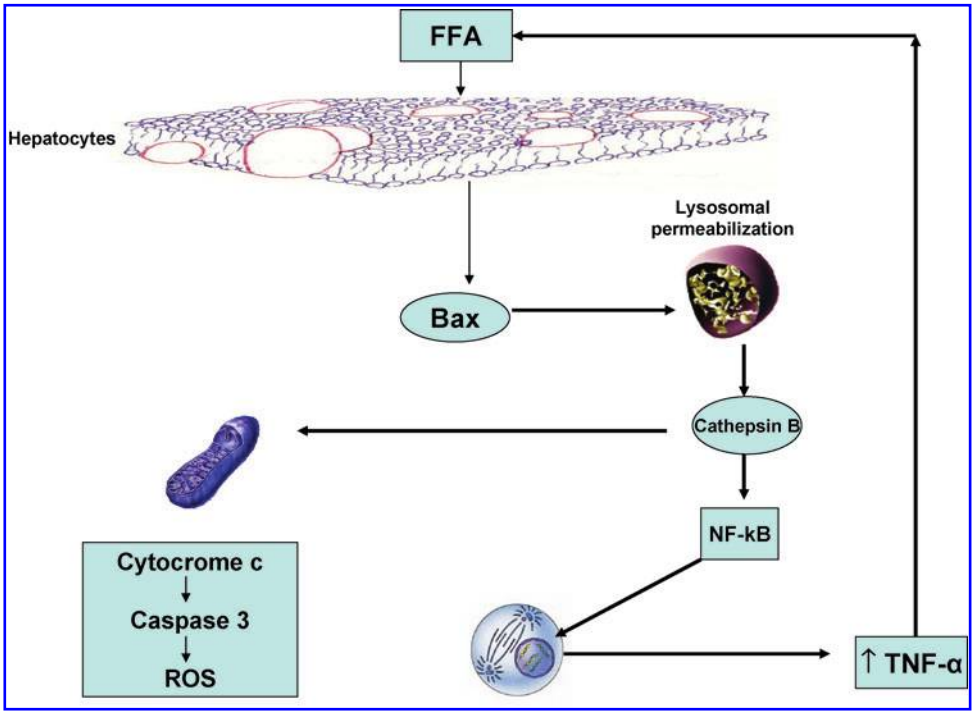

FIG. 2. Molecular mechanisms linking FFA and lysosome to liver damage in nonalcoholic fatty liver disease. In the setting of obesity and insulin resistance, high levels of FFAs are delivered to the liver. Hepatocyte stimulation by FFAs leads to the intracellular translocation of Bax to the lysosome. Lysosomal permeability is increased, leading to release of cathepsin B. The presence of capthesin B in the cytosol causes NF-κB translocation into the nucleus with increased production and release of TNF-α. Cathepsin B also causes mitochondrial dysfunction leading to hepatocyte apoptosis. FFA, free fatty acid; TNF-α, tumor necrosis factor-α. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

despite a 30% increase in food intake compared with their wild-type littermates, upon feeding a high-fat or a high-fat/high-carbohydrate diet (305). Enhanced mitochondrial β-oxidation was coupled with an increased CPT-I activity and an increased expression of UCP-2 and UCP-3 in heart, adipocytes, and muscle, further contributing to energy expenditure. Importantly, lipogenesis in these mice was unaffected, suggesting that malonyl-CoA generated by mitochondrial ACC2 is not required for progression of FA synthesis.

Whole-body deleted ACC1$^{-/-}$ knockout mice are not viable and die at embryonic stage (2), underscoring the importance of lipogenesis in early embryonic development.

Liver-specific ACC1 knockout (ACC1$^{-/-}$) mice fed a fat-free diet showed a 70% reduction in hepatic malonyl-CoA concentrations compared with controls, a 50% decrease in *de novo* lipogenesis, and a 40% decrease in hepatic triglycerides (TG) concentration without affecting FA oxidation and glucose homeostasis, thus further confirming the existence of distinct malonyl-CoA pools (168). However, upon feeding a high-fat diet, the liver-specific ACC1 deletion did not prevent the development of obesity, fatty liver, and insulin resistance. Consistently, liver-specific ACC1 mice generated by Harada *et al.* (97) showed virtually no alteration in hepatic lipogenesis, due to a compensatory effect af ACC2 activation: hepatic malonyl-CoA levels were not decreased, suggesting that malonyl-CoA synthesized by ACC2 is involved not only in mitochondrial FA oxidation but also in cytosolic *de novo* lipogenesis, at least in these genetically engineered models.

Alternative to gene knockout, Savage *et al.* (254) injected intraperitoneally ASO inhibitors to suppress ACC1 and ACC2 expression, either independently or synergistically, to test the hypothesis that inhibition of ACC1 would decrease the rate of lipogenesis and inhibition of ACC2 would stimulate mitochondrial β-oxidation.

They found that in high-fat-fed rats the synergic inhibition of both ACC1 and ACC2 was required to significantly reduce hepatic malonyl-CoA concentrations, reverse hepatic steatosis, and improve hepatic insulin sensitivity, the latter being associated with an increase in both Akt and forkhead box subgroup O1 phosphorylation.

Besides ASO technique, a number of ACC inhibitory compounds have been recently developed and tested in animal models, including CP-640186 (91) and Soraphen (83). CP-640186 inhibits both isoenzymes ACC1 and ACC2 reversibly and nonselectively. Rats treated with CP-640186 showed a reduction in FA and TG synthesis. Malonyl-CoA levels of lipogenic and oxidative tissues were decreased and paralleled an increase in whole-body FA oxidation rate (91). However, in another experiment with diabetic ob/ob mice, an increase in plasma glucose and TG levels and a worsening in glucose tolerance were observed (299). In mice with diet-induced insulin resistance, CP-640186 treatment reduced body-weight gain and improved peripheral insulin sensitivity (260) Soraphen is a more potent ACC inhibitor than CP-640186 (269): by binding to the biotin carboxylase domain of ACC, it disrupts the oligomerization needed for the activity. In high-fat-fed mice, treatment with Soraphen (50 or 100 mg/kg/day) caused a reduction in body-weight gain, a 70% reduction of *de novo* lipogenesis, and a major improvement in peripheral insulin sensitivity (269).

Other modulators of ACC enzyme activity include AMPK and transcription factors sterol regulatory element binding protein 1c (SREBP1c) and carbohydrate responsive element binding protein (ChREBP).

AMPK is a major regulator of ACC1 and ACC2 activities. When energy is required, AMPK is activated by several protein kinases that phosphorylate AMPK at specific serine and thyrosine residues. Phosphorylated AMPK, in turn, phosphorylates ACC, thereby leading to its inactivation (269, 327). This cascade can be reversed by protein phosphatases. At metabolic level, citrate allosterically stimulates both isoforms of ACC (327). Thus, subtle changes in energy requirement can finely modulate ACC activity in the short-term.

Transcription factors SREBP1c and ChREBP, modulating the expression of both isoforms of ACC, provide regulation at the mid- and long-term. After dietary intake of cholesterol and upon stimulation by insulin, ER-bound SREBP1c is cleaved and its activated form is transported into the nucleus, where it promotes transcription of both ACC1 and ACC2 (199). The ChREBP is also involved in the regulation of Acc1 and ACC2 expression: in animal models of genetic ChREBP deletion, exposure of hepatocytes of wild-type mice to high glucose concentrations resulted in induction of mRNA levels of genes involved in lipogenesis, including ACC1, whereas high-glucose concentrations failed to induce this response in hepatocytes of ChREBP$^{-/-}$ mice (98). ACC gene expression is

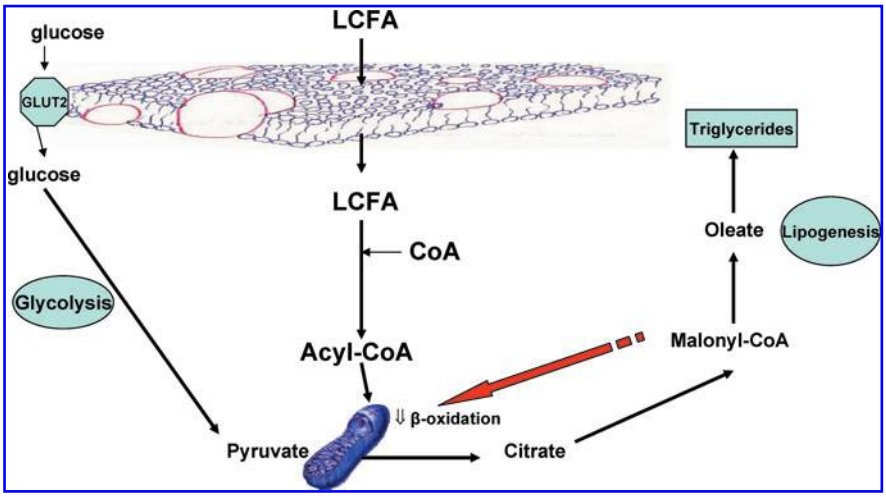

FIG. 3. Metabolic pathways modulating the synthesis and oxidation of FAs in the mitochondrion. The rate of FFA oxidation is critically modulated by the availability of malonylCoA in the proximity of mitochondrion, which inhibits carnitine palmytoiltransferase I. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

also sensitive to fibrates and the induction of ACC2 yields significant elevations in mitochondrial CPT-I activity in mouse liver (305). Altogether, the results of these studies show that ACC is a potentially useful target of future pharmacological approaches targeting hepatic steatosis, insulin sensitivity, and associated metabolic disorders. A major issue still unanswered by these experiments is whether enhanced FFA oxidation may lead to an increased ROS production and enhanced liver injury, thereby aggravating necro-inflammatory changes and steatohepatitis despite a reduction in liver fat content (100).

B. Modulation of PPAR-γ coactivator 1α gene activity

PGC-1α is a transcription coactivator, that is a protein or protein complex that increases the probability of a gene being transcribed by interacting with transcription factors but does not itself bind to DNA in a sequence-specific manner. PGC-1α interacts with transcription factors involved in a broad range of biological functions, including adaptive thermogenesis, mitochondrial biogenesis and function, glucose and FA synthesis and oxidation, fiber type switching in skeletal muscle, and heart development [reviewed in ref. (151)]. The nuclear receptors PPARs (PPAR-α, PPAR-δ, and PPAR-γ) are all subject to transcriptional coactivation by PGC-1α. PPAR-γ is essential for adipogenesis and differentiation, whereas PPAR-α and PPAR-δ play important roles in the control of FA oxidation. In addition, PPAR-γ is the target of thiazolidinediones, which are drugs that are widely utilized for increasing insulin sensitivity in diabetes.

The PGC-1α gene is located on chromosome 5 in mice (chromosome 4 in humans) and encodes a protein containing 797 (mouse) or 798 amino acids (human). PGC-1α has two putative nuclear localization signals and is located in the cell nucleus. It is expressed at high levels in tissues where mitochondria are abundant and oxidative metabolism is active, including brown adipose tissue, cardiac and skeletal muscle, brain, and kidney, whereas its expression level is lower in the liver and very low in white adipose tissue. A peculiarity of PGC-1α is that of being highly inducible by physiologic conditions known to increase the demand for mitochondrial ATP or heat production, including cold exposure, short-term exercise, and fasting. PGC-1α does not have histone acetyltransferase (HAT) activity itself, but the conformational change it induces after docking on specific transcription factors increases the affinity of the transcription complex to additional coactivators possessing HAT activity, such as steroid receptor coactivator-1 and cAMP response-binding element (CREB)-binding protein and p300. The result is acetylation of histone proteins, which produces conformation alterations that increase the accessibility of DNA to the transcription complex (149).

There is a growing interest in PGC-1α because recent evidence strongly suggests that it is a powerful regulator of energy metabolism under conditions of both health and disease (149). PGC-1α enhances adaptive thermogenesis in both brown fat and skeletal muscle, through the stimulation of mitochondria biogenesis, increased FA oxidation, and the uncoupling of oxidative phosphorylation. The essential role of PGC-1α in adaptive thermogenesis is demonstrated by the observation that the PGC-1α-deficient mice are unable to withstand a cold stress (4°C) for longer than 6 h due to a continuous decrease of core body temperature. Wild-type control mice, on the other hand, were able to tolerate the cold stress by keeping their core body temperature at 36.5°C, after an initial drop of 1.5°C (149, 155).

Mechanisms whereby PGC-1α induces adaptive thermogenesis involve increased transcription of nuclear respiratory factor (NRF)1 and NF-E2-related factor (NRF2), leading to the increased expression of mitochondrial transcription factor A (mtTFA) (331) as well as other nuclear encoded mitochondria subunits of the electron transport chain complex such as β-ATP synthase, cytochrome c, and cytochrome c oxidase IV (256). mtTFA translocates to the mitochondrion, where it stimulates mitochondrial biogenesis as manifested by stimulation of mitochondrial DNA replication and mitochondria gene expression (78). PGC-1α also interacts with other nuclear hormone receptors such as PPAR-α, thyroid receptor, and retinoic acid receptor, in brown adipose tissue to enhance the expression of brown fat-specific UCP-1 (17, 213). UCP-1 action leads to dissipation of the proton gradient and the uncoupling of oxidative phosphorylation, thereby increasing heat production. Under these conditions, there is a marked increase in the rate of energy metabolism.

In the liver, PGC-1α is expressed at low levels under normal fed conditions (223), but fasting produces a significant increase of PGC-1α expression, leading to stimulation of hepatic gluconeogenesis and FA oxidation (103, 353). During fasting, the expression of PGC-1α is activated by glucagon and catecholamines *via* the stimulation of the cAMP pathway and the CREB transcription factor. PGC-1α subsequently coactivates a variety of transcription factors such as hepatic nuclear factor-4α, glucocorticoid receptor, and forkhead box subgroup O1. These transcription factors bind to the promoter regions of those genes encoding key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (G-6-Pase).

The importance of PGC-1α in the regulation of hepatic gluconeogenesis, mitochondrial biogenesis and function, and FA oxidation has been confirmed in several cellular and animal models. In cultured primary hepatocytes, overexpression of PGC-1α is sufficient to drive the expression of key gluconeogenic genes, whereas knockdown of PGC-1α expression in the mouse liver dramatically reduced the expression of phosphoenolpyruvate carboxykinase and G-6-Pase and was associated with fasting hypoglycemia in response to impaired gluconeogenic gene expression and hepatic glucose production (155, 200).

PPAR-γ coactivator 1α gene null (PGC-1α$^{-/-}$) mice displayed increased body fat and altered skeletal muscle mitochondrial structure and function, coupled with a reduction in the expression of nuclear genes encoding proteins involved in mitochondrial electron transport (cytochrome c and cytochrome oxidase IV) and oxidative phosphorylation (beta subunit of ATP synthase). In addition, the expression of Tfam, a known PGC-1α target involved in mitochondrial DNA replication/transcription, was diminished in PGC-1α$^{-/-}$ muscle, providing one potential mechanism for defective mitochondrial biogenesis (151). These mitochondrial changes were associated with increased muscle fatigability in response to repetitive stimulation, indicating that PGC-1α is necessary for functional adaptation of skeletal muscle to physiologic demands.

In a parallel way with the muscle, the liver of PGC-1α$^{-/-}$ mice developed hepatic steatosis after a 24-h fasting period.

There were no differences in plasma TG or free FAs between the genotypes in fed or fasted states, rather hepatic steatosis derived from a combination of reduced mitochondrial FA oxidation capacity and an increased expression of lipogenic genes in the liver. The reduced FA oxidation of knockout mice was due to a mitochondrial respiratory dysfunction in state 2 and state 3 respiration rates, whereas the hepatic expression of PPAR-α target genes involved in cellular FA oxidation were not significantly different between the genotypes under fed or fasted conditions. An enhanced lipogenesis also contributed to steatosis in PGC-1$\alpha^{-/-}$ mice: the fasting-mediated downregulation of SREBP1c and its target gene stearoyl-CoA desaturase was abolished in PGC-1$\alpha^{-/-}$ mice. Further, expression of the gene encoding diglyceride acyltransferase, which catalyzes the last step in TG synthesis, was activated at baseline and induced by fasting to a greater level in PGC-1$\alpha^{-/-}$ mice. These results suggest that, in addition to a defect in oxidation, components of the TG synthesis pathway are activated in the PGC-1$\alpha^{-/-}$ mice and contribute to the fasting-induced hepatic steatosis in these animals.

These experimental data have been recently supported by a cross-sectional human study by Yoneda *et al.* (349), who found the functional PGC-1α single-nucleotide polymorphism rs2290602 to be associated with the presence and severity of NAFLD in 556 human subjects. Future studies will evaluate the effects of functional PGC-1α manipulation on whole-body, muscle, and hepatic energy homeostasis and function.

C. Modulation of PPAR-α activity

PPARs play a key role in modulating hepatic TG accumulation. PPARs are parts of the nuclear receptor superfamily (54) and they can be classified into three isotypes: PPAR-α, PPAR-β, and PPAR-γ (197). Lipids are natural PPAR ligands, leading to regulation of lipid metabolism and fuel partioning (54). PPARs form a heterodimer with the retinoid X receptor (RXR). The PPAR:RXR heterodimer binds to DNA PPAR response elements, resulting in gene transcription (16, 80).

PPAR-α is expressed in the liver and other metabolically active tissues, including striated muscle, kidney, and pancreas (116), where it regulates FA β-oxidation. In particular, PPAR-α regulates many genes encoding enzymes involved in the mitochondrial and peroxisomal FA β-oxidation. Peroxisome proliferators activating PPAR-α induce the gene transcription of the enzymes operating in both mithocondrial and peroxisomal FA β-oxidation, such as the acyl-CoA synthetase, the carnitine palmitoyl transferase I, the very long-chain acyl-CoA dehydrogenase, the tri-functional protein, the straight-chain acyl-CoA oxidase, the L-bifunctional protein, and the 3-ketoacyl-CoA thiolase (234, 236).

PPAR-α is also a regulator of the inflammatory response. In PPAR-α-deficient mouse inflammation is prolonged (55). There are several *in vivo* and *in vitro* studies about this issue. In vascular cells culture PPAR-α activation inhibits the production of endothelin-1, vascular cell adhesion molecule (VCAM)-1, IL-6, and in endothelial cells culture, factor tissue, smooth muscle cells, and macrophages (172). *In vivo* experiments showed that an exasperated inflammatory response to lipopolysaccharide stimulation occurred in aorta from PPAR-α-deficient mice (52). PPAR-α regulates also acute phase protein (APP) genes in liver cells. CRP, fibrinogen, serum amyloid A (SAA), and α2-macroglobulin belong to the APPs (107). The levels of these APPS are down-modulated by fibrates through a PPAR-α dependent mechanism (130). Therefore, such markers of inflammation are lowered after fibrate treatment, whereas albumin rises since it is a negative acute phase protein.

Loss of expression of the PPAR-α gene in mice results in hepatic steatosis when FA metabolism increases during fasting or high-fat diet (129). The hepatic steatosis decreases after administration of a potent PPAR-α agonist (187).

PPAR-α interacts with other factors, such adiponectin, which, upregulated by PPAR-γ, activates in turn PPAR-α (337). This mechanism aims at increasing hepatic FA oxidation (Fig. 4).

PPAR-α can be a target for the treatment of NAFLD/NASH. Fibric acid derivatives available as lipid lowering agents serve as PPAR-α activators (326). In a mouse model of fatty liver disease, fenofibrate improved steatosis and increased expression of genes involved in FA metabolism (178). Omega-3 polyunsaturated FAs (PUFA) present in fish oil, and their metabolites provide another source of PPAR-α ligands. Omega-3 PUFA also inhibit lipogenesis by antagonizing activation of liver X receptor (27, 189), thus reducing expression of SREBP1c (238), which results in the downregulation of key enzymes involved in hepatic lipid biosynthesis. In mouse models, ω-3 PUFA supplementation was associated with improvement in hepatic steatosis and insulin sensitivity, as well as lower fasting free FA concentration and lower serum TG levels (7).

D. Modulation of PPAR-γ activity

PPAR-γ is most highly expressed in adipocytes and to a lesser degree in hepatocytes in humans, but can be also found in vascular endothelium, pancreatic β-cells, hepatic stellate cells (HSCs), and macrophages. PPAR-γ activation promotes adipocyte differentiation and FFA uptake and storage in subcutaneous adipose tissue, driving FFA away from visceral depots and reducing FFA delivery to the liver, muscle, and other insulin-sensitive tissues. Besides fat repartitioning, PPAR-γ activation inhibits macrophage activation and proinflammatory cytokine production, enhances adiponectin secretion, inhibits TNF-α and resistin secretion in adipocytes, increases hepatic adiponectin receptor expression, and inhibits stellate cell activation (133, 267, 286, 338).

Importantly, PPAR-γ is expressed in human liver at lower levels than adipocytes, whereas its hepatic expression is higher in rodent models of steatosis: this may explain the divergent effects of PPAR-γ activation between humans and mice, where rosiglitazone induce hepatic steatosis in lipoatrophic mice, virtually devoid of fat tissue, whereas it decreased hepatic lipid content in mice with normal fat depots, since lipid-storing effects of hepatic PPAR-γ activation were superseded by PPAR-γ activation of adipose tissue (79). The hepatic steatogenic and proinflammatory effects of thiazolidinediones in mice may be at least partially mediated by iNOS activation, as in high-fat-fed obese mice iNOS disruption increases the ability of rosiglitazone to raise plasma adiponectin levels, further improving glucose tolerance, hepatic insulin sensitivity, and fat accumulation in the liver and adipose tissue (48). For the reasons discussed above, the overall effect of PPAR-γ activation in humans are an increase in insulin

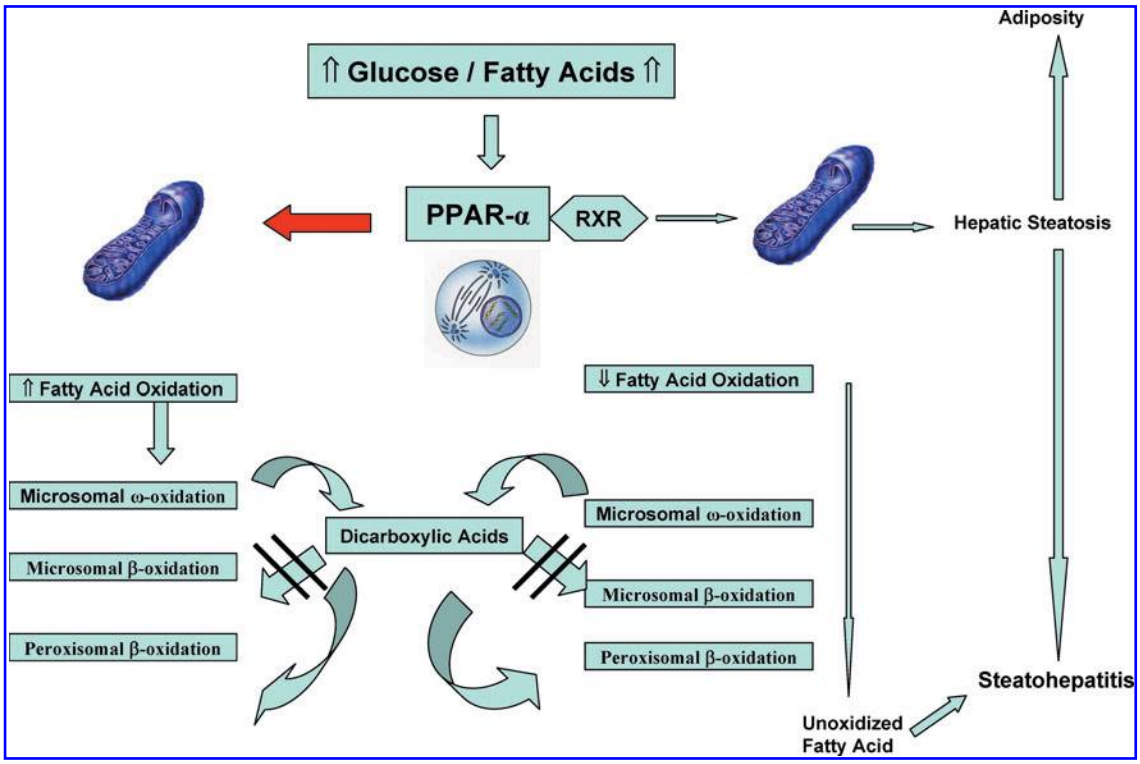

FIG. 4. Cross-talk between FA oxidation systems and PPAR-α in energy metabolism in the liver. In the liver, mitochondrial, peroxisomal, and microsomal FA oxidation systems are regulated by PPAR-α and metabolize energy. Increased PPAR-α sensing in the liver and the induction of the three FA oxidation systems results in increased energy burning and reduced fat storage. Decreased PPAR-α sensing and/or decreased FA oxidation capacity leads to a reduction in energy utilization and increased lipogenesis (PPAR-γ mediated), resulting in steatosis and steatohepatitis. Alcoholic and nonalcoholic steatohepatitis emanate from perturbations of FA oxidation systems in the liver. Abnormalities associated with different FA oxidation systems caused by genetic, toxic (including drug related), and metabolic perturbations also result in decreased energy burning in the liver, leading to lipid storage in liver cells. PPAR, peroxisome proliferator-activated receptor. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

sensitivity, coupled with a reduction in circulating free FAs, and a decrease in systemic and hepatic inflammatory and fibrogenic state. For these reasons, PPAR-γ agonists are an attractive therapeutic option as they reverse many of the pathophysiological abnormalities present in NAFLD.

E. Modulation of PPAR-δ activity

Among the three members of the PPAR nuclear receptor superfamily, PPAR-δ has been the last to receive attention and to be evaluated for the treatment of different metabolic disorders, including NAFLD.

Ubiquitously expressed, with high expression in liver and skeletal muscle, PPAR-δ has been implicated in lipid metabolism and energy homeostasis of various tissue types, including the liver. PPAR-δ agonists have been reported to increase high-density lipoproteins in animal models and regulate FA oxidation in cardiomyocytes and skeletal muscles (89, 219). The effect of PPAR-δ agonists on hepatic lipid oxidative metabolism and inflammation have been evaluated in three animal modes of NAFLD: the leptin-deficient (ob/ob) mice (240, 317), methionine- and choline-deficient diet mice (186), and low-density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice fed a Western diet (154).

In the methionine and choline deficient (MCD) dietary model of NASH, PPAR-δ agonist GW501516 reduced hepatic TG and thiobarbituric acid-reactants contents, liver inflammation, and number of activated HSCs. In this model, GW501516 increased hepatic expression of enzymes associated with FA β-oxidation [acyl-CoA oxidase (ACO), CPT-I, liver-FA binding protein, and peroxisomal ketothiolase], and it reduced the levels of those associated with inflammatory cytokines or chemokine (TGF-β1, IL-6, IL-1β, monocyte chemoattractant protein [MCP]-1, TNF-α, and nuclear factor [NF]-κB1)] (124).

Consistently with these findings, in the $LDLR^{-/-}$ mice, the synthetic selective PPAR-δ agonist L-165041 ([4-3-(4-Acetyl-3-dydroxyl-2-propylphenoxy propoxy) phenoxy] acetic acid) drastically reduced lipid accumulation in the liver. Gene expression analysis demonstrated that L-165041 lowered hepatic expression of PPAR-γ, apolipoprotein B, IL-1β, and IL-6.

Importantly, and differently from PPAR-α agonists, activation of PPAR-δ promotes FA β-oxidation and adaptive thermogenesis/mitochondrial uncoupling at extrahepatic sites,: PPAR-δ agonists in fact increased the expression of UCP-1 and UCP-3 in brown adipose tissue and of genes encoding enzymes for FA β-oxidation in both adipose tissue and muscle. Further, differently from PPAR-γ agonists, PPAR-δ activation had no effect on adipocyte lipogenesis (240, 287, 317) (Fig. 5).

It can be concluded from the data discussed above that PPAR-δ agonists show promise for the treatment of NASH, since they extend to extrahepatic tissues the beneficial effects of PPAR-α agonists without having the unwanted effects of

PPAR-γ agonists. Caution is warranted until these results are extended to humans, where different between-species tissue distribution of PPARs may substantially modify overall effect of PPAR-δ activation.

F. Summary

PPAR-δ has been implicated in lipid metabolism and energy homeostasis of various tissue types, including the liver. PPAR-δ agonists have been reported to increase high-density lipoproteins and regulate FA oxidation in cardiomyocytes and skeletal muscles. It can be concluded that PPAR-δ agonists could be a good option for the treatment of NASH.

V. Adipokines in NAFLD

Visceral adipocyte are now recognized as not simply a storage depot for excess energy, but rather an active endocrine organ secreting several molecules termed adipokines. The adipokines recently linked to the pathogenesis of obesity-associated NAFLD will be discussed hereafter.

A. Leptin

Encoded by "ob" gene, leptin is synthesized by mature adipocyte in response to changes in body fat mass and nutritional status. In obesity, plasma leptin levels are increased proportionally to body mass index (BMI) and acutely decrease in response to fasting or restriction of energy intake to a much larger extent than would be expected for smaller reductions of adiposity, thus signaling a negative energy balance. Adipocyte size and anatomical location (subcutaneous) appear to be the major determinants of leptin mRNA expression and secretion. *In vivo*, overfeeding and obesity, glucocorticoid treatments, glucose, and insulin increase leptin levels, whereas fasting, sustained exercise, cold exposure, and weight loss reduce leptin levels (170).

Besides its anorexigenic action in hypothalamus, leptin is an insulin-sensitizing hormone and reduces muscle, pancreas, and hepatic lipid content. In muscle, it inhibits malonyl-CoA synthesis, thereby increasing mitochondrial FFA oxidation. Further, leptin directly stimulates adenosine monophosphate kinase (AMPK), which activates ATP-producing catabolic pathways, such as β-oxidation and glycolysis, and inhibits ATP-consuming anabolic pathways.

Animals devoid of leptin expression, ob/ob mice (leptin gene mutation), db/db mice, and fa/fa rats (leptin receptor gene mutations) are obese, are insulin resistant, and have NAFLD, alterations reversed by leptin administration (63, 179). In lipoatrophic human diabetes, characterized by scarce mass, diminished leptin levels, and markedly elevated

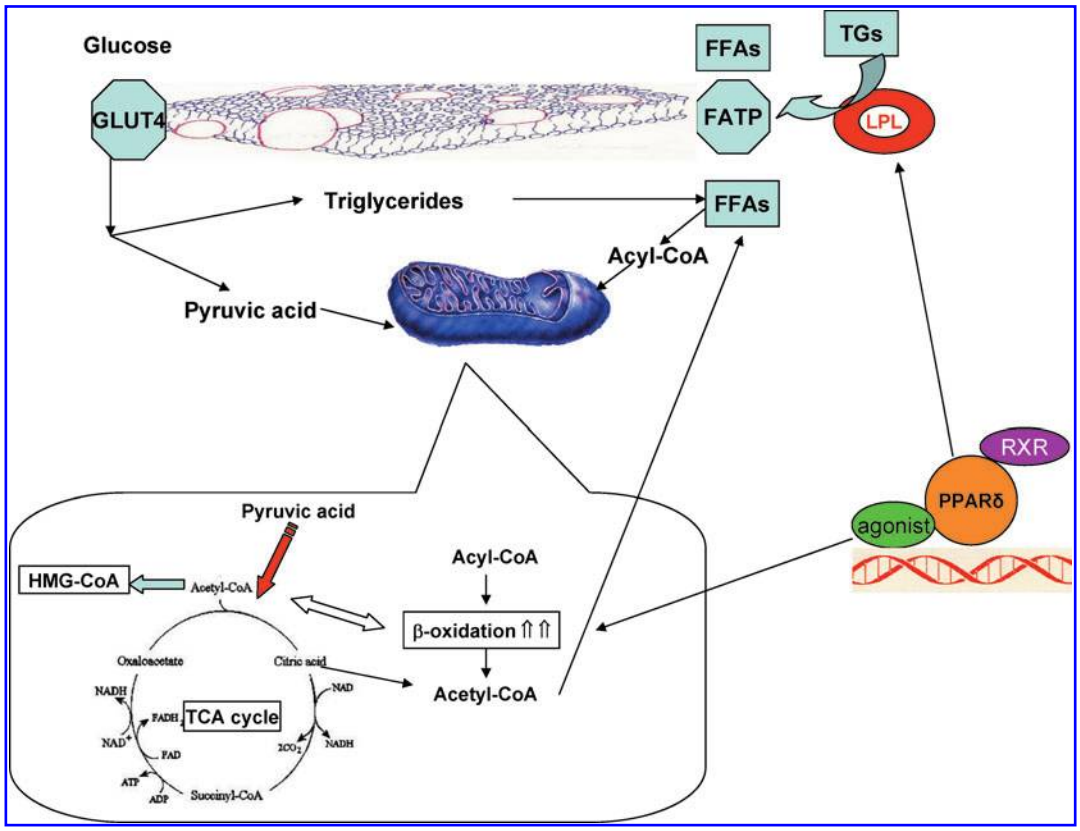

FIG. 5. Effects of PPAR-δ agonist on the cellular gene expression. PPAR-δ agonist increased the expression of genes encoding enzymes for FA β-oxidation in both adipose tissue and muscle. Differently from PPAR-γ agonism, PPAR-δ activation had no effect on adipocyte lipogenesis. ACAA2, acetyl-CoA acyl transferase2; ACS, acyl-CoA synthetase; CPT, carnitine palmitoyl transferase; DECR, 2,4-dienoyl CoA reductase; FATP, fatty acid transport protein; Glut4, glucose transporter 4; HADHA, mitochondrial trifunctional protein; HMGCS2, 3-hydroxymethyl-glutaryl-CoA reductase; HSL, hormone sensitive lipase; LCAD, long-chain acyl-CoA dehydrogenase; LPL, lipoprotein lipase; PDC, pyruvate dehydrogenase complex; PDK4, pyruvate dehydrogenase kinase; UCP, uncoupling protein. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

intrahepatic TG, leptin administration reduces liver enzymes, BMI, hepatic fat content, and histological steatohepatitis (146). However, in obese NAFLD patients leptin levels are elevated and directly correlate with hepatic fat accumulation, thus suggesting the presence of resistance to the antisteatotic and insulin-sensitizing actions of leptin. The reason for this phenomenon is poorly understood and may result from defects in leptin signaling or transport across blood brain barrier (35).

Leptin is also believed to play a significant part in hepatic fibrogenesis: ob/ob mice supplemented with daily intraperitoneal leptin injections develop significant hepatic fibrosis and higher levels of collagen I mRNA (142, 255). This action may be mediated by TGF-β secretion by Kupffer cells or may involve direct HSCs activation. Upon activation, HSC produce leptin, which further stimulates fibrogenesis, as HSC express leptin receptors, thus perpetuating the vicious cycle (293). At present, the contrasting effects of leptin do not allow to suggest that increasing leptin levels may be suitable for treating obesity-associated NAFLD.

B. Tumor necrosis factor-α

Upon secretion by visceral adipocytes and activated macrophages, TNF-α interacts with TNF receptors (TNF-R1 and TNF-R2): TNF-R1 mediates apoptosis and lipolysis, whereas TNF-R2 induces insulin resistance (262). In adipocytes, TNF-α acts mainly in an autocrine/paracrine fashion, reducing expression and secretion of leptin and adiponectin and inducing insulin resistance by reducing GLUT-4 expression and lipoprotein lipase activity and by increasing expression of hormone sensitive lipase (293).

TNF-α also impairs insulin signaling in adipocytes and hepatocytes through activation of stress-related protein kinases, such as c-JUN N-terminal kinase 1 (JNK-1), and of the inhibitor kappa kinase β (IKKβ)/nuclear factor kappa B (NF-κB) pathway. Low-grade activation of the IKKβ/NF-κB pathway in hepatocytes results in a state of subacute chronic inflammation with increased production of cytokines, including TNF-α and IL-6, promoting hepatic and systemic insulin resistance (15, 71).

Serum TNF-α is increased in obese and diabetic subjects, whereas weight loss decreases its levels. Although the correlation between insulin resistance and plasma TNF-α is weak, adipocyte TNF-α mRNA levels correlate tightly with body fat stores and hyperinsulinemia.

In the liver, production of TNF-α by Kupffer cells is believed to play a crucial role in the pathogenesis of NASH. In ob/ob mice, NAFLD is significantly improved by inhibition of hepatic TNF-α production or by infusion of anti-TNF-α antibodies and TNF-α knockout could prevent the development of insulin resistance induced by a high-fat diet, suggesting a role of TNF-α in mediating fat-induced hepatic insulin resistance (71, 293).

C. Resistin

Secreted by visceral adipocytes, resistin induces low-grade inflammation by enhancing nuclear translocation of NF-κB and TNF-α secretion from macrophages; it also promotes insulin resistance by downregulation of GLUT-4 in adipocytes (293). Resistin levels are increased in NAFLD patients and are correlated with histological severity and hepatic insulin resistance (185, 206). Resistin gene expression and protein secretion are markedly reduced by thiazolidinediones, and administration of antiresistin antibody has been shown to improve blood glucose and insulin action in mice with diet-induced obesity, whereas weight loss significantly reduced circulating resistin in humans (293).

D. Adiponectin

Contrary to other adipokines, adiponectin is believed to play a protective role against the development of different metabolic disorders and decreased adiponectin concentrations strictly correlate with the incidence and severity of diabetes, obesity, metabolic syndrome, and NAFLD (185, 293).

Adiponectin has antilipogenic effects that may protect nonadipocyte tissues like liver and muscle: it stimulates mitochondrial β-oxidation by activating AMP-dependent protein kinase (AMPK) and inhibits lipogenesis by downregulating SREBP1c. This, in turn, reduces malonyl-CoA levels and the inhibition on carnitine palmityl transferase-1, enhancing FFA oxidation and reducing hepatic TG accumulation (339). Adiponectin also has antioxidative and anti-inflammatory properties by antagonizing the effects of inflammatory mediators like TNF-α and attenuates fibrosis progression of NAFLD by reducing proliferation and increasing apoptosis of HSCs. Consistently, administrations of recombinant adiponectin markedly improved NASH in ob/ob mice (333, 363).

In human NAFLD plasma adiponectin is reduced compared with BMI and waist-matched healthy controls and closely correlates with liver histology, despite comparable levels of other proinflammatory adipokines, including TNF-α, leptin, and resistin, suggesting that hypoadiponectinemia may be an early event in the pathogenesis of NAFLD (36, 184).

E. Retinol binding protein 4

Retinol binding protein 4 (RBP4) was identified as an adipokine whose expression is increased in the adipose tissue of mice rendered insulin resistant by adipose-specific inactivation of the glucose transporter GLUT4 (341). RBP4 is highly expressed in liver and adipose tissue, and its circulating levels correlate with obesity, insulin resistance, and liver fat in rodents and humans (215, 281). Increased serum RBP4 levels might promote insulin resistance by impairing insulin-stimulated glucose uptake in muscles, elevating hepatic glucose production, and interfering with insulin signaling in adipocytes, although the mechanism is not fully clear (203). The relation of RBP4 with hepatic steatosis was independent of total, subcutaneous/visceral fat and insulin resistance, suggesting that other mechanisms, possibly involving hepatic retinol metabolism, may link RBP4 to steatosis (203, 215).

F. Renin-angiotensin system

Adipocytes express all components of renin-angiotensin system, including angiotensinogen, and renin-angiotensin system is overactivated in visceral adipose tissue of obese subjects (259).

Angiotensin II type-1 receptors have been found in adipocytes, hepatocytes, Kupffer cells, and HSCs (82). In adipocytes, angiotensin-II induces the release of proinflammatory adipokines, including IL-6 and TNF-α, and inhibits adipo-

nectin secretion. In the liver, angiotensin-II promotes insulin resistance, proinflammatory cytokine production, and fibrogenesis through ROS generation and NF-κB pathway activation (163, 318, 319).

Animal studies demonstrated a marked decrease in hepatic, insulin resistance, steatosis, and fibrosis and stellate cell activation with ATII type 1 receptor blockers (73, 106, 165). In humans, angiotensin II type 1 receptor polymorphisms have been associated with the presence and severity of NAFLD (348).

G. Interleukin-6

IL-6 inhibits adiponectin secretion and lipoprotein lipase promotes hepatocyte insulin resistance by enhancing SOCS-3 secretion and activated Kupffer cells in an autocrine manner. Consistently, intrahepatic and plasma IL-6 levels are increased in hepatocytes and Kupffer cells of human NASH and correlate with hepatic insulin resistance, necroinflammatory activity, and fibrosis (169, 265, 325). Chronic exposure to increased IL-6 levels promotes hepatocyte apoptosis (194).

H. Summary

Adipokines are crucial for the development of NASH and most of their actions are mediated by ROS generation. Leptin plays an important part in hepatic fibrogenesis through the action of TGF-β and causes direct hepatic HSC activation. TNF-α mediates fat-induced insulin resistance and infusion of TNF-α antibodies improves NAFLD in ob/ob mice. Resistin levels correlate with histological severity and hepatic insulin resistance. Adiponectin plays a protective role in NASH by enhancing FFA oxidative pathways and blunting inflammatory response triggered by pro-inflammatory adipokines. RBP4 might promote insulin resistance by impairing insulin-stimulated glucose uptake in muscles, elevating hepatic glucose production, and interfering with insulin signaling in adipocytes. Angiotensin-II promotes insulin resistance, proinflammatory cytokine production, and fibrogenesis through ROS generation and NF-κB pathway activation. Intrahepatic and plasma IL-6 levels are increased in hepatocytes and Kupffer cells of human NASH and correlate with hepatic insulin resistance, necroinflammatory activity, and fibrosis.

VI. Mechanisms of ROS-Induced Liver Injury in NAFLD

A. Inhibition of very low-density lipoprotein secretion from hepatocytes

An important concept recently emerged is the critical role of cellular redox balance in regulating post-ER presecretory proteolysis of apoB100 and very low-density lipoprotein (VLDL) TG secretion from hepatocytes. The regulation of the hepatic ApoB100 secretion occurs mainly by post-translational degradation of the newly synthesized protein (24, 25). There are three known pathways for post-translational degradation of apoB:

1. ER-associated degradation, which is stimulated by severe lipid deprivation: when conditions are not favorable for apoB assembly with lipids, apoB is ubiquitinylated and degraded by the proteasome. Most newly translated apoB is cotranslationally targeted to the proteasome.
2. Post-ER presecretory proteolysis, triggered by intracellular ROS levels and is believed to mediate the lipid-lowering effects of polyunsaturated FAs (207).
3. Receptor-mediated degradation, also known as reuptake, which occurs *via* cell surface or intracellular interactions of nascent apoB-particles with LDL receptors and heparan sulfate proteoglycans, targeting nascent apoB to the lysosome (47).

Several cellular and animal models have clearly shown that increasing hepatocyte oxidative stress by whatever means (polyunsaturated fat exposure, inactivation of antioxidant defense systems) enhances proteasomal and nonproteasomal apoB100 degradation, inhibits VLDL secretion, and leads to hepatocyte Tg accumulation, alterations totally reversed by antioxidant treatment (77, 121, 204, 301). Therefore, this mechanism is likely to contribute substantially to hepatic fatty infiltration in NAFLD and is an example of how the same mechanisms may be responsible for both steatosis and necroinflammation in NASH.

B. JNK pathway activation

ROS are potent inducers of JNK. Mitochondria are source of intracellulary generated ROS (8). Another important source of intracellular ROS is the small GTPase Rac-NADPH oxidase system, which pivots the respiratory burst and microbicidal function in phagocytes (323). JNKs belong to a subgroup of the mitogen-activated protein kinases (MAPK) superfamily. JNK has three isoforms encoded by three different genes. The *JNK1* and *JNK2* genes are ubiquitously expressed, whereas *JNK3* is found to be neural specific (181). JNK expression has been implicated in hepatocyte injury mediated by TNF-α (263) (Fig. 6), ischemia reperfusion (302), hepatitis (316) virus, and bile acids (227). In the ROS-induced JNK activation, a number of signaling pathways have been involved. One of them involves the MAP kinase kinase kinase (MAPKKK) ASK1. ASK1 activates both JNK and p38 by phosphorylating and activating respective MAPKKs (JNKK1/MKK4, JNKK2/MKK7, MKK3, and MKK6) (49, 111). The internal inhibitor of ASK1 is thioredoxin, an important cellular redox regulatory protein (196) whose redox-status modulates the activity of ASK1. The reduced form of thioredoxin is able to bind to ASK1 and to block its kinase activity. ROS oxidize thioredoxin, which dissociates from ASK1. ASK1 undergoes oligomerization and phosphorylation of a critical threonine residue within the active loop of ASK1 (88). Other functions of thioredoxin include ASK1 ubiquitation and degradation to inhibit ASK1-mediated JNK activation and apoptosis (158).

Another modulator of ASK1 activity is glutaredoxin. Like thioredoxin, glutaredoxin binds to ASK1 in the reduced form and suppresses ASK1 kinase activity (277). Increased levels of ROS cause glutaredoxin to disjoint from ASK1 and the ASK1-MKK4-JNK1 signal transduction pathway to be activated (276).

Protein phosphatase 5 (PP5) is an inhibitor of ASK1 and it plays an important role in the survival of cells in an ROS-rich environment. PP5 binds to the active form of ASK1 to block the ASK1-MKK4-JNK1 signaling cascade to protect against apoptosis (362).

There are other ASK1-independent signaling pathways of JNK activation mediated by ROS, such as the Src-Gab1 pathway, the glutathione S-transferase Pi (GSTπ) pathway,

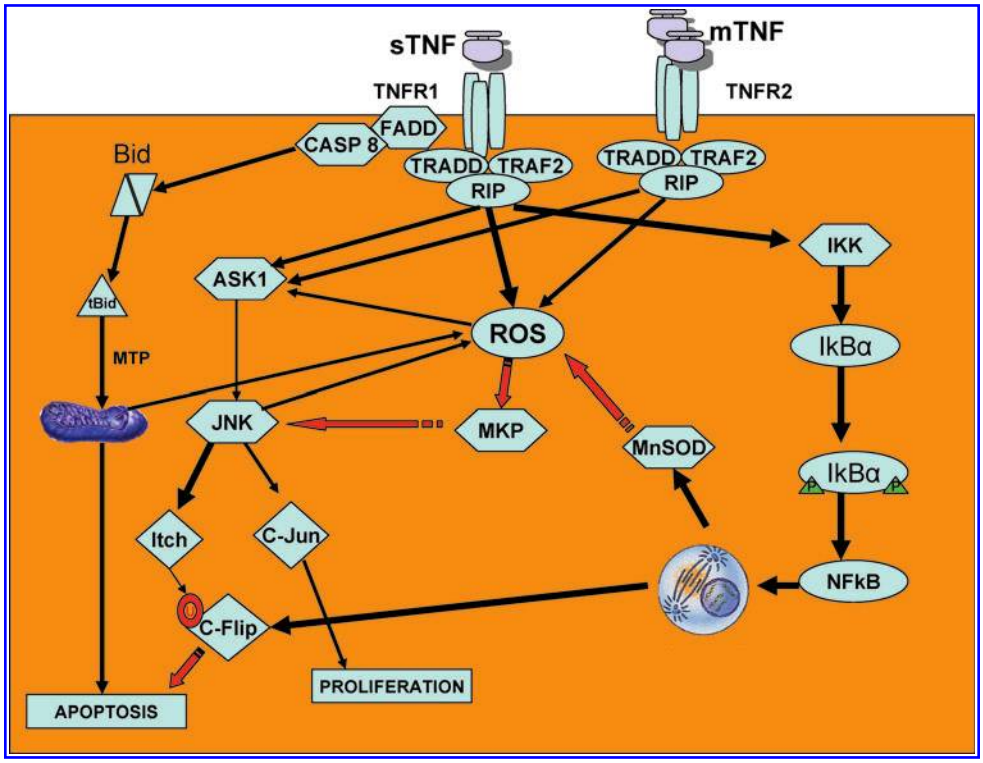

FIG. 6. Role of IKK, JNK, and ROS in TNF-α-induced cell death and proliferation. sTNF binds to TNF-R1, whereas mTNF predominantly binds to TNF-R2. After binding, both TNF receptors recruit the adapter molecules TRADD, TRAF2, and RIP to activate the IKK and JNK pathways. IKK phosphorylates IκB and p65 resulting in IκB degradation and NF-κB activation. Prolonged activation of JNK requires TNF-induced ROS production because ROS contribute to JNK activity by oxidizing and inactivating several members of the MKPs. Prolonged JNK activation shifts the balance toward cell death by inducing phosphorylation of the E3 ligase Itch and subsequent ubiquitination and degradation of the NF-κB-regulated caspase-8 inhibitor c-Flip. NF-κB activation prevents prolonged JNK activation and cell death by inducing transcription of the antioxidant manganese superoxide dismutase. Shorter activation of JNK induces proliferation through its target c-Jun. IKK, inhibitor kappa kinase; JNK, c-JUN N-terminal kinase; MKP, MAP kinase phosphate; mTNF, membrane TNF-α; RIP, receptor-interacting protein; sTNF, soluble TNF-α; TRAF2, TNF receptor-associated factor 2. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

and the receptor-interacting protein (RIP) and TNF receptor-associated factor 2 (TRAF2) and membrane lipid raft pathway. Upon H_2O_2 exposure, Gab 1 is phosphorylated and binds to Src homology 2-containing protein-tyrosine phosphatase, whereas GSTπ dissociates of GSTπ-JNK complex. The signaling ends up with JNK activation (3, 108). H_2O_2-induced JNK activation requires the interaction of JNK with RIP and TRAF2 to form a signaling complex at the cell membrane lipid rafts (268).

After activation, JNK carries out its proapoptotic activity through Bcl-2 family members. In particular, JNK can phoshorylate and either inhibit the antiapoptotic function of Bcl-2 (337) and myeloid cell leukemia 1 (114) or enhance the function of proapoptotic Bcl-2 family members, through conformational changes and mitochondrial translocation of Bax (300) and phosphorylation of Bim and Bmf (147). Bcl-2 family proteins are regulatory factors closely associated with mitochondria and the active JNK can induce the release of apoptogenic factors such as cytochrome c from isolated mitochondria in a cell-free assay (10). Therefore, the Bcl-2 family proteins may provide a molecular link between JNK and the mitochondrial apoptosis.

JNK pathway activation is also involved in the necrosis, or nonapoptotic caspase-independent cell death induced by cell death ligands, such as FasL and TNF-α (309, 310) especially in ROS-rich cellular environments. Treatment with TNF-α promotes ROS accumulation that mediates JNK activation and necrotic cell death (125, 249). In turn, JNK can fuel TNF-α-stimulated necrosis by increasing the production of ROS. Therefore, oxidative stress and JNK constitute a positive feedback loop in TNF-α-induced nonapoptotic cell death (308).

Transient JNK activation may be beneficial to the hepatocyte, whereas sustained JNK activation leads to cell death (157). In particular, JNK1 promotes and JNK2 attenuates hepatocytes injury from toxic bile acids (227). The involvement of JNK activation in the pathogenesis of NASH has been demonstrated in the MCD diet-induced model of mouse steatohepatitis (257). In this nutritional model of NASH, activation of hepatic JNK and of the transcription factor activator protein-1 signaling occurred in parallel with the development of steatohepatitis. Investigations in JNK1- and JNK2-knockout mice demonstrated that JNK1, but not JNK2, was critical for MCD diet-induced JNK activation: MCD diet-fed JNK1 null mice had significantly reduced levels of hepatic TG accumulation, inflammation, CYP2E1 overexpression, lipid peroxidation, liver injury, and apoptosis compared with wild-type and JNK2 knockout mice (21, 257). Ablation of JNK1 led to an increase in serum adiponectin but had no effect on serum levels of TNF-α (271).

The differential roles of JNK1 and JNK2 in hepatic insulin resistance and weight regulation were further demonstrated in high-fat-fed mice (271): JNK1 knockout mice failed to develop excessive weight gain, insulin resistance, or steatohepatitis, whereas JNK2 knockout animals became obese and insulin resistant similar to wild-type mice and had increased liver injury. In mice with established steatohepatitis, an antisense oligonucleotide knockdown of JNK1 improved steatohepatitis and normalized insulin sensitivity. Knockdown of JNK2 improved insulin sensitivity but had no effect on

hepatic steatosis and markedly increased liver injury. The increase in liver injury in JNK2 knockdown was paralleled by the increased hepatic expression of the proapoptotic Bcl-2 family members Bim and Bax and the increase in liver injury resulted in part from a Bim-dependent activation of the mitochondrial death pathway.

This further experiment suggested that JNK1 and JNK2 both mediate insulin resistance in high-fat-diet-fed mice, but the JNK isoforms have distinct effects on steatohepatitis with JNK1 promoting steatosis and hepatitis and JNK2 inhibiting hepatocyte cell death by blocking the mitochondrial death pathway.

These models used a whole-body JNK deletion to demonstrate the role of JNK in the pathogenesis of NASH. More recent experiments showed that JNK signaling plays multiple roles in multiple organs and, as a consequence, its inhibition has different consequences for NASH depending on the site of inhibition.

Kodama *et al.* (131) assessed the functional contribution of JNK isoforms in Kupffer cells *in vitro* and *in vivo* using chimeric dietary choline deficient mice (CDAA) in which the hematopoietic compartment including Kupffer cells was replaced by wild-type, $JNK1^{-/-}$, or $JNK2^{-/-}$ cells. CDAA diet induced significantly less hepatic inflammation and less liver fibrosis despite a similar level of hepatic steatosis in $JNK1^{-/-}$ mice as compared with wild-type or $JNK2^{-/-}$ mice. CDAA diet-induced hepatic inflammation was chronic and mediated by Kupffer cells. Pharmacologic or genetic ablation *JNK1* but not *JNK2* repressed the expression of inflammatory and fibrogenic mediators in primary Kupffer cells. *In vivo*, CDAA diet induced less hepatic inflammation and liver fibrosis despite an equivalent level of hepatic steatosis in chimeric mice with $JNK1^{-/-}$ hematopoietic cells as compared with chimeric mice with wild-type or $JNK2^{-/-}$ hematopoietic cells, further confirming the critical role of these cells in mediating the proinflammatory and profibrogenic effects of JNK in NASH.

Finally, Sabio *et al.* (247) demonstrated that mice with selective inhibition of JNK1 in adipose tissue protected against the development of hepatic steatosis, whereas specific ablation of JNK1 in hepatocytes induced hepatic steatosis, insulin resistance, and glucose intolerance.

Collectively, these experiments demonstrate that JNK1 therefore serves different actions in liver hepatocytes, Kupffer cells, and adipocytes and have potential implications for the design of JNK1-selective drugs for the treatment of NASH and metabolic syndrome (247, 248).

C. TNF-α pathway activation

High levels of ROS take part in the apoptosis process induced by many factors, such as UV, ionizing radiation, cancer chemotherapeutics, and TNF-α (87). TNF-α elicits its biological effects through its cell surface receptor TNFR1 (Fig. 6) (262). After TNF-α has bound to its receptor, TNFR1 joints to a key adaptor protein, TNFR1-associated death domain protein (TRADD). Subsequently, two signaling complexes are formed. Complex I, consisting of TNFR1, TRADD, RIP, and TRAF2, is a membrane complex and it causes the activation of NF-κB and JNK pathways. Complex II is cytoplasmic and it contains TRADD, RIP, FADD, and caspase 8. Complex II is responsible for TNF-α induced apoptosis (177).

NF-κB is a transcription factor that is held in the cytoplasm by members of the inhibitor of kB (IkB) protein family. After forming complex I, the IkB kinase (IKK) is activated. The IKKβ subunit phosphorylates IkBα, leading to its ubiquitination and subsequent proteasomal degradation. This allows the NF-κB complex to translocate into the nucleus, where it can activate transcription of NF-κB responsive gene by binding to kB sites in their promoter regions (101).

NF-κB possesses strong antioxidant function that is performed through some antioxidant enzymes/protein such as MnSOD and ferritin heavy chain (FHC) (217, 253). Unfortunately, next to this cytoprotective effect NF-κB has a causative role in developing hepatic insulin resistance during NAFLD as observed in mice heterozygous for an IKKβ null allele, which are partially protected from insulin resistance on ob/ob background as well after a high-fat diet (32, 356).

Complex I is also responsible for the activation of the JNK pathway trough MKK. Therefore, upon TNF-α stimulation, JNK pathway is activated either by TRAF2 in an earlier and transient phase, or by ROS in a delayed and persistent phase (138, 308). TNF-induced ROS shift the balance toward prolonged activation of JNK by promoting the activation of ASK1 and inhibiting MAP kinase phosphatases that are essential for dephosphorylating activated JNK (125). JNK activation in turn favors TNF-α ROS production in a positive feedback loop. Complex II activates caspase-8, which cleaves Bid to tBid. tBid induces mitochondrial permeabilization through several mechanism. tBid mediates TNF-induced opening of the permeability transition pore in the outer mitochondrial membrane. This phenomenon is named as mitochondrial permeability transition. Specific inhibitors of mitochondrial permeability transition prevent the hepatotoxic effects of TNF *in vitro* as well as *in vivo* (278). Mitochondrial permeability is also increased through the release of cathepsin B from the lysosomes. Cathepsin B cleaves procaspase-2 into caspase-2, which modifies the mitochondrial permeability (99). Next to these mechanisms, tBid mediates the insertion of Bak and Bax in the mitochondrial outer membrane, leading to the formation of pores (360). These events allow cytochrome c and ROS to escape from the mitochondria.

NF-κB activation was linked to the development of the metabolic syndrome and to the progression of fatty liver to steatohepatitis, insulin resistance, and type 2 diabetes mellitus (13, 18). Transgenic mice expressing active IKKβ in their hepatocytes had high serum levels of circulating proinflammatory cytokines such as TNF, IL-1β, and IL-6, and displayed moderate systemic insulin resistance. Inhibition of NF-κB could reverse the metabolic state (18). However, mice lacking IKKβ in hepatocytes developed peripheral insulin resistance in muscle and fat in response to high-fat diet, obesity, or aging (13), but they were protected from liver insulin resistance. When myeloid-specific deletions of IKKβ were produced, a global improvement of insulin sensitivity in mice on a high-fat diet was observed (13) because these deletions regarded either macrophages or Kupffer cells, two cell types involved in the development of hepatic insulin resistance. So, insulin resistance allows free FA to accumulate into hepatocytes, which become important sites for inflammatory cytokine production (19). Another site of cytokine production is the abdominal fat tissue (270). These cytokines may activate Kupffer cells, which can mediate the progression to the metabolic syndrome (270). In NAFLD patients NF-κB activation is

also essential to protect hepatocytes against TNF-induced cell death and to promote regeneration of hepatocytes mass by stimulating hepatocyte proliferation (65). Further, a high-fat diet or obesity sensitizes mice to TNF-mediated liver injury by diminishing NF-κB activation (242). In NAFLD excessive NF-κB activation triggers peripherical insulin resistance and slows TNF-induced cell death (332).

D. ER stress in the pathogenesis of NASH

Another mechanism connecting oxidative stress to hepatocyte injury and death in NASH is the unfolded protein response (UPR), a stress response to different insults that triggered in the ER and it has been recently linked to the pathogenesis of obesity-related disorders, including NAFLD (224, 315). The ER is a specialized organelle synthesizing, folding, assembling secretory, and membrane proteins. In the ER posttranslational maturations such as N-glycosylation, disulfide bond formation and oligomerization, require the presence of ER-resident chaperone proteins, specific redox environment and calcium levels. The concentration of proteins within the ER lumen is very high, $\sim$100 mg/ml. The protein synthesis rate is also incredibly high in hepatocytes, approaching 13 million secretory proteins per minute (272). Fine modulation of this huge energy-consuming process is achieved in the ER lumen.

Any insult (ER stress) perturbing ER folding ability, including alterations in cellular redox balance or in calcium concentration or excessive protein synthesis, triggers a physiological response, the UPR, which leads to an increase of ER folding capacities by enhancing the transcription of ER-resident chaperones and protein foldases, to a downregulation of the protein load in the ER lumen by slowing down protein synthesis and to an ER-associated degradation (ERAD) of irreparably misfolded proteins (245, 246). Accumulation of unfolded proteins is initially sensed by three ER transmembrane proteins, the kinase and endonuclease inositol requiring enzyme 1 (IRE1), the RNA-activated protein kinase (PKR)-like ER kinase (PERK), and the transcription factor activating transcription factor 6 (ATF6), which are normally maintained inactive by the binding of intraluminal ER chaperones, including glucose-regulated protein 78 (GRP78 or BiP) (235, 261) (Fig. 7). During UPR, BiP dissociates from these three transmembrane proteins to deal with the unfolded proteins, loosing IRE-1α, PERK, and ATF6, which self-activate themselves either by dimerization and auto-phosphorilation (IREl and PERK) or transfer (ATF6) to the Golgi apparatus.

The endonuclease IREl splices X box-binding protein 1 (XBP1) mRNA; the resultant spliced XBPl activates transcription through UPRE (UPR element) in promoters of genes that control ERAD. Another function of the nuclease IREl is to degrade mRNA of a variety of genes, which encode for secretory and membrane proteins, thus limiting *de novo* protein synthesis (109).

ATF6 translocates to the nucleus and interacts with ER stress response element (ERSE), which upregulates chaperones/foldases such as GRP78, homocysteine-induced ER protein (HERP), calreticulin, and calnexin, which increase the folding ability of the ER (120).

The activated PERK phosphorylates eukaryotic translation initiation factor 2, alpha subunit (eIF2α) to globally inhibit protein translation halting protein loading while selectively increasing translation of certain mRNAs including ATF4, which upregulates chaperones and antioxidant response genes and enhances damaged ER repairing (261).

Thus, by decreasing the load of nascent proteins, rapid unloading of unfolded proteins, increasing capacity of folding, or clearing out the damaged ER, the early UPR reverses ER stress resulting from accidental perturbations in the ER environment or from transient accumulation of large amounts of secretory proteins under physiological conditions in secretory cells, including hepatocytes.

If the prosurvival efforts are exhausted and ER folding capacity cannot be restored, prolonged or sustained UPR activation leads to pathological consequences, most importantly the initiation of cell apoptosis (ER-stress-related apoptosis): all three UPR pathways are able to trigger cell apoptosis through the activation of C/EBP homologous protein transcription factor (CHOP), JNK, and of caspases apoptotic pathway.

Sustained PERK-induced ATF4 upregulation enhances expression of C/EBP-homologous protein (CHOP or GADD153) (261, 340). CHOP is a transcription factor whose increased expression is a hallmark of the UPR and ER stress response. Pathways mediating CHOP-induced apoptosis are under investigation. Increased expression of CHOP upregulates ER oxidase 1 (ERO1), which promotes oxidative stress and inflammatory response, and GADD34, which dephosphorylates eIF2α, reversing the translational attenuation by the early UPR and thus intensifying ER stress, and downregulates the antiapoptotic gene Bcl-2. In addition to ER stress, TNF activates PKR, and amino acid deprivation activates general control of nitrogen protein kinase (GCN2). Both PKR and GCN2 phosphorylate elF2α kinases, which block translation, promoting apoptosis *via* the ATF4/CHOP pathway and sensitizing to TNF killing by inhibiting synthesis of nuclear factor (NF)-κB-dependent protective proteins. IRE1 is also involved in ER stress-induced apoptosis: IRE1 activation recruits TNF receptor-associated factor-2 (TRAF-2), which activates apoptosis signal-regulated kinase 1 (ASK1) and/or JNK, which promotes apoptosis (see above).

Finally, caspases also participate in ER stress-induced apoptosis. In mice, procaspase-12 is localized on the cytoplasmic side of the ER and is cleaved and activated specifically by ER stress (188). Calpains, a family of Ca^{2+}-dependent cysteine proteases, play a role in caspase-12 activation, and calpain-deficient embryonic cells display reduced ER stress-induced caspase-12 activation and apoptosis (288). In addition, elevation of cytoplasmic Ca^{2+} level caused by tunicamycin and thapsigargin leads to the accumulation and activation of m-calpain at the ER membrane, where it can activate caspase-12 (288). Caspase-7, which translocates from the cytosol to the cytoplasmic side of the ER membrane in response to ER stress, has been reported to interact with and cleave caspase-12, leading to its activation. In that study, a dominant negative mutant of caspase-7 inhibited caspase-12 activation and apoptosis (232).

In addition to the role of calpains and caspase-7 in the activation of caspase-12, TRAF2 has been shown to promote the clustering of procaspase-12 at the ER membrane, thus contributing to activation of this apoptotic pathway (351).

Evidence for an activation of UPR in the steatotic liver has been provided in obese and nutritional rodent models of NAFLD, including the methionine choline-deficient diet mice

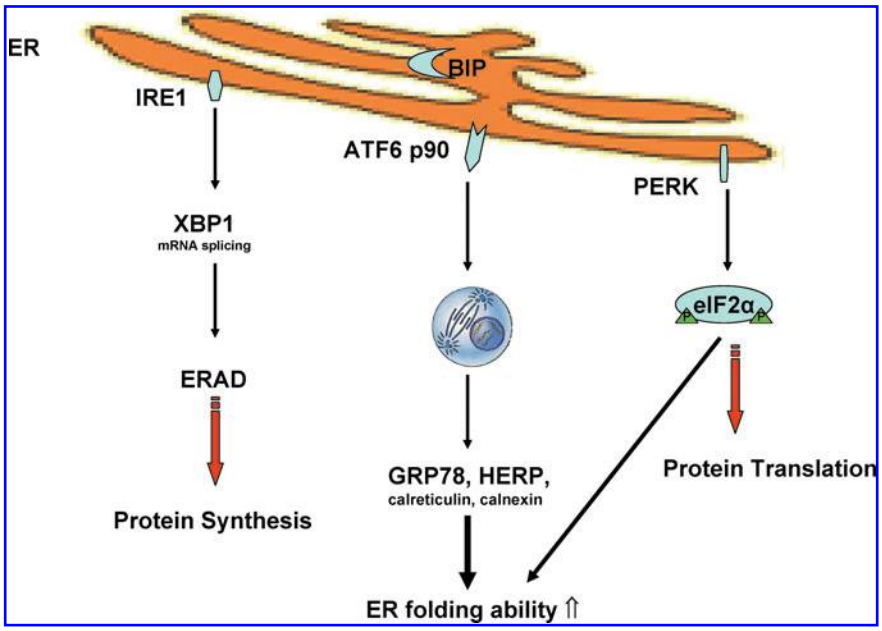

FIG. 7. Pathways of ER stress. Accumulation of unfolded proteins is initially sensed by three ER transmembrane proteins, the kinase and endonuclease IRE1, the RNA-activated protein kinase (PKR)-like ER kinase (PERK), and the transcription factor ATF6, which are normally maintained inactive by the binding of intraluminal ER chaperones, including glucose regulated protein 78 (GRP78 or BiP). During UPR, BiP dissociates from these three transmembrane proteins to deal with the unfolded proteins, loosing IRE-1α, PERK, and ATF-6, which self-activate themselves either by dimerization and auto-phosphorylation (IREl and PERK) or by transfer (ATF6) to the Golgi apparatus. The endonuclease IREl splices X box-binding protein 1 (XBP1) mRNA; the resultant spliced XBPl activates transcription through UPRE (UPR element) in promoters of genes that control ERAD. Another function of the nuclease IREl is to degrade mRNA of a variety of genes that encode for secretory and membrane proteins, thus limiting *de novo* protein synthesis. ATF6 translocates to the nucleus and interacts with ER stress response element (ERSE), which upregulates chaperones/foldases such as GRP78, homocysteine-induced ER protein (HERP), calreticulin, and calnexin, which increase the folding ability of the ER. The activated PERK phosphorylates eukaryotic translation initiation factor 2 and alpha subunit (eIF2α) to globally inhibit protein translation halting protein loading while selectively increasing translation of certain mRNAs, including ATF4, which upregulates chaperones and antioxidant response genes and enhances damaged ER repairing. ATF6, activating transcription factor 6; ER, endoplasmic reticulum; IRE1, inositol requiring enzyme 1; UPR, unfolded protein response. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

(94, 230, 243, 244). These data have been recently confirmed in humans: Puri *et al.* found that increased eIF2α and JNK pathways are activated in the liver of obese patients with NAFLD compared to insulin resistant subjects without NAFLD and their activation correlated with the histologic severity of the disease (224). Gregor *et al.* (90) provided evidence of activation of ER stress in the liver and adipose tissue of obese patients undergoing bariatric surgery. The improvement of hepatic steatosis after surgery is associated with a decrease of ER stress markers in the liver (97).

Recent data highlighted the mechanisms whereby ER stress activation promotes steatosis, inflammation, and fibrogenesis in NAFLD.

E. Role of TG cellular stores in mitochondrial dysfunction and liver injury in NASH

Increased TG stores in the steatotic liver may derive from increased uptake of free FAs coming from adipose tissue lipolysis or from the diet, from increased *de novo* lipogenesis, from decreased VLDL secretion, and from decreased FFA oxidation. Kinetic models using isotope technique found that in obese patients with NAFLD, 59% of hepatic triacylglycerol arose from circulating FFA pool, 26% from *de novo* lipogenesis and 15% from the diet. Notably, *de novo* lipogenesis was constitutively elevated in the fasting state and failed to increase postprandially, contrary to healthy subjects, where it rose from 5% to 23% postprandially (59, 294).

1. ER stress and FFA overflow to the liver. An activation of ER stress has been found in the adipose tissue of obese rodents and humans (42, 97, 279). The IRE1 branch of the UPR can directly promote lipolysis in cultured adipocytes by promoting degradation of the lipid droplet-coated protein perilipin A and can inhibit insulin signaling *via* JNK activation in adipocytes (122, 303, 364). An elevated flux of FFA, especially saturated FAs, can further activate ER stress in hepatocytes *via* a redistribution of calcium stores, thus amplificating the vicious cycle (320).

2. ER stress and hepatic *de novo* lipogenesis. The transcription factor SREBP1c is a key activator of hepatic lipogenesis and has been found to be activated in animal and human NAFLD (12, 104). SREBP1c is synthesized as an inactive precursor in ER membranes and must be cleaved in the Golgi to be activated. Ye *et al.* (344) first demonstrated that ATF6 and SREBP1 shared the same proteolytic process in the Golgi to be transcriptionally active. Kammoun *et al.* (126) recently showed that ER stress induces SREBP1c proteolytic cleavage and the subsequent transcription of lipogenic enzymes, leading to hepatic steatosis; in the same experiment, ER stress inhibition by overexpression of the chaperone BiP/GRP78 profoundly decreased SREBP1c and lipogenic enzymes expression, improving hepatic steatosis and insulin sensitivity in ob/ob mice.

ER stress may also promote hepatic *de novo* lipogenesis by enhancing hepatic overexpression of another transcription factor, C/EBPβ (CCAAT/enhancer binding protein β), whose activation has recently been proposed to play a role in the development of hepatic steatosis through an activation of the lipogenic pathway, whereas its inhibition abrogates fatty liver in db/db mice (41, 230).

Other genetic deletion studies support the link between overactivation of different elements of the UPR, *de novo* lipogenesis, and hepatic steatosis in animal models of fatty liver (144, 260).

3. ER stress and hepatic VLDL secretion. VLDL synthesis occurs in the ER lumen, where the first step is the synthesis of apolipoprotein B100 (apoB100) followed by its lipidation by microsomal TG transfer protein. Recent data in rat hepatoma cells and mice suggest that ER stress activation reduces VLDL synthesis by promoting apoB100 degradation by ER-associated protein degradation complex (ERAD system) and by PERK-mediated inhibition of apoB translation (204, 229).

ER stress can also play a role in promoting progression from steatosis to necroinflammation: UPR activates the hepatic transcription factor cyclic-AMP-responsive-element-binding protein H (CREBH), which triggers the acute inflammatory response (359). Similarly, to SREBP1c, CREBH is an integral protein of the ER that needs to be proteolytically processed in the Golgi to be activated. The proteolytic cleavage ofCREBH is induced by ER stress together with proinflammatory cytokines.

Further, CREBH and the ATF6 branch of the UPR synergistically activate genes of the acute phase response, including the transcription of C-reactive protein (CRP), which is a marker and possibly a mediator of necroinflammation in NASH (350).

Another link between UPR and hepatic inflammation is the kinase JNK, which is activated by the IRE1 branch, as previously discussed. JNK promotes hepatic necro-inflammation and has been implicated in the transition from hepatic steatosis to steatohepatitis, whereas JNK1 inhibition protected mice fed a choline-deficient diet from progression to steatohepatitis and fibrosis (117, 135).

F. Role of free cholesterol in mitochondrial dysfunction and liver injury in NASH

It is increasingly recognized that an important determinant of the transition from steatosis to NASH is the type rather than the amount of fat infiltrating hepatocytes, and that hepatic TG accumulation is not *per se* toxic, but rather it would protect the hepatocyte by buffering the accumulation of toxic FFA: inhibiting hepatic TG synthesis improved hepatic steatosis, but exacerbated liver injury, necroinflammation, and fibrosis in obese mice with NASH (336). In this model, the severity of liver injury paralleled the extent of hepatic FFA accumulation, of cytochrome P450 2E1 activation, and of lipid peroxidation. Consistently, an altered lipidomic profile can be found in NASH subjects as compared to steatosis and healthy controls, and hepatic FFA uptake inhibition with acipimox acutely improved hepatic injury and insulin sensitivity, without affecting liver TG stores (225, 239). Besides the higher lipotoxicity of saturated FAs as compared to unsaturated fat, the toxic effect of free cholesterol (FC) accumulation in hepatocyte mitochondria has recently emerged. Using nutritional and genetic models of hepatic steatosis, Mari *et al.* (171) showed that FC loading, but not FFA or TG, sensitized to TNF- and Fas-induced development of NASH. FC distribution in ER and plasma membrane did not cause ER stress or alter TNF signaling. Rather, mitochondrial FC loading accounted for the hepatocellular sensitivity to TNF due to mitochondrial glutathione (mGSH) depletion. Selective mGSH depletion in primary hepatocytes recapitulated the susceptibility to TNF and Fas seen in FC-loaded hepatocytes; its repletion rescued FC-loaded livers from TNF-mediated SH. Moreover, hepatocytes from mice lacking Niemann-Pick type C1, a late endosomal cholesterol trafficking protein, or from obese ob/ob mice, exhibited mitochondrial FC accumulation, mGSH depletion, and susceptibility to TNF. These findings have been replicated in other animal models of NASH (31, 330) and are confirmed by preliminar data in humans (343). Collectively, these data suggest the pharmacological modulation of FC uptake by intestine and hepatocytes by ezetimibe may have potential therapeutic implications in NASH (361).

G. Summary

ROS can promote NAFLD by acting at different cellular steps of different hepatic and extrahepatic cells and tissues: they inhibit VLDL secretion from hepatocytes, thus favoring steatosis; they activate JNK and TNF pathway in hepatocytes, Kupffer cells, and visceral adipocytes, thus promoting hepatic insulin resistance and necroinflammation; finally, they trigger ER stress promoting hepatocyte apoptosis. Eventually, FC accumulation may deplete mGSH. All these sites and mechanisms are potential target for treatment.

VII. UCPs and Mitochondrial Dysfunction

UCPs are inner mitochondrial membrane proteins that act as a proton carrier to allow the dissipation of the proton electrochemical gradient, which results in the uncoupling of oxidation and ATP synthesis and eventually decreases mitochondrial ATP production (213). Specifically, UCP-2 modulates the coupling between substrate oxidation and ATP synthesis by dissipating the proton-motive force used by the complex V for producing ATP (214). Experimental models suggest that the main physiological functions of UCP-2 are an increase in adaptive thermogenesis and the protection against excessive generation of superoxide radicals during fatty-acid oxidation by mitochondrial respiratory chain (214). Superoxide directly or indirectly, through oxidation of unsaturated fatty acyl chains of membrane phospholipids, leads to massive production of 4-HNE and other reactive alkenals. HNE can directly activate the proton conductance of UCPs, lowering proton-motive force and attenuating the original superoxide production, thus providing a local feedback by which UCP-2 controls superoxide mitochondrial production (213, 214).

Under physiological conditions, UCP-2 is expressed in hepatic Kupffer cells, but not in hepatocytes (314). However, hepatocyte UCP-2 has been demonstrated to be overexpressed in animal and human NAFLD livers (314, 334). In steatosis, hepatocytes are supplied by large amounts of FFAs, and they elevate mitochondrial β-oxidation and respiratory chain activity to limit FFA accumulation. This, in turn, increases mitochondrial ROS production and enhances hepatic oxidative stress. Increased superoxide and HNE production upregulate UCP-2, which acts as protective mechanism against excessive ROS production but exposes the liver to chronic depletion of ATP, increasing hepatocyte susceptibility to conditions of acute energy demand, like ischaemia–

reperfusion injury (46, 314, 334). Importantly, evidence from animal and human livers suggests that hepatic ATP depletion occurring early during the development of NASH is due to mitochondrial uncoupling action of UCP-2 and not to a dependent dysfunction on the ATP synthase, which was increased upon NASH development (334). Further, hepatocyte death and necroinflammatory changes occurring during acute stress conditions (*i.e.*, the ischemia-reperfusion injury) were reversed by antisense plasmid or guanosyl dyphosphate inhibition of UCP-2 (314, 334), further supporting a role for increased UCP-2 activity in the development of NASH. These concepts are represented in Figure 8.

A. Summary

UCP-2 is overexpressed in hepatocytes during progression of NASH, causing a proton leak and allowing mitochondria to elevate substrate oxidation in response to FFA accumulation, acting as a protective mechanism. Despite these favorable effects, progressive ATP depletion exposes the hepatocytes to increased susceptibility to noxious stimuli when the hepatic energy requirement increases. Further research is required to define the potential effect of modulation of UCP-2 activity coupled with the control of mitochondrial redox balance as a therapeutic tool for the treatment of NASH.

VIII. Mitochondrial Dysfunction as a Link Between Maternal Fat Overnutrition and NASH in Adult Offspring

There is increasing evidence that maternal exposure to poor nutrition in the developmental environment increases the risk of developing noncommunicable disease, specifically features of the metabolic syndrome such as dysglycemia, type 2 diabetes, and obesity in the offspring (280).

Recent data connected maternal overnutrition to the risk of NAFLD and NASH in adulthood and mechanicistically linked mitochondrial dysfunction to an increased susceptibility to develop progressive steatohepatitis later in life.

Bruce *et al.* (29) fed female C57 BL6J mice either a high-fat (HF) or control chow (C) diet before and during gestation and lactation. The resulting offspring were fed either a C or HF diet postweaning to generate four offspring groups: HF/HF, HF/C, C/HF, and C/C. At 15 weeks of age, liver histology revealed that whereas the C/HF offspring developed simple steatosis, the HF/HF offspring developed NASH. At 30 weeks, liver histological analysis revealed that both the HF/C and C/HF groups had NAFLD, whereas the HF/HF group had a more severe form of NASH.

These histological changes were associated with important changes in several cellular functions. At 15 weeks of age, hepatic mitochondrial electron transport chain (ETC) enzyme complex activity (I, II/III, and IV) was reduced in both groups

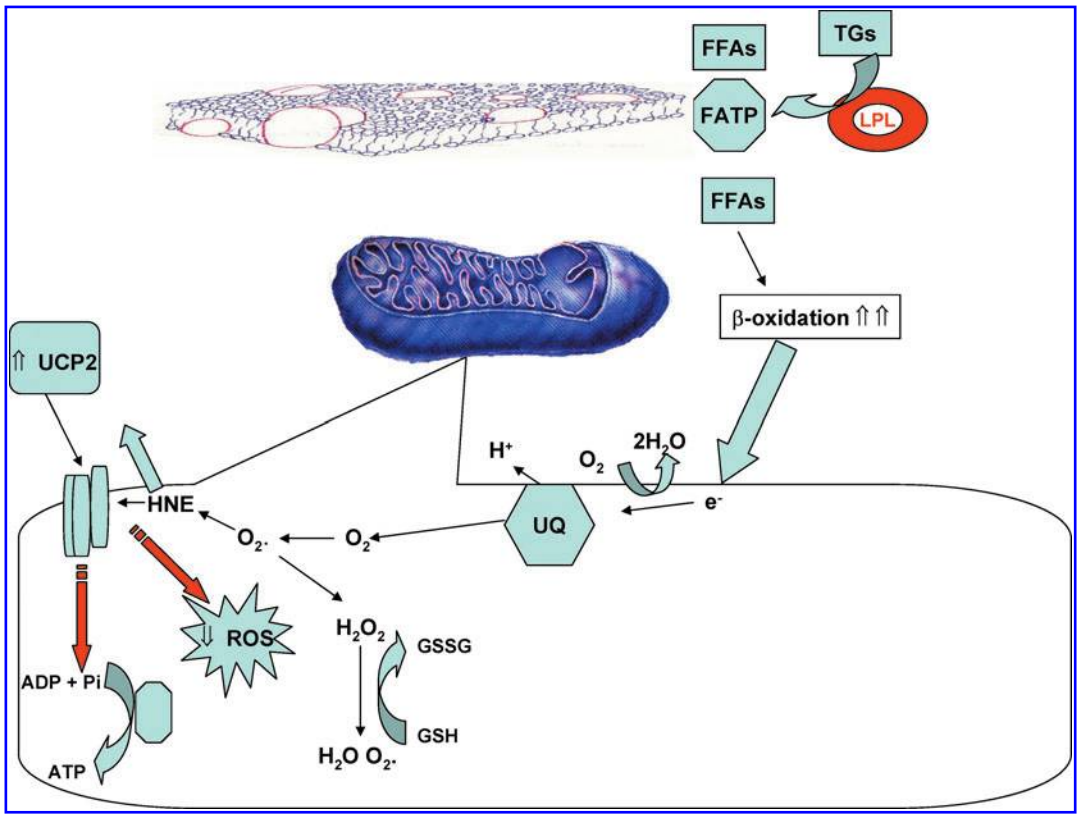

FIG. 8. Role of UCP-2 mitochondrial dysfunction and antioxidant system in ROS generation and ATP depletion in the pathogenesis of liver injury in NASH. Steatosis increases the FFA supply over the hepatocyte energy requirements; this increases the O_2 pressure in the mitochondrial matrix and the rate of production of ROS O_2·, which, in turn, produces oxidation of mitochondrial protein and release of 4-hydroxy-2-nonhenal (HNE). HNE acts as positive signal on the expression and activity of UCP-2. Uncoupling substrate oxidation from ATP synthesis not only reduces redox pressure on the respiratory chain and ROS formation but also induces chronic ATP depletion. When hepatocytes face acute injury (*e.g.*, ischemia-reperfusion), ATP synthesis is compromised and, despite the favorable effects, UCP-2 activation exposes the hepatocytes to increased susceptibility when the hepatic energy requirement changes. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

of offspring from HF-fed mothers (HF/C and HF/HF). Plasma β-hydroxybutyrate, as a proxy measure of mitochondrial flux of acetyl-CoA to keto-acid production, was highest in the C/C group, whereas animals from all other groups have significantly lower levels of β-hydroxybutyrate, the lowest being the HF/HF group, suggesting a failure of hepatic β-oxidation. In addition, microarray analysis revealed hepatic expression of genes involved in oxidative stress (iNOS, endothelial NOS, glutathione S-transferase mu 6, and lipocalin 2), lipogenesis (fatty acid synthase, ACC, ATP citrate lyase, and Diacylglycerol O-acyltransferase 1), and inflammatory (tumor necrosis factor ligand 1, C-reactive protein, monocyte to macrophage differentiation factor, and IL-12b) pathways were all upregulated in the 15-week-old HF/C and HF/HF offspring compared to the C/C animals. By contrast, genes involved in mitochondrial FA transport and β-oxidation (Carnitine Palmitoyl Transferase-1, Acyl-Coenzyme A dehydrogenase, and hydroxyacyl-Coenzyme A dehydrogenase) were downregulated in the same animals. The authors conclude that maternal fat intake contributes to the development of NASH in adult offspring, an effect that is mediated through impaired hepatic mitochondrial metabolism and upregulated hepatic lipogenesis. Specifically, this study provides the first evidence in a mammalian system showing that exposure to a maternal HF diet can lead to developmental induction of impaired ETC enzymes, which persists into adulthood and contributes to the adult onset of NAFLD and the progression to NASH when the HF exposure is continued into adulthood. Further studies are needed to investigate these priming mechanisms and to fully elucidate the timing of events on the causal pathway to NAFLD onset and progression to NASH. If further confirmed, this study has important implications for to educate women of the possible consequences of overnutrition and obesity during pregnancy, that is, altered mitochondrial function and metabolic pathways in the fetus, which may prime the offspring to the onset on NASH later in adulthood. These data emphasize the importance of a balanced diet during pregnancy, lactation, and the consequences for incidence of chronic disease if an unhealthy diet is consumed across generations.

A. Summary

increasing experimental evidence suggests that maternal overnutrition can predispose offsprings to the development of NASH through impairment of hepatic mitochondrial electron transport chain, with relevant preventive implications for nutrition in pregnancy.

IX. Myeloperoxidase Involvement in Liver Injury in NASH

Recent human evidence suggests that, besides cytochrome P450 activity, peroxisomal β-oxidation, and mitochondrial electron leak, increased hepatic oxidative stress in NAFLD can result from the activity of recruited inflammatory cells. One of the principal molecules released after recruitment and activation of phagocytes is myeloperoxidase (MPO), an enzyme involved in the generation of ROS. In the presence of physiological chloride concentrations, MPO reacts with hydrogen peroxide (H_2O_2, formed by the respiratory burst) to catalyze formation of hypochlorous acid/hypochlorite ($HOCl/OCl^-$) and other oxidizing species [reviewed in ref. (306)]. These oxidants may contribute to host tissue damage at sites of inflammation through reactions with a wide range of biological substrates, including DNA, lipids, and protein amino groups. In the absence of physiological chloride concentrations, the MPO-H_2O_2 system can also form reactive nitrogen species that may initiate lipid peroxidation or form protein tyrosine residues, another posttranslational modification found in many pathological conditions.

Besides its abundant presence in neutrophils, where MPO makes up 5% of the total cell protein content, lower levels of MPO have been found in monocytes, macrophages, and in a subpopulation of Kupffer cells (28). The presence of MPO in different macrophage populations may reflect a fine modulation of inflammatory status, which may alternatively promote or ameliorate obesity-associated chronic low-grade inflammatory status: M2 macrophages with tissue-remodeling and antiinflammatory capacities were depleted in obese adipose tissue in mice, whereas accumulation of proinflammatory M1 macrophages, which can generate high amounts of ROS and RNS, was associated with obesity and insulin resistance (162). Further, attenuation of the M2 differentiation program of Kupffer cells was associated with fatty liver (198). Recently, MPO-containing M1 macrophages/Kupffer cells have been proposed to play a pathogenetic role in NAFLD: Rensen *et al.* (237) investigated by immunohistochemistry the distribution of MPO-containing inflammatory cells in liver biopsies of 40 severely obese subjects with either NASH or simple steatosis. MPO-derived oxidative protein modifications (hypochlorite-modified and nitrated proteins) were also identified and correlated to hepatic gene expression of the chemotactic chemokines IL-8 and growth-related oncogene (GRO)-α, which recruit neutrophils to inflammatory sites in response to accumulation of chlorinated or nitrated proteins, and to M1/M2 macrophage markers as determined by quantitative PCR.

They found that the number of hepatic neutrophils and MPO-positive Kupffer cells was increased in NASH and was accompanied by accumulation of hypochlorite-modified and nitrated proteins.

Hepatic expression of IL-8 and GRO-α was higher in patients with NASH compared to simple steatosis with accumulation of MPO-mediated oxidation products and correlated with hepatic neutrophil sequestration. Further, hepatic M2 macrophage marker gene expression was increased in the liver of NASH patients. Since M2 macrophages produce TGF-β and other proteins involved in tissue remodeling, such as metalloproteinases 1 and 12 (358), these cells may play a relevant contribution to hepatic fibrosis in these patients.

A. Summary

In vivo human studies suggest that an increased Kupffer cell MPO activity represents an additional source of oxidative damage in NASH through the accumulation of MPO-mediated oxidation products, thereby contributing to the transition from simple steatosis to NASH. Moreover, the hepatic upregulation of M2-phenotype Kupffer cells suggests that part of the inflammatory response is directed toward tissue remodeling in NASH.

X. Nitrosative Stress in the Pathogenesis of NASH

Nitric oxide (NO) and reactive nitrogen species (RNS) are formed in normal hepatocytes and are critical for normal physiological processes, including insulin signaling, growth,

apoptosis, and dilatation of blood vessels (58). Moreover, NO regulates mitochondrial respiration through reversible binding at the redox-active heme site in cytochrome c oxidase and modulates mitochondrial biogenesis through interactions with soluble guanylate cyclase (58, 201). When the levels of oxidation products exceed the capacity of normal antioxidant systems, nitrosative stress occurs, resulting in damage of mitochondrial respiratory chain and several cellular functions. Specifically, NO can react with cytochrome *c* oxidase (complex IV) and competitively block oxygen binding and electron transfer (58). In the presence of superoxide radicals generated by hepatic Kupffer cells, NO forms peroxynitrite ($ONOO^-$), nitrosoperoxycarbonate ($ONOOCO^{-2}$), and other nitrogen species (201), which can damage DNA and bind protein tyrosine residues to form nitrotyrosine. Nitrotyrosine, in turn, causes dysfunction and degradation of many functional proteins. In the liver, NO and peroxynitrite induce mitochondrial dysfunction (58), and increased iNOS expression in the steatotic liver of ob/ob mice correlates with nitration of mitochondrial proteins and mitochondrial respiratory chain dysfunction (77). Increased formation of 3NT has been demonstrated in the liver and plasma of NASH patients, closely paralleling the severity of histological necroinflammation (66, 183, 251). In a sample of 115 Japanese subjects, four functional single-nucleotide polymorphism of the iNOS gene (rs2297510, rs2297511, rs2797512, and rs1060822) have been associated with the presence of NAFLD, one of which (rs1060822) also correlating with hepatic iNOS mRNA expression and fibrosis severity (347).

The contribution of intrahepatic NO and peroxynitrite to the pathogenesis of NASH has been further demonstrated in the obese, leptin-deficient ob/ob mouse model (66, 77). In the liver of these animals, mitochondrial respiratory chain (MRC) activity was reduced, coupled with marked 3-tyrosine nitration of mitochondrial proteins, particularly the ND4 subunit of complex I and cytochrome *c*, whereas intrahepatic iNOS protein and TNF-α expression were markedly increased, correlating with the severity of histological steatosis and inflammation. Treatment of these animals with uric acid, a peroxynitrite scavenger, decreased tyrosine nitrated proteins, improved the activity of MRC complexes, and led to a marked regression of hepatic steatosis and inflammation, strongly supporting the involvement of NO and nitrotyrosine as the second hit in the progression of NASH.

Fujita *et al.* (72) found that the levels of NO and its metabolites were markedly higher in the liver and visceral fat of NASH rats than in controls, correlating with the severity of hepatic histological inflammation and fibrosis; NO species were also significantly increased in the arterial, caval, and, most consistently, portal blood of the NASH rats as compared with controls. A three- to fourfold increase in the iNOS expression in the visceral fat, especially mesenterium, was found in the NASH model. These results suggest that, at least in this animal model of NASH, mesenterium visceral fat, and not the liver, is the main site of NO overproduction; NO then overflows to the liver, resulting in increased nitrotyrosine formation and subsequent inflammation, apoptosis, and fibrosis of NASH. This intriguing hypothesis needs to be tested in other animal models of NASH, as well as in humans.

A. Summary

There is increasing evidence from *in vivo* animal and human data that an increased NO and other RNS production may contribute to mitochondrial dysfunction and liver injury in NASH, and that a strategy targeting nitrosative stress may improve liver disease in these patients. For a more effective therapeutic approach, future research will have to elucidate the exact mechanisms and sites (hepatic *vs.* visceral fat) of production of RNS, which are most relevant for hepatic injury in NASH.

XI. Oxidized LDL and Hepatic Fibrogenesis in NAFLD

Oxidatively modified low-density lipoproteins (LDL)s are increasingly recognized to play an important part not only in atherosclerosis, but also in the development of diabetes and metabolic syndrome. Growing experimental and human data suggest that oxLDL may also have profibrogenic properties. Hepatic fibrogenesis is characterized by the accumulation of extracellular matrix (ECM) components. HSCs are the major effector cells during hepatic fibrogenesis and are the primary source of ECM production in the liver. During liver injury, quiescent HSCs undergo dramatic phenotypic changes from vitamin A, fat-storing cells to proliferative myofibroblast-like-cells with acquisition of fibrogenic properties (70). This process is coupled with the activation of signaling pathways for pro-fibrogenic TGF-β pro-mitogenic platelet-derived growth factor-beta (PDGF-β), and Wnt signaling, as well as the depletion of PPAR-γ (43, 69).

Schneiderhan *et al.* (258) investigated the effects of oxLDL on collagen and fibronectin synthesis of cultured human and rat HSC. They found that oxLDL stimulated the synthesis of collagen types I and III and fibronectin of cultured HSC in a dose-dependent manner, and this stimulation was reduced by 56% by preincubation of cultured human HSC with the monoclonal antibody OKM5, a known inhibitor of CD36-mediated oxLDL uptake. Other authors (112, 304) confirmed these findings in liver tissue samples of patients with NAFLD: they found that patients with NASH exhibited higher amounts of oxidized LDL compared with other chronic liver diseases, and the degree of CD36 expression on activated stellate cells paralleled the expression of the profibrotic cytokine TGF-β and the severity of fibrosis deposition in NAFLD patients. In nonobese nondiabetic normolipidemic subjects with NASH, oxidized LDL levels are comparable to those of healthy controls in fasting conditions, but they dramatically increase postprandially and such increase correlates tightly with the degree of histological fibrosis (182). Altogether, these findings suggest that CD36 expression in stellate cells is a substantial mediator of HSC activation and hepatic fibrogenesis in NAFLD.

Besides CD36, the uptake ox-LDL into cells is mediated by a variety of scavenger receptors, including lectin-like oxidized LDL receptor-1 LOX-1, SR-AI/II, and SR-BI.

CD36 is a multiligand scavenger receptor that recognizes and binds many ligands, including ox-LDL and long chain-free FAs. Compared with that of CD36, the expression of LOX-1 in activated HSCs is more inducible and highly expressed at both transcript and protein levels (127), suggesting that LOX-1 might have a more important role in the uptake and transport of extracellular ox-LDL into activated HSCs. Kang *et al.* (127) evaluated the effects of LOX-1-mediated uptake of oxLDL on

human HSCs *in vitro*. In addition, ox-LDL stimulated the synthesis of α(I) collagen and alpha-smooth muscle actin (a-SMA), the markers of activated HSCs, in a dose-dependent manner. Additionally, pro-fibrogenic genes, including connective tissue growth factor and type I and II TGF-β receptors (Tb-RI & Tb-RII), as well as pro-mitogenic genes, including receptors for PDGF-b (PDGF-bR) and EGF (EGFR), as well as cyclin D1, a key regulator in cell cycle progression are activated. Pretreatment with the LOX-1 antagonist k-carrageenan dramatically eliminated the stimulatory effects of ox-LDL on HSCs activity.

A. Summary

These data collectively indicate that exogenous ox-LDL markedly induced expression of genes closely relevant to HSC activation, suggesting the role of ox-LDL in the stimulation of HSC activation *in vitro*. If confirmed *in vivo*, these data suggest that reducing oxLDL levels or uptake by HSC may be an effective antifibrotic treatment in NASH.

XII. Antioxidant Systems in NAFLD

In NASH, more ROS are generated in liver by the mitochondrial respiratory chain and at other sites, including microsomal CYP2E1. Another potential source in liver is the NADPH oxidase of Kupffer cells (132). This abundant formation of endogenous ROS may start to oxidize the unsaturated lipids deposited within the hepatocytes to trigger extensive lipid peroxidation (262). Lipid peroxidation also occurs in animals with hepatic steatosis due to a choline/methionine-deficient diet (141, 205) and in subjects affected with NASH (251). Lipid peroxidation release reactive aldehydes, such as 4-HNE and MDA, that can damage mitochondria (53, 75, 124, 152).

Also cytokines may contribute to the genesis of the liver lesions in NASH.

ROS activate NF-κB pathway (297), which favors the synthesis of TNF-α. TNF-α enhances ROS formation and sets off hepatocytes apoptosis (68). TGF-β is released by Kupffer and stellate cells after these cells are overloaded with apoptotic bodies. In turn, TGF-β transforms stellate cells into collagen-producing myofibroblastic cells and stimulates tissue transglutaminase, which creates cross-links between cytokeratins (74) inducing the formation of Mallory bodies. ROS may deplete some antioxidants to further aggravate ROS-induced damages. Glutathione s-transferase activity was found low within the cytosol and mitochondria in ob/ob fatty liver. Lower hepatic expression of the μ-class glutathione s-transferase has been reported in obese patients with steatosis in comparison with obese individuals without NAFLD (355). Low serum levels of vitamin E are found in some obese children with steatohepatitis (284), and supplementation with vitamin E can decrease transaminase in obese children (140).

A. Vitamin E

Vitamin E is the most widely studied antioxidative, particularly in NASH. Soden *et al.* (273) have examined the role of vitamin E in an animal model of liver disease. There is a number of protective mechanisms that ameliorate or attenuate the effect of ROS, including the upregulation of a range of endogenous antioxidants, such as coenzyme Q10 and ubiquinones. There is also a range of exogenous antioxidants, including vitamins E and C, carotenoids (beta carotene and lycopene), and polyphenols (flavonoids). Vitamin E is of particular interest because it can limit membrane injury by ROS. There are eight naturally occurring vitamin E compounds, four tocopherol homologs and four tocotrienols, which are synthesized by plants from homogenistic acid. The four tocopherol homologs (dα-, dβ-, dγ-, and dδ-) have a saturated 16-carbon phytol side chain, whereas the tocotrienols (dα-, dβ-, dγ-, and dδ-) have three double bonds on the side chain. All are derivatives of 6-chromanol and differ in the number and position of the methyl groups on the ring structure. The term "vitamin E" can be used to describe any combination of compounds; however, α-tocopherol is the most abundant in nature and in human plasma, and the most widely used in supplements and biomedical research studies. The structure of vitamin E makes it a highly effective antioxidant, readily donating the hydrogen from the hydroxyl group on the ring structure to free radicals, and rendering them inactive. Vitamin E is fat soluble and is primarily located within the phospholipid bilayer of the cell membranes, where it has a major biological role in protecting polyunsaturated fats and other components of the cell membranes from oxidation by free radicals. Vitamin E supplementation with d1-α-tocopherol (900 mg/day for 4 months) improves total body glucose disposal and nonoxidative glucose metabolism in lean subjects, and in type 2 diabetes (208).

Vitamin E may have dual benefits by decreasing oxidative stress and improving insulin sensitivity and is an attractive therapeutic agent for NASH and other liver diseases.

A number of studies examined the role of supplemental vitamin E in liver disease with conflicting results. In the first prospective, double-blind, randomized placebo-controlled trial with histological end-points, 49 patients with NASH were randomized to receive either vitamins E and C (1000 IU and 1000 mg, respectively) or placebo daily for 6 months. Additionally, all patients were given standard weight-loss counseling and encouraged to follow a low fat diet. At the end of treatment, vitamins E+C were no better than placebo at improving any histological or metabolic feature (98). In another RCT, 2 years of vitamin E plus UDCA significantly improved steatosis compared to either agent alone or placebo (324). The results of these and other trials led Cochrane reviewers to conclude that data were too limited to assess the effectiveness of Vitamin E in NAFLD (156). After that review, another large, well-designed RCT has been completed, the multicenter placebo-controlled, double-masked "Pioglitazone *versus* vitamin E *versus* Placebo for the treatment of nondiabetic patients with NASH" (PIVENS) trial, where 247 nondiabetic patients with NASH were randomized to 96 weeks of either pioglitazone, vitamin E (800 IU daily) or placebo (252). At the end of the study, histological improvement was reached by 19% of patients on placebo, 34% of patients on pioglitazone, and 43% of patients with vitamin E ($P = 0.04$ vit E *vs*. pioglitazone). The authors concluded that vitamin E is superior to pioglitazone and placebo for the treatment of adult, nondiabetic patients with NASH.

Why could vitamin E supplementation not translate into clear clinical benefit? First, the term "vitamin E" is used collectively for eight compounds, each potentially with its own biological effects. Most research used tocopherol (264) rather than tocotrienols. However, tocotrienols exhibit powerful neuroprotective, anticancer, and some cholesterol-lowering

properties that are not necessarily shared by tocopherols. Pretreatment with α-tocopherol can ameliorate, but not abrogate liver injury. This may be mediated through reduction in ROS and lipid peroxidation (275).

A range of experimental studies have shown that vitamin E can play a role in protecting cells from injury caused by ROS and lipid peroxidation. ROS and depleted antioxidants have been implicated in insulin resistance and obesity and may play a role in the pathogenesis of disease such as NASH. However, to date there is little data to support the use of supplemental vitamin E in treating liver disease. The possible therapeutic utility is made complex because vitamin E is a group of compounds, and issues related to bioavailability and interactions between these related compounds need to be resolved.

Vitamin E (α-tocopherol) is relatively safe and readily available. The rationale for using vitamin E in patients with NASH is derived mostly from its antioxidant properties. Oxidative stress probably acts as a second hit to promote inflammation in the steatotic liver and to cause progressive fibrosis (2). In *in vitro* and animal studies, vitamin E supplementation decreased levels of the profibrogenic cytokine TGF-β, ameliorated liver necrosis and fibrosis (209), and prevented HSC activation (110).

B. Coenzyme Q10 (ubiquinone)

The ubiquinones, also known as the coenzymes Q, are quinone derivatives that have isoprene-derived hydrocarbon chains (Fig. 9). These moleculess are found in the mitrochondria of all living cells (hence the name: *ubi*quitous *quinones*), as well as in other organelles such as the ER, peroxisomes, lysosomes, and vesicles; in humans the isoprene chain is 10 units long, and is known as Coenzyme Q10. The ubiquinones carry electrons through the electron transport chain in mitochondria, and assist the mitochondria to produce energy (Fig. 9); they also have direct antioxidants properties due to their polyphenolic structure (81, 292). The effects of Coenzyme Q10 supplementation on different parameters related to oxidative stress, ER stress, and intracellular VLDL processing have been assessed in high-fat-induced rodent models of obesity and NAFLD.

In the first (34), dietary CoQ supplementation caused significant increases in apolipoprotein B mRNA and microsomal TG levels and altered the phospholipid content of microsomal membranes, suggesting that it can modulate intrahepatocyte VLDL assembly and secretion. In the second, CoQ10 supplementation decreased the hepatic expression of prooxidative enzyme NADPH oxidase and inflammatory markers six transmembrane protein of prostate 2 (STAMP2), C-reactive protein, cyclooxygenase 2 (COX2), IL-6, and TNF-α without changing obesity and tissue lipid peroxides in high-fat-fed mice (274).

C. Mitochondrial MnSOD

SODs are major antioxidant enzymes that are classified into three subgroups, cytosolic CuZn-SOD (Sod1), mitochondrial Mn-SOD (Sod2), and extra-cellular EcSOD (Sod3) (357). MnSOD is synthesized in the cytosol and translocated into the mitochondria after posttranscriptionally modifications (328). In the mitochondrion, it catalyzes the dismutation of two superoxide radicals, producing H_2O_2 and oxygen. MnSOD is induced with free radical challenge (243) and cigarette smoke (85).

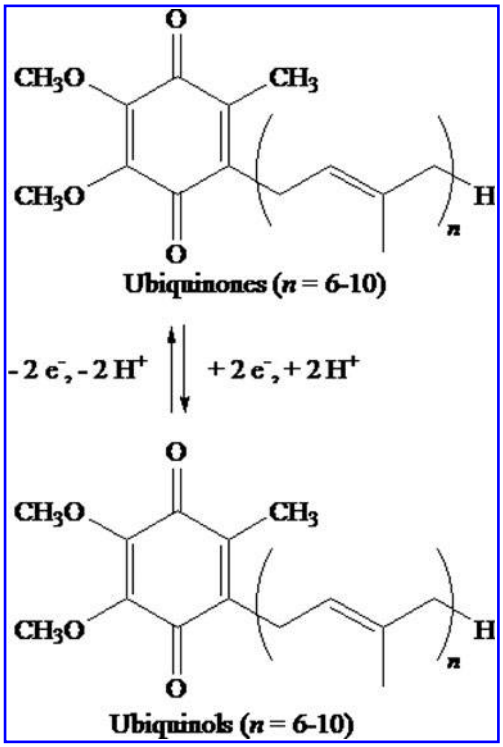

FIG. 9. Ubiquinones: structure and chemical properties. The ubiquinones, also known as the coenzymes Q, are quinone derivatives that have isoprene-derived hydrocarbon chains.

MnSOD gene is characterized by several polymorphisms in the mitochondrial targeting sequence. For instance, the T/C polymorphism is a valine-to-alanine change in the mitochondrial targeting sequence (180). This amino acid substitution enhances transport of MnSOD into the mitochondrial matrix for an alteration of the helical structure (282). Functional MnSOD polymorphisms have been associated with the development of different diseases in humans, including heart failure (6), colorectal carcinoma (283), rheumatoid arthritis (173), diabetic nephropathy (195), and NASH (190).

MnSOD is a scavanger for ROS and the presence of the T allele makes the targeting of MnSOD to mitochondria less effective, decreasing its capacity to detoxify superoxide anion produced in mitochondria and increasing susceptibility to excessive oxidative damage inside hepatocytes (190). Several studies reported that SOD activity markedly decreased in the liver of patients with NAFLD (311, 345). Then, higher oxidative stress may result in a higher prevalence of NASH. Further, CuZn-SOD-deficient mice show a marked degradation of apolipoprotein B (apoB) in the liver and plasma (228). Generation and removal of ROS is likely to be connected with lipoprotein metabolism.

D. Mitochondrial glutathione peroxidase

Glutathione is synthesized in cytosol and then transferred into other cellular compartments. GSH transport can be conventionally subdivided in intracellular and intercellular (60). GSH transport into mitochondria in hepatocytes is characterized

by its dependence on the membrane fluidity. mGSH levels depend on activity of some enzymes, especially glutathione peroxidase (136): overexpression of mitochondrial glutathione peroxidase protects different cells against apoptosis caused by various prooxidants, including H_2O_2, oxidized phospholipids, FC, and respiratory chain inhibitors (113). Overexpression of mitochondrial glutathione peroxidase inhibits production of organic hydroperoxides and loss of the membrane potential and integrity of the plasma membrane, preventing the decrease in the synthesis of ATP and the release from mitochondria of cytochrome *c,* the event leading to activation of caspase 3 and cell apoptosis (113). The involvement of GSH in the pathogenesis of liver injury in NAFLD has been demonstrated *in vivo* by Videla *et al.* (311): they showed that the hepatic antioxidant defenses were reduced in NAFLD, and more consistently in NASH than in simple steatosis, as indicated by a more pronounced reduction in SOD, catalase, and glutathione peroxidase activities, leaving the hepatocyte with little resistance toward attack by prooxidant species. The reduced hepatic antioxidant activity was also present at systemic level, as suggested by a diminished plasma total antioxidant capacity.

E. Heme oxygenase

Heme oxygenase (HO) is involved in the initial reaction in heme catabolism and it is the rate limiting reaction. Two HO isoenzymes for the degradation of the heme are contained in the liver. HO-1 is the enzyme that is induced by cytokines heavy metals and ROS. The antioxidant properties of HO are due to the elimination of pro-oxidant heme and to its reaction products that are biliverdin, bilirubin, and iron. Biliverdin and bilirubin are an antioxidative defense mechanism against lipoprotein oxidation (166). It was found that the HO-1 expression was significant increased in NASH patients. The induction of HO-1 was considered an adaptative defense mechanism against oxidative species released by lipid peroxidation (166).

F. Summary

Several antioxidant systems have been implicated in the pathogenesis of NASH, including vitamin E, Coenzyme Q, MnSOD, mitochondrial glutathione peroxidase, and HO-1. Among all these systems, vitamin E supplementation has recently evaluated in several randomized clinical trials in human NASH, with mixed results: while early trials were negative, the recent 2-year long PIVENS yielded positive results on histology.

XIII. Newer Antioxidants with Potential Therapeutic Applications in NASH

A. Silibinin/silymarin

Milk thistle (Silybum marianum) is medicinal plant used for its hepatoprotective properties in chronic liver disease since the 4th century. Two commercially available formulations are the crude extract, silymarin, and the semipurified product, silibinin. Silymarin consists of at least seven flavonolignans, of which the most prevalent (60% of overall weight) are the diastereoisomers silybin A and silybin B; silibinin consists only of silybin A and silybin B. The beneficial effects of these compounds have long been ascribed exclusively to their potent ROS scavenging activity. Recently, silymarin has been reported to have anti-inflammatory activity through inhibition of T-cell inflammatory cytokines and hepatocyte NF-κB signaling (220). In cultured human HSCs silybin has been shown to exert further beneficial effects (298):

- Marked reduction in intracellular ROS generation, as assessed by 2′,7′ dichlorodihydrofluorescein diacetate (DCFH-DA).
- Dose-dependent anti-inflammatory action through Ik-B dephosphorylation-mediated reduction in IL-1-induced synthesis of MCP-1 and IL-8, two critical mediators of intrahepatic leukocyte recruitment of circulating monocytes and neutrophils in NASH (218, 335).
- Direct and indirect antifibrotic properties by reducing platelet-derived growth factor (PDGF)-induced HSC proliferation and migration, and by reducing TGF-β1-induced *de novo* synthesis of collagen type I.

These *in vitro* beneficial effects of silibinin were explored in animals and human NAFLD. In a high-fat-induced rat model of NASH (92) treatment with silibinin yielded a significant improvement in hepatic steatosis and inflammation, coupled with a decrease in hepatic markers of lipid peroxidation malondialdehyde and superoxide anion (O_2^-) and in systemic insulin and TNF-α levels (345). However, silibinin failed to normalize hepatic mitochondrial ATP production, PPAR-α, and cytochrome P450 CYP2E1 activity.

In a controlled trial of 59 patients with NAFLD, 6 months of treatment with silybin conjugated with vitamin E and phospholipids significantly improved ultrasonographic steatosis, liver enzymes, and circulating markers of liver fibrosis TGF-β, hyaluronic acid (HA), and metalloproteinase 2 (MMP-2) (159).

B. Curcumin

Curcumin is the primary constituent of turmeric, the common name for *Curcuma longa*, an Indian spice derived from the rhizomes of the plant of the Zingiberaceae family, which is cultivated in India and other parts of Southeast Asia. Turmeric comprises a group of three curcuminoids: curcumin (diferuloylmethane), demethoxycurcumin (17% of overall content), and bisdemethoxycurcumin (3% of overall content) (Fig. 10). Curcumin is a lipophilic polyphenol that is nearly insoluble in water but is quite stable in the acidic pH of the stomach. Animal studies have shown curcumin is rapidly metabolized, conjugated in the liver, and excreted in the feces, therefore having limited systemic bioavailability. A 40 mg/kg intravenous dose of curcumin given to rats resulted in complete plasma clearance at 1 h postdose.

An oral dose of 500 mg/kg given to rats resulted in a peak plasma concentration of only 1.8 ng/ml, with the major metabolites identified being curcumin sulfate and curcumin glucuronide (115).

In the last 10 years, almost 2000 more publications on curcumin have appeared in the National Institutes of Health PubMed database. These studies have revealed that curcumin has antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, antiproliferative, and pro-apoptotic effects. How a single agent could exhibit all these effects is an enigma under intense scrutiny. Numerous molecular targets for curcumin have been identified over the years. These targets fall into two categories, namely, targets to which curcumin di-

rectly binds and modulates their activity, and those of which modulation of activity is indirect or secondary (4, 5, 23). The first category (direct interaction) includes COX2, lipoxygenase (LOX), glycogen synthase kinase (GSK)-3β, phosphorylase-3 kinase, xanthine oxidase, N-aminopeptidase, amyloid protein, human a1-acid glycoprotein, autophosphorylation activated protein kinase, DNA polymerase, focal adhesion kinase (FAK), glutathione S-transferase, albumin, P glycoprotein, pp60 src tyrosine kinase, thioredoxin reductase (TrxR), tubulin, topoisomerase II, ubiquitin isopeptidase, and toll-like receptor (TLR) 4.

The molecular targets indirectly affected by curcumin might be upregulated or downregulated depending on the particular target. Curcumin has been shown to activate various nuclear transcription factors, including PPAR-γ, p53, NRF2, CHOP, and ATF3. Induction of p53 mediates the expression of cell-cycle-dependent kinase inhibitor p21; and NRF2 activation by curcumin has been linked with induction of GST and NADP(H):quinone oxidoreductase (NQO) and hemeoxygenase-1. Curcumin is also known to induce the expression of death receptor 5 (DR5), glutathione reductase, transferrin receptor 1, iron regulatory protein and ferritin H and L through a mechanism that is not fully understood. Curcumin has been also shown to downregulate various transcription factors involved in cellular proliferation, metabolism and inflammation (*e.g.*, NF-κB, hypoxia inducible factor [HIF]-1a, activator protein [AP]-1, signal transducers and activators of transcription protein [STAT]-3, specificity protein [SP]-1, and β-catenin), protein kinases (*e.g.*, protein kinase [PK]A, PKC, Src, and FAK), growth factor receptors (*e.g.*, EGFR and human epidermal growth factor receptor 2 [HER2; also known as p185 neu and ErbB2]), chemokines (*e.g.*, chemokine ligand [CXCL]1 and CXCL2), chemokine receptors (*e.g.*, CXCR4), antiapoptotic proteins (*e.g.*, cellular FLICElike inhibitory protein [cFLIP], inhibitor of apoptosis protein [IAP], X-linked IAP [XIAP], Bcl-2, and Bcl-xL), cell-cycle regulatory proteins (*e.g.*, cyclin D1, cyclin E, and c-myc), invasion and angiogenesis biomarkers (*e.g.*, matrix metalloproteinase [MMP]-9, vascular endothelial growth factor [VEGF], and urokinase plasminogen activator [uPA]), and inflammatory biomarkers (*e.g.*, TNF-α, IL-1, IL-6, COX2, 5-LOX, prostate specific antigen, and CRP).

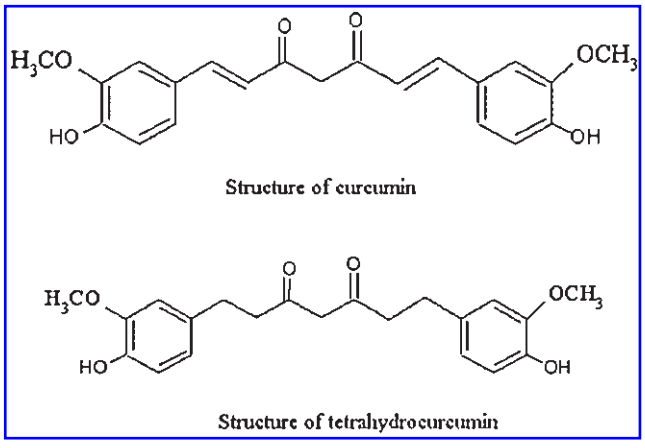

FIG. 10. Molecular structure of curcumin and tetrahydrocurcumin, the two main components of turmeric.

Several experimental datasets recently suggested that the antioxidant, anti-inflammatory, and antifibrotic properties of curcumin may be useful in NASH. In cultured HSCs, curcumin has been shown to inhibit cell activation by leptin and by oxidized LDLs (127, 290).

Leptin is able to activate HSC through an increased oxidative stress, which is a major factor to stimulate HSC activation and hepatic fibrogenesis: incubation of HSCs with leptin induced a dramatic increase in cellular levels of ROS and lipid peroxides, and reduced the content of cellular GSH and the ratio of GSH to GSSG (335). Coincubation of HSC with leptin and curcumin abrogated the stimulatory effect of leptin on HSC activation by interrupting leptin signaling and attenuating leptin-induced oxidative stress. Curcumin interrupted leptin signaling by reducing phosphorylation levels of leptin receptor (Ob-R) and its downstream intermediators. In addition, curcumin suppresses gene expression of Ob-R through the activation of endogenous PPAR-γ and of glutamate-cysteine ligase to increase *de novo* synthesis of glutathione in HSCs (290).

Another independent mechanism whereby curcumin exerts antifibrogenic properties is the inhibition of HSC uptake of oxidized LDL through the downregulation of lectin-like oxidized LDL receptor-1 (LOX-1) gene expression. Curcumin treatment of human activated HSCs led to inhibition of their activation, parallel with a fall in extracellular oxidized LDL particle uptake by the HSCs. This suppressive effect of curcumin results from the interruption of canonical Wnt signaling pathway and the activation of PPAR-γ. Preliminary data suggest that curcumin can also inhibit gene expression of CD36 and other scavenger receptors of oxidized LDL particles in HSCs, which may further contribute to the curcumin-caused reduction in the level of intracellular ox-LDL and HSC inhibition (112).

In mice fed an MCD diet for 10 weeks, intraperitoneal administration of curcumin significantly reduced intrahepatic 8-OH deoxyguanosine content, a marker of oxidative stress, and histological steatosis, necroinflammation, and fibrosis (313). These changes were paralleled by a reduced intrahepatic gene expression of monocyte chemoattractant protein-1, CD11b, procollagen type I, and tissue inhibitor of metalloprotease (TIMP)-1, together with protein levels of alpha-smooth muscle-actin, a marker of fibrogenic cells. In addition, curcumin reduced the generation of ROS in cultured HSCs and inhibited the secretion of TIMP-1 both in basal conditions and after the induction of oxidative stress. These findings were confirmed in the same mouse model, where curcumin also prevented the MCD-induced activation of NF-κB and decreased downstream induction of intercellular adhesion molecule, COX2, and MCP-1 (143).

In a rabbit model of high-fat-induced NASH, oral curcumin administration led to rabbits with a lower NASH grade and lower levels of aminotransferases, higher values for mitochondrial antioxidants, lower mitochondrial ROS, an improved mitochondrial function, and lower levels of TNF-α protein levels compared to vitamin E treatment (231).

In conclusion, curcumin might be useful in the management of NASH through a mechanism involving the antioxidant, anti-inflammatory, and mitochondrial-protective potential of curcumin. Preliminary human evidence suggests an effectiveness of curcumin in different inflammatory diseases. Phase I clinical trials have shown that curcumin is

safe even at high doses (12 g/day) and exhibits promising anti-inflammatory effects in different inflammatory conditions, including cancer, cardiovascular diseases, diabetes, arthritis, neurological diseases, and Crohn's disease. A major factor limiting its effectiveness in systemic diseases is its poor systemic bioavailability due to poor absorption and rapid metabolism through intestinal and hepatic glucuronidation and sulforilation. To improve the bioavailability of curcumin, numerous approaches have been undertaken, including the use of adjuvant like piperine (an inhibitor of glucuronidation), the use of curcumin phospholipid complex, the use of liposomal curcumin, and the use of curcumin nanoparticles or of structural analogs of curcumin (*e.g.*, novel synthetic curcumin analog, EF24) (9). Further human studies are needed to confirm the effectiveness of these approaches in human NASH.

C. Resveratrol

Resveratrol is a phytoalexin polyphenolic compound occurring in various plants, including grapes, berries, and peanuts. Multiple lines of evidence suggest its beneficial effects on neurological, hepatic, and cardiovascular systems (Fig. 11). The potential mechanisms responsible for its biological activities include downregulation of the inflammatory response through inhibition of the synthesis and release of pro-inflammatory mediators, modification of eicosanoid synthesis, inhibition of Kupffer cells and adhesion molecules, and inhibition of iNOS and COX2 *via* its inhibitory effects on NF-κB or activator protein-1 (51). Resveratrol would also act by scavenging ROS, thus decreasing lipid peroxidation and subsequent inflammatory response and liver injury (148). Mouse models have recently suggested at least two further protective mechanisms of resveratrol in NAFLD, involving the activation of AMP-activated protein kinase and of sirtuin pathway. Sirtuin is an NAD^+-dependent protein deacetylase that has been involved in resveratrol-mediated protection from high-fat diet-induced liver injury through due to at least three mechanisms: induction of antioxidant proteins MnSOD and nuclear respiratory factor 1, possibly *via* stimulation of PGC-1α, induction of PPAR-α-regulated genes of FA oxidation, and lowering activation of proinflammatory cytokines, such as TNF-α and IL-6, *via* down-modulation of NF-κB activity (216, 226, 266).

Bujanda *et al.* (30) investigated the effects of oral resveratrol on liver histology, on hepatic cytokines (TNF-α) and markers of lipid peroxidation (MDA) and of oxidative stress (superoxide dismutase, glutathione peroxidase, catalase, and NOS) in a rat model of diet-induced NAFLD. Compared to controls, resveratrol group showed significant lower histological steatosis, hepatic TNF-α, and MDA levels; reduced NOS activity; and increased antioxidant activity of superoxide dismutase, glutathione peroxidase, and catalase.

D. Viusid

Viusid, a nutritional supplement, is a compound of different molecules (ascorbic acid, zinc, and glycyrrhizic acid) with recognized antioxidant properties. The protective effects of glycyrrhizic acid had been formerly evaluated in CCl_4-induced model of liver injury. CCl_4 is not toxic *per se* but is responsible for oxidative stress and lipid peroxidation

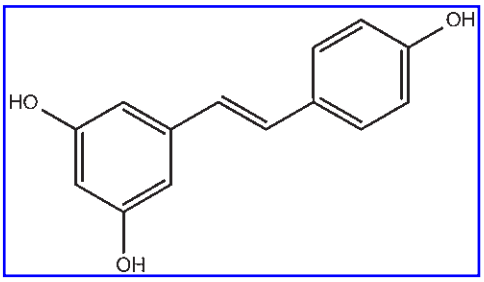

FIG. 11. Molecular structure of the polyphenolic compound resveratrol.

through the cytochrome P450-mediated generation of the highly reactive CCl3 , eventually leading to cellular damage characterized by hepatocellular necrosis. The subsequent chloromethylation, saturation, peroxidation, and the progressive destruction of the unsaturated FAs of the membrane phospholipids are collectively known as lipid peroxidation, which leads to a functional and structural disruption. Glycyrrhizic acid has been shown to protect the liver from CCl_4-induced lipid peroxidation and necroinflammatory injury thought the induction of heme oxygenase-1 and the downregulation of TNF-α, iNOS, and COX2 mRNA pathway activation (145). In mice models, zinc supplementation reverses ethanol-induced hepatic oxidative stress and steatosis by restoring the activity of hepatocyte nuclear factor-4α and PPAR-α and it prevented hepatocyte apoptosis and necroinflammation by attenuating hepatic TNF-alpha, TNF-R1, and Fas activation in the liver (128, 365). In dietary methionine and choline deficient mice, a widely used model of NASH, the supplementation of polaprezinc, a zinc-carnosine chelate compound, did not influence the development of steatosis but attenuated lipid peroxidation and suppressed HSC activation, eventually improving fibrosis through by reducing TNF-α, TGF-β1, procollagen α1(I), and tissue inhibitor of metalloproteinase (TIMP)-1 expression in the liver (285).

Based on the experimental data, Viusid was evaluated in association with diet and exercise in a randomized, controlled trial in 60 patients with biopsy-proven NAFLD (312).

After 6 months, Viusid significant improved steatosis and necroinflammation, but not fibrosis, compared with lifestyle intervention alone. The insulin resistance index improved by 35%–40% and body weight decreased consistently (by 12% in both arms) and similarly in both arms. Further experiments are required to evaluate the efficacy of this compound alone, that is, without other insulin-sensitizing measures, and for prevention of fibrosis progression in NASH.

E. Summary

Newer antioxidants have been developed and tested in experimental NASH with encouraging results. Among these, silymarin and the nutritional supplement Viusid revealed consistent benefit on liver disease when added to lifestyle intervention in NAFLD.

XIV. Concluding Remarks and Future Prospects

NAFLD and NASH are very common conditions affecting negatively on liver, metabolic, and cardiovascular health.

Imbalance between pro- and antioxidant mechanisms at cellular and extracellular levels is a hallmark of this condition and tightly connected to the hepatic inflammatory and fibrogenic process characterizing the progressive form of NAFLD, that is, NASH. Currently, no established therapy for NASH exists, since the most promising pharmacological drug class, that is, insulin sensitizer thiazolidinediones, has no significant effect on fibrosis, has important side-effects, including weight gain, and an unknown long-term safety profile. Further systematic investigation is needed to better understand the role of ROS/RNS in NAFLD, especially to elucidate the molecular mechanisms linking oxidative stress to inflammation and fibrogenesis. To this purpose, redox proteomic and microarray gene analysis may be able to clarify the complex links between oxidative stress molecules and signaling pathways involved in hepatocyte energy homeostasis, mediating cellular injury and triggering the necroinflammatory cascade, such as c-JNK and NF-κB. From a clinical standpoint, recent antioxidants, mostly of natural origin, showed encouraging safety and efficacy results in preclinical models, and some in clinically well-designed randomized trials, but overall results were contradicting. These trial results are difficult to interpret due to heterogeneous patient populations, different types and amounts of antioxidant therapy, and differing outcome measures, but some of these studies show the promise of antioxidants for amelioration of hepatic injury arising from oxidative stress. Further well-designed clinical and animal studies will be necessary to identify the exact physiological roles of redox imbalance *in vivo* and its contribution to the progression of steatohepatitis.

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Abbreviations Used

4-HNE = 4-hydroxynonenal
ACAA2 = acetyl-CoA acyl transferase2
ACC = acetyl-CoA carboxylase
ACS = acyl-CoA synthetase
AMPK = AMP-activated protein kinase
ASO = antisense oligonucleotides
ATF6 = activating transcription factor 6
BMI = body mass index
CHOP = C/EBP homologous protein transcription factor
ChREBP = carbohydrate responsive element binding protein
COX2 = cyclooxygenase 2
CPT-I = carnitine palmitoyltransferase 1A
CYP2E1 = cytochrome P450 enzymes 2E1
CYP4A = cytochrome P450 enzymes 4A
DCF = dichlorofluorescin
DCFH-DA = 2′,7′-dichlorofluorescin diacetate
DECR = 2,4-dienoyl CoA reductase
ER = endoplasmic reticulum
FA = fatty acids
FATP = fatty acid transport proteins
FC = free cholesterol
FFA = free fatty acids
Glut4 = glucose transporter 4
GSH = glutathione
HADHA = mitochondrial trifunctional protein
HMGCS2 = 3-hydroxymethyl-glutaryl-CoA reductase
HO = Heme oxygenase
HSCs = hepatic stellate cells
HSL = hormone sensitive lipase
IKKβ = inhibitor kappa kinase β
IL = interleukin
iNOS = inducible nitric oxide synthase
IRE1 = inositol requiring enzyme 1
JNK = c-JUN N-terminal kinase
LCAD = long-chain acyl-CoA dehydrogenase
LDL = low-density lipoproteins
LPL = lipoprotein lipase
MCD = methionine and choline deficient
MCP1 = monocyte chemoattractant protein 1
MDA = malondialdehyde
mGSH = mitochondrial glutathione
MnSOD = manganese superoxide dismutase
MPO = myeloperoxidase
MTPr = mitochondrial trifunctional protein
NAFLD = nonalcoholic fatty liver disease
NASH = nonalcoholic steatohepatitis
NF-κB = nuclear factor (kappa)B
NRF2 = NF-E2-related factor
PDC = pyruvate dehydrogenase complex
PDK4 = pyruvate dehydrogenase kinase
PGC-1α = peroxisome proliferator-activated receptor-γ coactivator 1α
PPAR = peroxisome proliferator activated receptor
PUFA = polyunsaturated fatty acids
RBP4 = retinol binding protein 4
RIP = receptor-interacting protein
ROS = reactive oxygen species
RXR = retinoid X receptor
SREBP = sterol regulatory element binding protein
TG = triglycerides
TGF-β = transforming growth factor-β
TNF-α = tumor necrosis factor-α
TRAF2 = TNF receptor-associated factor 2
UCP = uncoupling protein
UPR = unfolded protein response
VLDL = very low-density lipoproteins

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