Molecular basis of obesity-related hepatic steatosis

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ABSTRACT

Non-alcoholic fatty liver disease is a chronic inflammation liver condition that is currently highly relevant because of its strong association with increasingly incident diseases such as obesity and type-2 diabetes mellitus. The primary purpose of this paper is to discuss the best part of current knowledge on the molecular mechanisms involved in hepatic steatosis development, the condition’s initial stage, and on progression to steatohepatitis. Special attention has been paid to clinical and experimental obesity-related fatty liver. In the latter, the fa/fa rat is assessed, which constitutes an animal model for obesity with phenotype features similar to human obesity, including insulin resistance and dyslipemia. Hepatic steatosis is a complex, mainly metabolic condition where apparently non-compatible metabolic processes concur, in addition to oxidative stress, endoplasmic reticulum stress, mitochondrial dysfunction, and decreased expression of survival genes. Extrahepatic signals underlie the disorder, such as those arising from peripheral insulin resistance associated with an increase in adipose mass and systemic free fatty acids, together with intrahepatic signals leading to derangement of liver glycostatic and lipidostatic functions, as well as to greater vulnerability to other aggressions.

Key words: Lipogenesis. Insulin resistance. Metabolic deregulation. Non-alcoholic fatty liver.

RESUMEN

La enfermedad del hígado graso no alcohólico es una enfermedad inflamatoria hepática de carácter crónico de gran relevancia en la actualidad por su fuerte asociación con enfermedades de incidencia creciente como la obesidad y la diabetes mellitus tipo 2. En este trabajo se recoge buena parte del conocimiento existente sobre los mecanismos moleculares implicados en el establecimiento de la steatosis hepática, el primer estadio de la enfermedad, y en su progreso a steatohepatitis. Se ha prestado una atención especial al hígado graso asociado a la obesidad, clínica y experimental. En este caso, se valora la rata fa/fa, un modelo animal de obesidad con rasgos fenotípicos similares a los de la obesidad humana, incluyendo la resistencia a la insulina y la dislipemia. La steatosis hepática se revela como una situación compleja, eminentemente metabólica, en la que se simultanean procesos metabólicos aparentemente contradictorios, así como estrés oxidativo, estrés de retículo endoplasmático, disfunción mitocondrial y desenfreno en la expresión de genes de supervivencia. En buena medida, en su base se sitúan señales extrahepáticas, como las producidas en una situación de resistencia periférica a la insulina asociada a un aumento de la masa adiposa y de ácidos grasos libres sistémicos, e internas, causantes de un desajuste de las funciones glucostática y lipidostática del hígado y de una mayor vulnerabilidad a otras agresiones.


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**INTRODUCTION**

Non-alcoholic fatty liver disease (NAFLD) (1-6) is a chronic inflammatory condition of the liver that includes a range of alterations from simple deposition of fat or liver steatosis through non-alcoholic steatohepatitis (NASH) and fibrosis, to final stages such as cirrhosis (7-12). Liver steatosis has been traditionally considered a benign, reversible, asymptomatic condition with few associated clinical complications (11,13,14). However, it is a mandatory step in the development of NAFLD, which is characterized by lipid deposition in liver cell cytoplasms in the form of microvesicular (Fig. 1A) or macrovesicular (7,15,16) (Fig. 1B) lipid vacuoles. The latter case, where a huge fat-containing vacuole displaces the nucleus towards the cell’s periphery (6), is the type of steatosis most commonly found in individuals with NAFLD (3,7). Lipid bodies are no static fat storage but exhibit highly active metabolism and their lipid composition closely resembles a lipoprotein – a core of hydrophobic lipids, mainly triglycerides (15,17,18) and esterified cholesterol, and a single layer of polar lipids, phospholipids, and free cholesterol to which a range of constitutional and facultative proteins become associated (6,19-21). NASH is a necroinflammatory complication of chronic liver steatosis, and its major histological characteristics include macrovesicular steatosis and inflammatory infiltration with polymorphonuclear leukocytes and/or neutrophils (Fig. 1C). Mallory bodies may also be found, as well as glycogenated nuclei, ballooning hepatocyte degeneration, and increased cell death (20,22,23). This chronic inflammation of the liver induces the development of fibrosis to various extents, mainly in perivenous and perisinusoidal areas in zone 3 of the hepatic acinus (24), thus distorting liver architecture. In end-stage NAFLD fibrosis may degenerate into cirrhosis (25) and hepatocarcinoma (26,27).

NAFLD has become a most common cause of chronic liver disease and impaired liver function in industrialized countries (11,13,18,28-30), where 10-23% of the adult population is estimated to have it (3,29,31,32) with a frequency that is 3-fold that of type-2 diabetes mellitus (T2DM) and 5-10-fold that of hepatitis C. While the real prevalence of this condition is difficult to ascertain given a lack of serial analyses of liver biopsies and consensus histological diagnosis (33,34), its increased prevalence parallels that of other strongly associated diseases such as obesity, T2DM, and metabolic syndrome (35-40). Epidemiological studies have shown that the incidence of NAFLD among individuals with these conditions is much higher than in the general population (35,39,41-49). So much so that steatosis is currently considered the manifestation of metabolic syndrome in the liver (37-40,50), with said syndrome being defined as an association of at least 3 of the following disturbances: insulin resistance, central obesity, arterial hypertension, and dyslipemia, whether hypertriglyceridemia or low HDL-cholesterol levels (31,50).

Only a percentage of individuals with liver steatosis progress to more advanced stages of the disease (51-53). Nearly 53% of subjects diagnosed with simple steatosis keep a stable histology, whereas 43% progress to steatohepatitis (54,55); of these, 7-16% develop cirrhosis (56), and some patients ultimately need a liver transplant (31,57,58). The double hit theory is most widely accepted by the scientific community to explain response individuality and enhanced predisposition to NAFLD progression with disease advance (11,59,60) (Fig. 2). This theory posits that a first hit induces fat storage in the liver, and this renders hepatocytes more susceptible to a second hit, which results in inflammatory response leading to steatohepatitis (51,61). Current research suggests that a combination of environmental, genetic, and metabolic agents induce the development and progression of the condition (2). Insulin resistance is considered a most significant pathophysiological factor in steatosis development (62-64). Upon failed adaptation hepatocytes become dysfunctional and cell death may ensue through necrosis and apoptosis, and subsequent liver failure (59,60). Should adaptation occur, hepatocytes preserve their functional viability but become more vulnerable to inflammation-triggering stimuli. The extent of inflammation and cell death will depend on the severity and duration of these stimuli (51). This second hit may involve autocrine, paracrine and endocrine factors with a potential to trigger oxidative stress, lipid peroxidation (64), abnormal cytokine production, or to induce mitochondrial dysfunction and fatty-acid metabolism disorders (60). Chronic liver tissue inflammation may also lead to increased proinflammatory mediators, which activates fibrogenesis in hepatic stellate cells in a paracrine manner (12,34).
PATHOGENESIS

The fact that liver steatosis is reversible while steatohepatitis is not (65) makes this transition a non-return point regarding disease progression. Understanding the mechanisms involved in fatty liver development, and which processes and defense agents become affected by fat storage as a factor for liver cell vulnerability is therefore important. Epidemiological studies reveal that 30% to 97% of individuals with NAFLD are obese (49), and 60-90% of patients with morbid obesity exhibit some of the histological characteristics of NAFLD (66). One study found that 80% of obese subjects have a fatty liver, 33% portal inflammation, 24% fibrosis, and 3% cirrhosis (16). This paper reviews the molecular basis of NAFLD in obesity, and given the scarce number of studies in patients, most knowledge derives from studies in animals with either diabetic or genetic obesity. In the latter case, most common models include those with hypothalamic appetite-regulating system dysfunction whether from leptin deficiency (ob/ob mouse) or leptin-receptor deficiency (fa/fa rat) (67,68). Some results obtained in our laboratory in fa/fa rats will also be discussed – in addition to phenotypal obesity with liver steatosis that does not spontaneously progress to NASH, this model exhibits a number of associated clinical manifestations, including insulin resistance, hypertriglyceridemia, and cholestasis (69-71).

Peripheral insulin resistance underlying obesity-associated liver steatosis

Insulin resistance – a disorder of insulin’s ability to regulate glucose and lipid metabolism – is an early crucial defect associated with obesity and T2DM (72-74), as well as other metabolic disturbances (36-40). The etiology of insulin resistance is partly unknown, but a strong association between insulin resistance and excessive lipid deposition outside the adipose tissue exists, particularly in the muscle and the liver (73,75,76).

Under postprandial conditions, with high cell energy loads and high glycemia, insulin activity predominates. In the fat tissue insulin delays lipolysis for stored triglycerides upon the inactivation of hormone-sensitive lipase (HSL) (77), increases glucose uptake and glycolytic oxidation (which provides glycerol-3-phosphate, the 3-carbon backbone needed for triglyceride synthesis), and activates lipoprotein lipase (LPL) (77) in the vascular endothelium (which removes triglycerides from chylomicrons (CM) and VLDL, thus releasing fatty acids that diffuse into liver cells). In skeletal and cardiac muscle insulin increases glucose entry by increasing the expression of GLUT4 transporters at the plasma membrane (7); oxidation is enhanced by glycolysis, and glucose is stored as glycogen while fatty-acid β-oxidation and glycogenolysis are inhibited. Essentially, insulin represents an anabolic hormone paradigm in these tissues, and via sev-
eral routes increases fat content and adipose tissue mass, as well as glucose and fatty-acid storage in muscles at the expense of postprandial glycemia and lipemia reductions. In contrast, in the presence of hypoglycemia, glucagon attempts to counter low energy loads and reverses the aforementioned effects by switching on processes that mobilize energy reserves while promoting the fueling use of fatty acids by tissues instead of glucose. Glucagon, via protein kinase A (PKA), activates HSL in the fat tissue (77), thus generating significant glycerol amounts, that the liver will use in the synthesis of glucose, and free fatty acids (FFAs), usable as fuel by most tissues. At a molecular level, a drop in the energy load in cells activates the fuel sensor par excellence, namely AMP-activated protein kinase or AMPK (78), which in turn activates lipogenesis.

Insulin works through activation of specific signalling cascades (Fig. 3). Its binding results in the cross phosphorylation of tyrosine residues in the receptor’s intracellular chains providing anchorage sites for insulin receptor substrates such as IRS-1 and IRS-2, or for Grb-2. From IRS proteins signalling ensues via a number of membrane-bound molecules (including phosphoinositide 3-kinase or PI3K) to a protein kinase that finally leaves the membrane. Akt is the kinase that moves through the cell to phosphorylate targets including components that control glucose receptor GLUT4 traffic, as well as enzymes regulating glyco-

![Diagram](image-url)
gen and protein synthesis, and other metabolic processes. From Grb-2 signals are transmitted via a number of small G proteins to a mitogen-activated protein kinase (MAPK), which mediates cell proliferation and growth processes by targeting transcription factors (7).

Adipose tissue is a relevant source of molecular signals for insulin action regulation (49); highlights include TNF-α (12,49), IL-6 (79), resistin (80), fatty acids (11), leptin (81), adiponectin (81), and plasminogen activator inhibitor (80). Robust evidence suggests that TNF-α, IL-6 to a lesser extent (82), is the adipocytokine responsible for the development of insulin resistance by somehow impairing receptor cross-phosphorylation (12). Given the elevated production of TNF-α in obese individuals, and that visceral adipose tissue exhibits higher TNF-α production when compared to subdermal fat tissue (12), increased insulin resistance and steatohepatitis are to be expected in individuals with central obesity (83). A polymorphism in the TNF-α promoter has also been found in patients with NAFLD that is associated with insulin resistance (84-86) and higher amounts of cytokine transcripts in the liver and adipose tissue (87).

In peripheral insulin resistance, insulin cannot revert hypoglycemia-induced HSL activation (88), and FFAs are released from the fat tissue into blood in a dysregulated manner (7) (Fig. 3A). High serum fatty-acid and adipose glycerol concentrations (12) have been seen in obese patients under postprandial conditions (8) or in NAFLD (16,48). FFAs block intracellular insulin signalling in adipose tissue itself (11), but also stimulate, together with hyperglycemia, insulin secretion by pancreatic β cells (89) thus inducing hyperinsulinemia (7) and, should the pancreas become exhausted, T2DM. Patients with T2DM commonly have high serum FFA levels (16), insulin resistance and hyperinsulinemia by TNF-α (12).

High FFA levels may also impair lipid metabolism in other tissues (16), for instance blocking LPL activation in skeletal muscle (16), which prevents triglyceride-rich lipoprotein –CM and VLDL– metabolism, and hence increases their time of residence in the circulation. Hypertriglyceridemia is a factor most commonly associated with peripheral insulin resistance and NAFLD, which may aggravate when liver VLDL production is also increased as described for some patients (16).

Liver energetic metabolism and liver insulin resistance

Insulin plays a lipogenic and antigluconeogenic role in the liver cell. Under physiological conditions insulin increases glucose uptake by activating its phosphorylation to glucose 6-phosphate, and its use by the hepatocyte for glycogen formation first, and then for oxida-
ual addition of triglycerides, phospholipids, and cholesterol esters to the nascent particle (105,106). Therefore, the liver secretes a range of VLDL particles with highly variable maturation extents, sizes, and triglyceride contents. VLDL production is mainly regulated at a post-translational level by mechanisms involving ubiquitin and the proteasome, hence apoB100 is destroyed when a minimum amount of lipids is not recruited. It is currently accepted that high intracellular lipid availability increases the likeliness that a high proportion of apoB100 may reach a competent particle shape for secretion (107). Despite this, however, the role of insulin—a lipogenic hormone—in the regulation of VLDL production is controversial. High insulin concentrations have been described to promote apoB degradation and decreased VLDL secretion in primary cultures involving human and murine hepatocytes (108). Along this line, a lower secretion of VLDL-apoB100 has also been recorded for patients with non-alcoholic steatohepatitis (52). However, other studies related both hyperinsulinemia and insulin resistance to increased apoB100 synthesis and secretion (109,110). An in vivo measurement of VLDL production in fa/fa rats with hepatosteatosis in our laboratory corroborates the latter. Recent findings showing that ER stress may result in liver steatosis and insulin resistance, and conversely that steatosis may cause ER stress, with opposing effects on triglyceride secretion depending on its extent, may help reconcile this apparent conflict (111). Prolonged exposure to oleate of the liver or McA-RH7777 liver cells results in ER stress and steatosis; however, while low doses of oleate increase apoB100 secretion, higher doses result in reduced secretion upon the promotion of apoB100 proteolysis as

Fig. 4. An overview of fatty acid metabolism in hepatocytes under insulin resistance conditions. FFA, free fatty acids; CMR, chylomicron remnants; ACS, acyl-CoA synthetase; CPT, carnitine palmitoyltransferase; TAC, tricarboxylic acid cycle; PDH, pyruvate dehydrogenase; OAA, oxaloacetate; CS, citrate synthase; PC, pyruvate carboxylase; ACL, ATP:cytrate lyase; ME, malic enzyme; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; TG, triglycerides; EC, esterified cholesterol; PL, phospholipids; FC, free cholesterol; MTP, microsomal triglyceride transfer protein; apoB100, apolipoprotein B100.
part of the response to excessive lipid-induced ER stress, which further exacerbates steatosis on restricting triglyceride secretion. ER stress involves both protease-dependent and protease-independent proteolytic mechanisms (111).

There is evidence suggesting liver insulin resistance in specific NAFLD stages, with active gluconeogenesis, fatty acid β-oxidation, and a lower respiratory coefficient being concurrent with high insulin levels. Adipocytokines, FFAs, and hyperglycemia, in addition to the aforementioned ER stress, may contribute to the development of said liver insulin resistance on modulating insulin response, lipid metabolism, or liver inflammatory/immune response (112-114). As with adipose tissue (93), TNF-α may alter intracellular insulin signalling in the liver, and the expression profile of other cytokines that also distort this hormone’s signals in liver cells (112,115). Main FFAs in patients with NAFLD include palmitic (C16:0) and oleic (C18:1) acids (116,117). While the latter promotes steatosis, the former –similar to other saturated fatty acids– favors hepatocyte lipopoptosis as mediated by JNK (Jun N-terminal kinase). Polyunsaturated fatty acids (PUFAs) may induce liver insulin resistance both directly and indirectly. Indirectly as they change the composition and fluidity of mitochondrial and plasma membranes, and therefore receptor activity, associated protein recruitment, and protein sensitivity to regulators (93,118). In this respect CPT modifications via AMPK have been described (119) that render it resistant to malonyl-CoA inhibitory effects. PUFAs also inhibit liver SREBP-1c expression and activity (120), and compete with insulin in controlling the expression of genes involved in glucose metabolism and de novo fatty acid synthesis (118). In contrast, saturated and monounsaturated fatty acids do not seem to act on SREBP-1c or have any effects on lipogenic gene expression (121). However, even in severe liver insulin resistance, this transcription factor—which becomes activated by sterol-deficiency induced proteolysis– remains active and sustains de novo fatty acid synthesis, contributing to 50% of total triglycerides in animal models (32).

As a model of obesity with steatosis that does not spontaneously progress to steatohepatitis, fa/fa rats have indetectable serum TNF-α levels, and in contrast to patients exhibit high levels of adiponectin, a hormone with anti-inflammatory effects that improves liver sensitivity to insulin (122). Therefore, a normal liver response to high circulating insulin levels could be expected. However, a transcriptomic analysis in isolated hepatocytes (Table I) reveals overexpression of genes involved in fatty-acid oxidation, including those coding for peroxisomal acyl-CoA oxidase, acetyl-CoA acyltransferase, enoyl-CoA hydratase, numerous respiratory chain components, and uncoupling protein 2 (UCP-2), which suggests a certain degree of hepatic insulin resistance and an initiation of compensatory mechanisms. These animals, with considerable hyperinsulinemia and high serum FFAs (123), as well as overexpressed lipogenic and complex lipid-forming genes, have an exacerbated secretion of triglyceride-rich VLDL, which reflects moderate ER stress (111). Their hepatocytes, with a higher expression of fatty acid translocase CD36 at the basolateral membrane, are expected to show a higher uptake of fatty acids into the cytoplasm. A transcriptomic, statistical, and functional analysis of this model reveals two pieces of evidence: 1) that hepatocyte steatosis occurs because the uptake and endogenous synthesis of FFAs and complex lipids exceeds the also increased hepatocyte’s ability to handle them and secrete them as VLDLs; 2) that established steatosis entails not only changes in the (all activated) biological processes involved in energetic metabolism, but also in those related to immunity and defence – coagulation, detoxification, and stress response, all of them repressed. There is also a concomitant activation of cell cycle, nucleic acid metabolism, transcription, and transcription regulation, whereas chromatin remodeling decrease, thus suggesting that epigenetic factors may also play a role in the etiopathogenesis of steatosis.

**On the edge of steatohepatitis. A combination of mitochondrial dysfunction and oxidative stress**

In a liver parenchymal cell with activated oxidation processes mitochondrial β-oxidation represents the primary route for fatty acid oxidation (3). Reduced coenzymes FADH₂ and NADH, generated by the process itself and during acetyl-CoA oxidation in the tricarboxylic acid cycle (TAC), donate their electrons to the respiratory chain, and ATP results from ADP phosphorylation by ATP synthase (Fo and F1), propelled by the return to the matrix of protons previously pumped by respiratory complexes into the intermembrane space (Fig. 5). However, mitochondria are also the primary source of reactive oxygen species (ROS) (32). It is thus inevitable that a small percentage of electrons will directly react with molecular oxygen to yield superoxide anions (O₂·⁻), H₂O₂, and other by-products included in the ROS category (Fig. 5). ROS emerging from this and other processes are then neutralized by enzymatic systems such as superoxide dismutase, catalase, and y glutathione peroxidase, and by cell defence vitamins, mainly vitamins E and C. Mitochondrial dysfunction, ultrastructural abnormalities (11,64), decreased respiratory chain complex activity (5,32), deficient oxidative phosphorylation, decreased ATP synthesis capability, reduced intracellular ATP concentration (124) –which compromises cell response to any cell damage (34)– and damaged mitochondrial DNA have all been found in patients with NASH (7). No ultrastructural abnormalities have been seen in patients with simple steatosis (7). It may be simplistically considered that the causes...
Table I. Biological processes significantly altered in hepatocellular steatosis according to PANTHER (129,130). The statistical significance of the difference between the expected number of sequences for each process following Affymetrix RGU34A matrix normal distribution and the number obtained experimentally is included.

<table>
<thead>
<tr>
<th>Biological process</th>
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<th>P-value repressed sequences</th>
<th>P-value overexpressed sequences</th>
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<td>Lipid, FA, steroid metabolism</td>
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<td>9.11E-04</td>
<td>5.42E-06</td>
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<td>– FA metabolism</td>
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<td>– Lipid, FA transportation</td>
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<td>4.86E-02</td>
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<tr>
<td>– Lipid, FA binding</td>
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<td>– Other carbohydrate metabolism</td>
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<td>– Chromatin packaging and remodeling</td>
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of decreased ATP production include: a) an interruption or blockade of the respiratory chain at any point, which results in reduced electron transport for all upstream complexes, which would then be forced to donate electrons to molecular oxygen; and b) a dissipation of the proton gradient across the inner mitochondrial membrane, which drives ATP synthase. As with other cell types, hepatocytes may develop adaptive mechanisms to keep electron flow similar to normal tissues. Some mechanisms involve increased mitochondrial and mitochondrial crest size, which entails an increase in the synthesis of respiratory complexes and coenzyme Q availability, whereas others involve an activation of the mitochondrial biogenesis process (124).

In a liver cell with steatosis and dysfunctional mitochondria a considerable portion of fatty acids will be processed by the peroxisomal and microsomal oxidative systems. Peroxisomes perform several β-oxidation cycles to shorten (Fig. 4) the length of long-chain and very-long-chain fatty acids, long-chain dicarboxylic acids, eicosanoids, and CoA esters of bile acid by-products, which results in acetyl-CoA and H₂O₂ (101,102, 125). Microsomal oxidation is under the control of the cytochrome P450 system, specifically of CYP2E1 and members of the CYP4A family (126). These cytochrome-related activities also result in reduced molecular oxygen (12) and ROS production (127). CYP2E1 seems to be the primary microsomal source of H₂O₂ and lipid peroxidation (126); furthermore, it shows greater expression and/or activity in obese individuals with central fat deposition, T2DM, or hyperlipidemia (126) under insulin resistance conditions and also in NASH patients and animal models (2); all these situations exhibit both oxidative stress and mitochondrial damage. In addition, CYP2E1 has a higher expression in the perivenous (zone 3) region of liver acini, this being the area with highest hepatocellular damage in steatohepatitis (126). There is indication of potential complementariness and/or redundancy in the function of CYP2E1 and CYP4A during lipid oxidation, as a reduction in CYP2E1 expression and a compensatory increase in CYP4A expression may be seen in fa/fa rats and ob/ob mice (126).
ROS production is therefore inherent in the activation of cell systems for fatty acid oxidation. Also massive glucose oxidation as occurs in diabetes mellitus is a relevant source of ROS. These are short-lived substances with local effects (32) that may also induce oxidative stress in cells when defensive systems cannot manage to counter them adequately. Oxidative damage by ROS has been described in patients with steatosis, NASH, or alcoholic steatohepatitis, and in animal models with NASH (126). ROS may exert multiple effects including increased TNF-α synthesis (which may in turn induce insulin resistance, necrosis, and apoptosis), natural antioxidant and both ATP and NAD+ depletion, DNA damage, changes in protein stability, membrane distortions, and proinflammatory cytokine secretion (32).

A relevant effect of ROS is lipid peroxidation (124), which is mostly demonstrated in PUFAs. Lipid peroxidation ultimately leads to reactive aldehydes (12), compounds with a half-life longer than that of ROS that easily diffuse across cell membranes, and may reach extracellular targets and carry oxidative damage to tissues. Lipid peroxidation seemingly plays a crucial role in hepatocellular damage as in NASH (11). There is a strong association between steatosis severity and NASH development risk (60), which correlates to lipid peroxidation extent. This fact is not surprising – the huge amount of lipids stored in a steatotic hepatocyte are a source of ROS through oxidative systems, and represent a suitable substrate for reactive aldehyde generation (12). These products can block the synthesis of nucleotides and proteins, and increase tumor growth factor β1, which promotes Mallory body formation (124). In addition, they are powerful inflammatory cell chemo-attractors and stellate cell activators (2), and may perpetuate inflammatory response by enhancing proinflammatory cytokine and chemokine secretion (12), and the release of adhesion molecules such as ICAM-I, E-selectin, and P-selectin (2). All these phenomena may result in cell death through apoptosis and necrosis, inflammation, and fibrosis, all of them the histological mark of NASH. The evidence pool renders indisputable the statement that massive oxidative damage by reactive aldehydes underlies progression to steatohepatitis. The mechanism through which oxidative stress leads to cell damage is seemingly chronic NF-κB activation, the latter factor being also activated by growth factors, nitric oxide, and selected cytokines.

This will also enter a self-perpetuating cycle where hardly a chance for improvement may be found. In the mitochondria reactive aldehydes may interact with membrane phospholipids, proteins such as the adenine nucleotide transporter (ANT) (124), which exports the newly synthesized ATP, and respiratory chain complexes whose properties they change, thus compromising electron transportation and proton pumping to the inter-membrane space (Fig. 5). A consequence of blocked mitochondrial β-oxidation is the processing of a higher percentage of fatty acids in microsomes, whose products – dicarboxylic acids – worsen mitochondrial damage (32). The incorporation of protons into the mitochondrial matrix as promoted by fatty acids themselves and by excess ROS and UCP-2, bypasses ATP synthase; the driving force promoting ATP synthesis declines, which compromises both its synthesis and subsequent ANT-mediated export. A defective electron transport exacerbates ROS production, which will enhance lipid peroxidation and mitochondrial dysfunction. The paradox in the organic metabolism of a diabetic individual is replicated in ROS-damaged mitochondria. Energetic efficiency and the respiratory quotient obtained from the mitochondrial oxidation of nutrients is low, and hepatocytes consistently perceive hunger signals that may eventually lead to systemic satiation.

To avoid oxidative stress, the cell has to make up for oxidizing agent production by increasing the expression of genes involved in antioxidant defense, enhancing reduced glutathione levels via augmented γ-glutamylcysteine synthase activity and both glutathione S-transferase and catalase overexpression (126). Vitamin defenses may become depleted. Under chronic oxidative stress, as occurs in fatty liver and other NAFLD-related conditions, a decrease in antioxidant vitamins has been found, including α-tocopherol and β-carotene, because of depletion (12). Lipoproteins play a crucial role in the distribution of antioxidant vitamins across tissues. Regarding fa/fa rat hepatocytes, the expression of many genes involved in detoxification and stress response has been found to be repressed, including those coding for catalase, sulphotransferases, carboxylesterases, glutamine synthase (to clear ammonium), and a great variety of cytochrome P450 family members with monoxygenase activity. In such setting, while no apparent cell damage or immune response can be found in tissues, any stimulus, such as changing to a fat-rich diet (128), represents a second hit that triggers steatohepatitis.

Other factors, including endotoxins, ischemia-reperfusion, acute ethanol therapy, or iron, enhance oxidative stress. Forty percent of patients with NAFLD exhibit mild iron deposition in cells, this being a metal that catalyzes ROS formation, activates Kupffer cells, and enhances NF-κB production, hence patients with higher intracellular iron levels have a greater risk of fibrosis (12). Ethanol, in turn, leads increased ROS in two ways – as a result of its metabolism the NADH:NAD+ ratio increases, and so does the passage of Fe2+ to Fe3+, which in turn induces both direct and indirect ROS formation upon CYP2E1 activation. There are NASH forms secondary to drugs, jejunoileal bypass, or total parenteral nutrition where steatohepatitis is more severe and clinical implications are more serious (124). Table II summarizes some of the most important causes of NASH, and the primary factors contributing to disease progression (34). All this seems to suggest a combination of multiple factors, its...
Table II. Factors involved in the development of steatohepatitis. Based on Harrison et al. (34).

<table>
<thead>
<tr>
<th>Factor</th>
<th>1st hit</th>
<th>2nd hit</th>
</tr>
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<tbody>
<tr>
<td>Liver parenchymal cell steatosis</td>
<td>↓ Circulating insulin, ↑ adipose lipolysis, ↑ fatty acid synthesis, ↓ mitochondrial β-oxidation</td>
<td>Changes in PPARG, CYP2E1/CYP3A4 polymorphisms</td>
</tr>
<tr>
<td>Susceptible genotype</td>
<td></td>
<td>Lipid peroxidation, ↑ CYP2E1</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>↑ Lipid peroxidation, ↑ CYP2E1</td>
<td>↑ Lipid peroxidation, ↑ VLDL production</td>
</tr>
<tr>
<td>Depletion of essential antioxidants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial dysfunction</td>
<td>Low ATP synthesis, ↑ UCP-2, ↑ oxidative stress, and ↑ FFAs</td>
<td></td>
</tr>
<tr>
<td>Liver cell adaptations for survival</td>
<td>Lowered defense systems, blocked regeneration</td>
<td></td>
</tr>
<tr>
<td>Cytokine upregulation</td>
<td>TNF-α, IKK-β, and NF-κB activation</td>
<td></td>
</tr>
<tr>
<td>Kupffer cell dysfunction</td>
<td>Susceptibility to endotoxins, ↓ phagocytic capacity, abnormal cytokine production profiles, ↑ fibrogenesis</td>
<td></td>
</tr>
<tr>
<td>Fibrogenesis</td>
<td>Fibrogenic cytokines and growth factors in stellate cells</td>
<td></td>
</tr>
</tbody>
</table>

central axis being the generation of reactive oxygen species and lipid peroxidation products. However, inter-subject variability in the development of NAFLD leads to suspect a contribution of genetic factors such as polymorphisms described for CYP2E1 and TNF-α (2), as well as epigenetic and/or environmental factors that predispose individuals to this disease.

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