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POINT OF VIEW

Non-alcoholic fatty liver disease. From insulin resistance to mitochondrial dysfunction

J. A. Solís Herruzo, I. García Ruiz, M. Pérez Carreras and M. T. Muñoz Yagüe

Departments of Gastroenterology and Hepatology. Research Center. University Hospital "12 de Octubre". Universidad Complutense. Madrid, Spain

ABSTRACT

Non-alcoholic fatty liver disease represents a set of liver lesions similar to those induced by alcohol that develop in individuals with no alcohol abuse. When lesions consist of fatty and hydropic degeneration, inflammation, and eventually fibrosis, the condition is designated non-alcoholic steatohepatitis (NASH). The pathogenesis of these lesions is not clearly understood, but they are associated with insulin resistance in most cases. As a result, abdominal fat tissue lipolysis and excessive fatty acid uptake by the liver occur. This, together with a disturbance of triglyceride export as VLDL, results in fatty liver development. Both the inflammatory and hepatocellular degenerative components of NASH are attributed to oxidative stress. Mitochondrial respiratory chain loss of activity plays a critical role in the genesis of latter stress. This may be initiated by an increase in the hepatic TNFα, iNOS induction, peroxynitrite formation, tyrosine nitration and inactivation of enzymes making up this chain. Consequences of oxidative stress include: lipid peroxidation in cell membranes, stellate cell activation in the liver, liver fibrosis, chronic inflammation, and apoptosis.

Key words: Non-alcoholic fatty liver disease. Insulin resistance. Mitochondrial dysfunction.

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INTRODUCTION

Alcohol-induced liver lesions belong to three different categories (1-3): a) *fatty liver,* where hepatocytes are filled with a big fat vacuole displacing the nucleus and other organelles towards the cell's periphery *(macrovacuolar steatosis).* On occasion, hepatocytes contain multiple fat droplets that will not displace the nucleus peripherally, allowing it to remain in its central position *(microvacuolar steatosis);* b) *alcoholic hepatitis.* These patients exhibit, together with liver steatosis, hepatocyte ballooning degeneration, alcoholic hyaline or Mallory bodies, megamitochondria, mixed inflammatory infiltrates with predominant polymorphonuclear cells, and both pericentral and pericellular fibrosis. All these changes are more common and severe in the centrolobular zone 3; and c) *alcoholic liver cirrhosis,* primarily micronodular that may secondarily evolve to macro-micronodular cirrhosis. Also cirrhoses with this etiology may become complicated with hepatocellular carcinoma.

These lesions, mainly those corresponding to alcoholic hepatitis, have been considered highly suggestive of alcohol abuse. However, as early as in the 1950s Zelman (4) and Werswater and Fainer (5) described the presence of liver steatosis and fibrosis associated with inflammatory infiltrates in the liver of obese patients. Also Thaler reported on several occasions –during the 60s and early 70s– apparently alcoholic lesions in non-drinking subjects. Hence Thaler suggested that the term *"alcoholic hepatitis"* be replaced by *"fatty hepatitis", "Fettleberhepatitis",* or *"steatohepatitis"* (6-8). Similar cases were further described during the 70s in obese (9-11) and diabetic (12-14) individuals, as well as in patients undergoing enteric bypass for morbid obesity (15,16). All these reports were received with skepticism as most authors were convinced that these patients were in fact heavy drinkers. In 1980, Ludwig et al. (17) coined the term *"non-alcoholic steatohepatitis"* (NASH) to designate these lesions that mimic those induced by alcohol in individuals with no alcohol abuse. NASH is currently considered a part in a wider spectrum of lesions including –in addition to NASH itself– non-alcoholic fatty liver, fatty liver and inflammation, and

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Correspondencia: J. A. Solís Herruzo. Servicio de Medicina Aparato Digestivo. Centro de Investigación. Hospital Universitario 12 de Octubre. Universidad Complutense. Madrid. Ctra. de Andalucía, km 5,400. 28041 Madrid. e-mail: jsolis.hdoc@salud.madrid.org

probably a high number of cryptogenic cirrhoses (18,19). To designate all this range of lesions the term *"non-alcoholic fatty liver disease"* (NAFLD) was proposed. The prognostic relevance of all these lesions is heterogeneous. While steatosis is a stable lesion that only develops into more severe forms in 3% of cases, NASH evolves to cirrhosis in 15-25% of cases (18,20). Many cryptogenic cirrhoses probably originate in NASH, with steatohepatitis signs disappearing over time (21-23). As with cirrhosis from other etiologies, NASH-derived cirrhosis may also result in hepatocellular carcinoma (24).

The diagnosis of NASH is not based on the presence of a specific liver lesion, but on the existence of a constellation of lesions including steatosis, hepatocyte hydropic degeneration, and inflammatory infiltrates. In addition, hyaline Mallory bodies, megamitochondria, and fibrosis in varying degrees are commonly found. A scoring system has been recently suggested to assess the various hepatic lesions of NAFLD, and to establish the histological diagnosis of NASH (19,25). A conceptual and critical diagnostic feature of NAFLD is absence of alcohol abuse. There is no unanimous view on what may be considered *"non-abusive alcohol consumption"* regarding NAFLD, but consumption is usually considered non-abusive when ethanol ingestion remains below 20-40 g/day in males and 20 g/day in females (26,27).

NAFLD is a common lesion in Western populations, and will become commoner in the future, as it is associated with insulin resistance, diabetes, and obesity. After viral infection and alcohol abuse, it currently represents the third most common cause of hypertransaminasemia. It is estimated that 17 to 33% of the general population have NAFLD, and the lesion present in 5.7 to 17% of this same population is NASH (28,29). When hypertransaminasemia has been studied for a cause in subjects with no viral infection markers and no alcohol abuse, NAFLD lesions are found in 40 to 90% of cases (29). In a study by ourselves some 20 years ago we found that NASH lesions had a prevalence of 5/100,000 population, an incidence of 0.9/100,000 population/year, and a frequency in liver biopsies of 1.9% and one in twelve alcoholic hepatitis cases (30,31). More recent reviews of this problem have shown that such incidence and prevalence have been on the rise for the past few years.

NAFLD has been identified in association with a high number of metabolic, surgical, and toxic conditions *(secondary NAFLDs).* However, the primary factor associated with NAFLD is *metabolic syndrome,* defined as the associated presence of at least three of the following changes in one individual: blood hypertension (≥ 130/85 mmHg), central obesity (waist > 102 cm in males; > 88 cm in females), fasting hyperglycemia (≥ 110 mg/dl), hypertriglyceridemia (> 150 mg/dl), and reduced HDL (< 40 mg/dl in males; < 50 mg/dl in females) (32). A common pathophysiologic feature in this syndrome is insulin resistance (33-35). In fact, NAFLD would represent the hepatic component of insulin resistance syndrome.

FROM INSULIN RESISTANCE TO FATTY LIVER

Insulin is the primary anabolizing hormone in the body. Its effect brings about an increased synthesis of proteins, glycogen, and lipids, facilitates glucose uptake by cells, and decreases glyconeogenesis and lipolysis. The mechanisms for such varied effects are only partially understood. In adipocytes and skeletal muscle cells the binding of its specific receptor by insulin is known to activate the receptor's tyrosine kinase, the latter's self-phosphorylation, and the phosphorylation in tyrosine/activation of IRS-1 *(Insulin Receptor Substrate-1).* This is followed by the activation of PI3K (Phosphatidyl Inositol-3 Kinase). This kinase activates a glucose transporter that is usually found within vesicles in the cytoplasm –Gluc-4 *(glucose transporter 4)*– and moves it unto the plasma membrane, thus facilitating cell glucose uptake (34,36,37) (Fig. 1). Within cells glucose is used an energy source, or stored as glycogen when not required. In the presence of insulin resistance, IRS-1 phosphorylation in tyrosine does not take place; cell glucose uptake stops; glucose is retained in the extracellular space and hyperglycemia occurs, which in turn stimulates insulin secretion by pancreatic β cells (38). Once the pancreas is depleted and can no longer compensate for hyperglycemia, type-II diabetes mellitus develops.

The cascade phenomena following the binding of insulin to its receptor is more extensive than previously mentioned. PI3K activation after IRS-1 phosphorylation activates *phosphodiesterase*, and as a consequence AMPc degradation and depletion. Absence of AMPc precludes PKA *(Protein Kinase A)* activation, and hence lipoprotein lipase (LPL) activation too (39). That is, neither triglyceride hydrolysis nor free fatty acid (FFA) release from fat tissue occur (40). A consequence of insulin resistance in fat tissue is that cAMP remains high, which activates PKA, which in turn activates LPL. This results in triglyceride degradation and FFA release into the blood. The lipogenic and anti-lipolytic effects of insulin are coordinated by the hormone's PI3K-mediated effects on SREBP *(Sterol Regulatory Element Binding Protein),* a transcription factor that plays an essential role in the activation of various genes involved in lipogenesis (acetyl-CoA carboxylase; fatty acid synthase; glycerol-3 phosphate acetyltransferase, etc.) (41), and in VLDL excretion (MTP, *Microsomal Transfer Protein*). Hence, in the absence of insulin activity, all these genes are repressed, and so is lipogenesis (42) (Fig. 1).

The effects of *insulin on the liver* slightly differ from those exerted on fat tissue and skeletal muscle, as insulin receptor phosphorylates another substrate –IRS-2 (43)– into tyrosine, which through PI3K and Akt-2/PKB phosphorylates and inactivates GSK3 *(Glycogen Synthase Kinase-3),* and the latter stops inhibiting glycogen synthase thus allowing an increase in the latter's activity (44). As a result, insulin increases glycogen synthesis in the liver. Liver insulin resistance results in opposite effects. It de-

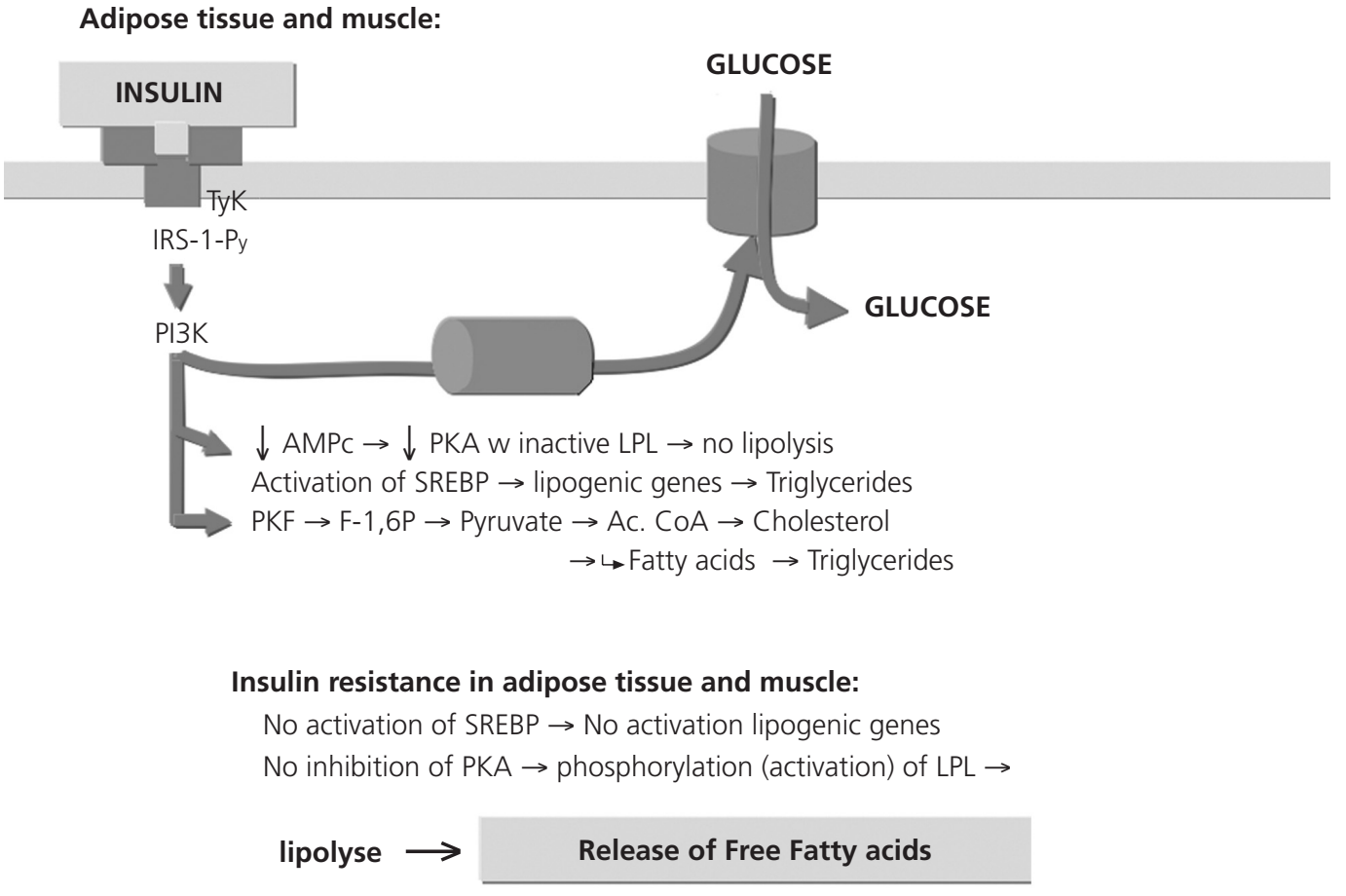

Fig. 1.- Mechanism of insulin action in the fat tissue and skeletal muscle.
Mecanismo de actuación de la insulina en el tejido adiposo y en el músculo esquelético.

creases glycogen synthesis and increases glycolysis, glyconeogenesis, and glucose release into the circulation. In addition, insulin stimulates –also through IRS-2 and SREBP activation– the expression of lipogenic genes determining the synthesis of fatty acids in the liver (45).

Factors playing a role in insulin resistance are probably multiple (46-48). Steatosis itself has been implied, as well as oxidative stress, FFAs, TNFα, and –as intracellular mediators– ceramide, IKKβ (49), NFκB, PKC-θ (Protein Kinase C-θ), JNK1 (Jun N-Terminal Kinase-1) (36,50-54), cytochrome CYP2E1 (55), and SOCS (56). The latter proteins interfere in insulin signal transmission, as they preclude IRS-1 and IRS-2 from coming into contact with insulin receptor (57) or induce proteasomal degradation for these substrates (58). Their overexpression in the liver induces insulin resistance and increased SREBP, which in turn originates steatosis (48). The role of *liver steatosis* in the pathogenesis of insulin resistance is supported by a number of observations. In the course of liver steatosis of any origin, insulin resistance also develops in a secondary manner. For instance, insulin resistance commonly develops in lipodystrophies (59,60), disturbed mitochondrial β-oxidation (61), or VLDL secretion defects (62). Similarly, the feeding of rats with fat-rich diets induces hepatic insulin resistance (47). In lipodystrophies, subcutaneous and visceral fat is mobilized, hypertriglyceridemia develops, and fat deposition in the liver occurs. On the other hand, mice lacking fat tissue develop severe liver and muscle steatosis, inability to activate PI3K through IRS-2, and hepatic insulin resistance (63). We also have a number of studies demonstrating that insulin resistance correlates with liver fat deposition (64). Both *FFAs* and *TNFα* are likely to interfere in the transmission of insulin-generated signals on inducing IRS-1 phosphorylation in serine 307 –rather than tyrosine (65-68). Phosphorylation in this serine is incompatible with simultaneous phosphorylation in tyrosine. Both TNFα and FFAs possibly bring about this phosphoryla-

tion following JNK1 *(Jun-N-terminal Kinase-1)* activation (65,69,70). JNK1 overactivation has been demonstrated in mice with NASH (71). NFκB release secondary to IKK-β activation has been involved in the pathogenesis of oxidative stress-induced insulin resistance (72).

As previously mentioned, huge amounts of FFAs are released into the circulation as a result of insulin resistance-associated lipolysis. Abdominal fat lipolysis is particularly important in the pathogenesis of NAFLD (73). Thus, for instance, almost two thirds of liver fat deposits in NAFLD have been seen to derive from circulating FFAs (74), and the severity of liver steatosis has been shown to correlate with visceral fat tissue rather than subcutaneous or peripheral fat tissue values (75). Removal of subcutaneous fat by liposuction solves none of NAFLD-related metabolic disorders (76). Indeed, insulin resistance, peripheral adiponectin, TNFα, IL-6, CRP, insulin, glucose, etc. remain all unchanged following such fat removal. In contrast, a reduction of visceral fat improves insulin resistance and other metabolic disturbances associated with NAFLD (77). Visceral fat has been shown to be particularly resistant to insulin activity (78), and is thus more easily hydrolyzed. In addition, based on its strategic position in the circulation of portal blood, the liver directly receives FFAs released during abdominal fat lipolysis. Fatty acid and glycerol plasma concentrations in patients and animals with NAFLD are strongly increased, and insulin can be seen to have a reduced capability in blocking the release of such lipolysis-derived products (79).

FFAs arriving in the liver activate nuclear receptor PPARα, which by forming a heterodimer with RXR (Retinoid X Receptor) induces the transcription of numerous genes involved in fatty acid catabolism and clearance (acyl-CoA oxidase, cytochrome P_{450}, fatty acid-binding protein, microsomal triglyceride transfer protein, apolipoprotein B100, etc.) (80-82) (Fig. 2). Specifically, these proteins play a role in FFA utilization, triglyceride (steatosis) and phospholipid synthesis, glyconeogenesis (hyperglycemia), or oxidation in mitochondria, peroxisomes, or microsomes. These three oxidation types are highly significant, as they may contribute to the cell's oxidative stress. *β-oxidation in mitochondria* may lead to ROS *(Reactive Oxygen Species)* formation, mainly superoxide anions (O_2^-), during oxidative phosphorylation (83). *β-oxidation in peroxisomes* leads to hydrogen peroxide formation, whereas oxidation in microsomes –with the involvement of cytochrome P_{450}– determines the for-

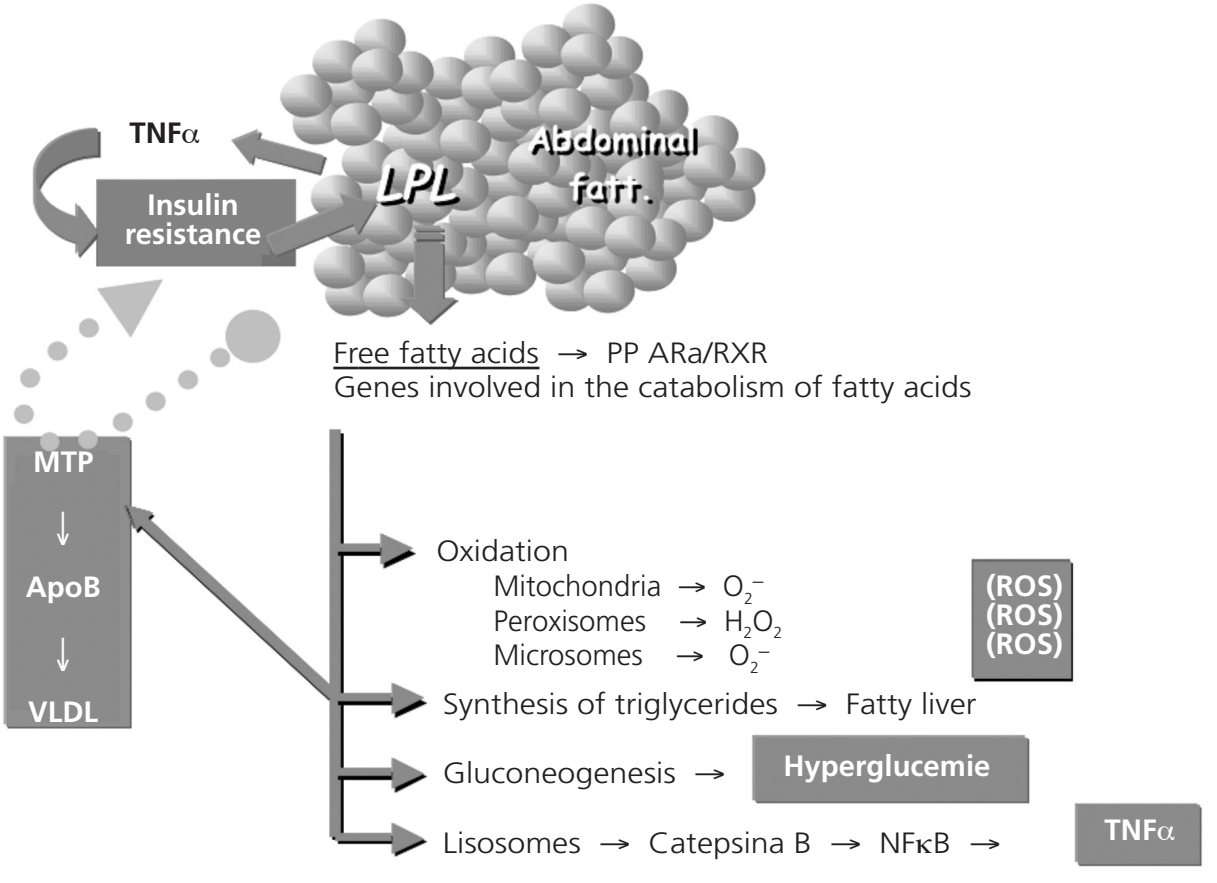

Fig. 2.- Consequences of increased free fatty acid uptake by the liver.
Consecuencias del aumento de la llegada al hígado de ácidos grasos libres.

mation of superoxide anions and dicarboxylic acids. Triglyceride buildup in liver cells would result from liver FFA uptake in amounts greater than those that may be used or exported into the blood as VLDLs. To this day no altered incorporation of FFAs into triglycerides, phospholipids, or cholesterol esters has been demonstrated in patients with NAFLD (79). On the contrary, some studies have shown that triglyceride exports as VLDLs are reduced in patients with NAFLD due to their lower incorporation into apolipoprotein B100 (84,85). Polymorphisms in the MTP (Microsomal Triglyceride Transfer Protein) promoter have been seen in these patients that may explain such lipid export defect (86,87). MTP incorporates triglycerides into apolipoprotein B in the endoplasmic reticulum and Golgi apparatus, thus giving rise to VLDL formation and facilitated lipid release from liver cells (88-90). When MTP activity is reduced, lipid export from hepatocytes decreases, cells retain their lipids, and liver steatosis ensues. Liver steatosis is a common occurrence in diseases with MTP mutations (91). In chronic HCV infection (mainly genotype 3), commonly associated with NAFLD, liver MTP activity is significantly decreased (92). Therefore, NAFLD's steatosis would on the one hand result from greater FFA uptake by the liver as a result of insulin resistance-derived lipolysis, and on the other hand from disturbed triglyceride export into the circulation as VLDLs (Fig. 2).

FROM STEATOSIS TO NON-ALCOHOLIC STEATOHEPATITIS

Oxidative stress

If insulin resistance plays a fundamental role in the pathogenesis of fatty liver, then oxidative stress is probably pivotal in the evolution from steatosis to NASH and the more advanced lesions of NAFLD. It has been posited that NASH would result from two aggressions. The first one would be represented by fatty liver; the second by oxidative stress (93,94). We have plenty of evidence suggesting that oxidative stress is present in NAFLD. Patients and animals with this lesion have increased liver levels of malonic aldehyde (MDA) (95,96), 4-hydroxynonenal (4-HNE) (97), 3-tyrosine nitrated proteins (79,95,96), and 8-hydroxydeoxyguanosine (97,98), all of them markers for lipid, protein, and DNA oxidative lesion, respectively. Furthermore, blood thioredoxin levels, another oxidative stress marker, are elevated in NASH (99), while those of antioxidizing factors are decreased (96,100,101). Genes coding for most antioxidizing factors have an ARE *(Antioxidant-Response Element)* in common that responds to transcription factors Nrf1 and Nrf2 *(Nuclear factor erythroid 2-related factor).* These two factors act as heterodimers, and make up complexes with Small-Maf and other bZIF proteins (102). Nrfs factors are normally sequestered in the cytoplasm (103). When a cell suffers from oxidative stress, Nrfs factors are translocated into the nucleus, bind AREs in antioxidizing genes, and induce their expression (104,105). The relevance of these antioxidizing factors in the pathogenesis of NAFLD is supported by studies in $Nrf1^{-/-}$ mice, which lack Nrf1 and develop a decreased expression of genes with AREs, steatosis, necrosis, apoptosis, liver inflammation, and pericellular and pericentral fibrosis, in addition to oxidative stress (106). Consistent with this is the finding that antioxidizing *(Glutathione S Transferase)* gene expression is diminished in patients with NAFLD (107).

The *consequences of oxidative stress* on cells are manifold. They induce cell membrane lipid peroxidation, and cell degeneration and necrosis, cell death by *apoptosis* (108,109), *proinflammatory cytokine* expression, liver stellate cell activation, and fibrogenesis (93,94,110).

Source of oxidative stress

The role of mitochondria

While the source of oxidative stress in NASH is probably multiple (fatty acid oxidation, microsomal cytochromes, siderosis, cytokines, Kupffer cells, etc.), mitochondrial dysfunction seems to play a predominant role. Mitochondria are involved in both FFA β-oxidation and ROS generation (83,111-114). Several studies have shown that mitochondria in patients with NASH are abnormal from both a morphologic and a functional perspective. In these patients mitochondria are big, swollen, with scarce criptae, and usually with paracrystalline inclusions (79,115). These changes are very similar to those found in mitochondrial myopathies arising from disturbances in the mitochondrial respiratory chain (MRC) (116). In addition, $[^{13}C]CO_2$ generation from ^{13}C-methionine and ATP resynthesis after fructose overload are severely reduced in patients with liver steatosis (117,118). Both problems suggest that mitochondrial function, in addition to mitochondrial morphology, is altered in patients with NASH.

Mitochondria are the primary site for FFA β-oxidation. A number of steps may be distinguished in this process (83,119):

(α) *FFA uptake by mitochondria.* An enzyme, CPT-1 *(Carnitine Palmitoyl Transferase-I)* and a translocase play a role in this process, and long-chain fatty acids must be previously bound to carnitine. Once the fatty acid has entered the mitochondria and is found in the mitochondrial matrix, carnitine is released back into the cytoplasm. Carnitine depletion (120-122), CPT-I deficiency (123), or a defective translocase may alter fatty acid uptake by mitochondria, and prevent their β-oxidation. This may contribute to fatty acid retention in the cytoplasm, and their subsequent re-esterification in triglycerides (Fig. 3).

In a study by ourselves in patients with NASH we found normal intrahepatic levels of both free and total

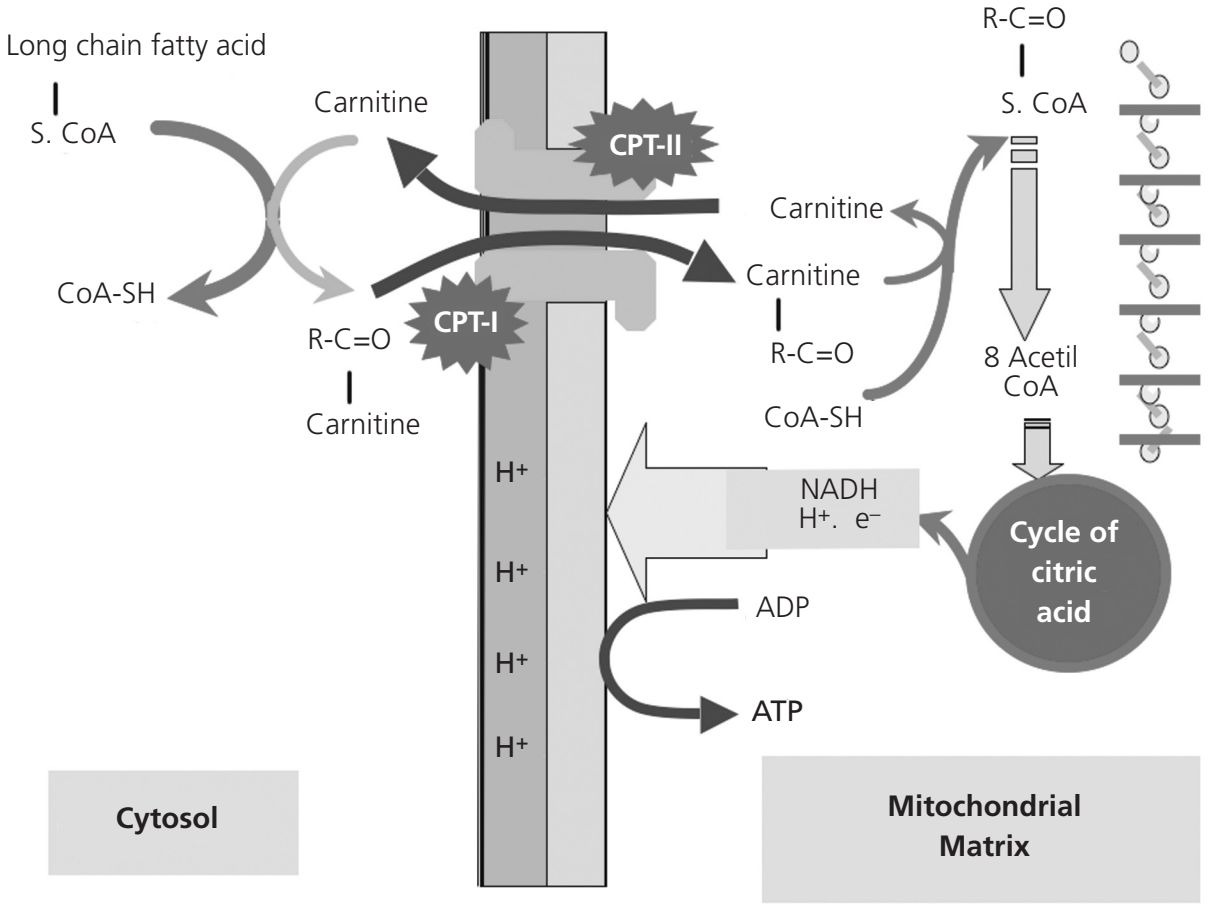

Fig. 3.- Mitochondrial metabolism of fatty acids.
Metabolismo mitocondrial de los ácidos grasos.

carnitine (124), which was consistent with other authors' findings in obese and alcoholic patients with fatty liver (122, 125); similarly, the measurement of CPT-I activity in the liver of patients with NASH revealed normal values (124), and hence we may not attribute cytoplasmic triglyceride build up to FFAs not entering the mitochondria.

(β) The second step in this *mitochondrial fatty acid oxidation* process includes a range of successive β-oxidations leading to acetyl-CoA and short-chain fatty acids-CoA formation, and NAD^+ to NADH conversion (126). Few studies have measured fatty acid β-oxidation in NAFLD. With indirect methods fatty acid β-oxidation has been presumed to be increased in these patients (79, 127). By directly measuring mitochondrial (palmitic acid) and peroxisomal (lignoceric acid) β-oxidation in *ob/ob* mice with NAFLD and NASH lesions we found that oxidation was significantly increased for both fatty acids (95). These results are consistent with the findings by Diehl's team in this same type of mice (128,129). Such β-oxidation increase has been attributed to insulin resistance, and hence to increased lipolysis and FFA uptake in the liver (41,79). FFAs play a role in the activation of transcription factor PPARα (Peroxisome Proliferator-Activated Receptor α), which in turn activates the expression of genes involved in fatty acid β-oxidation (130,131) (Fig. 3).

(χ) NADH resulting from β-oxidation is re-oxidized to NAD^+ in a process designated *oxidative phosphorylation,* which leads to ATP formation. The latter represents the only energy source that may be used by cells. This phosphorylation includes a number of enzyme complexes located at the inner mitochondrial membrane (complexes I to V), which are designated the *mitochondrial respiratory chain* (MRC). In this chain NAD^+ and $FADH_2$ electrons pass from one complex to the next, and eventually combine with oxygen and protons to form water. This process is coupled with another concomitant one where mitochondrial matrix protons are sent to the intermembrane space of mitochondria, thus generating an electro-

chemical gradient between the matrix and this space. When these protons go back to the mitochondrial matrix via ATP synthase (complex V), they determine the conversion of ADP into ATP, and hence the electrochemical energy built up in the intermembrane space is used in the formation of cell-usable energy (83,119,132). Along this oxidative phosphorylation process some electrons usually escape, and give rise to ROS –mainly O_2^-– formation after binding mitochondrial matrix oxygen (133,134). When oxidative phosphorylation is deficient due to low MRC activity, not only ATP formation decreases, but electrons escaping the system increase and ROS formation is higher (126) (Fig. 4). As is the case with NASH, such ROS formation would be enhanced when liver FFA uptake and β-oxidation are increased. It is also enhanced in diabetes mellitus, where glucose oxidation represents a significant provision of electrons to MRC.

Information available on the function of oxidative phosphorylation and MRC in patients with NASH is very limited. Caldwell et al. (115) found that MRC complex I and III activity was normal in platelet mitochondria from patients with NASH, and Sanyal et al. (79) found no defects in this chain's enzyme expression when studying muscle tissue from a patient with NASH. We have directly studied the activity of all MRC enzyme complexes in the liver of patients with NASH (124). In this study we were first to demonstrate that these complexes' activity was reduced by 30 to 50% *versus* control activity. This defect compromises both complexes with mitochondrial gene-encoded (complexes I, III, IV, V) and nuclear gene-encoded (complex II) components. Consistent with these findings were the reports by Haque et al. (135), who published that cytochrome c oxidase –one of MRC complexes– activity was reduced. While the cause of these enzymatic defects remained unexplained, we saw that these complexes' activity was inversely correlated to blood TNFα levels, body mass index, and HOMA index to assess insulin resistance (124).

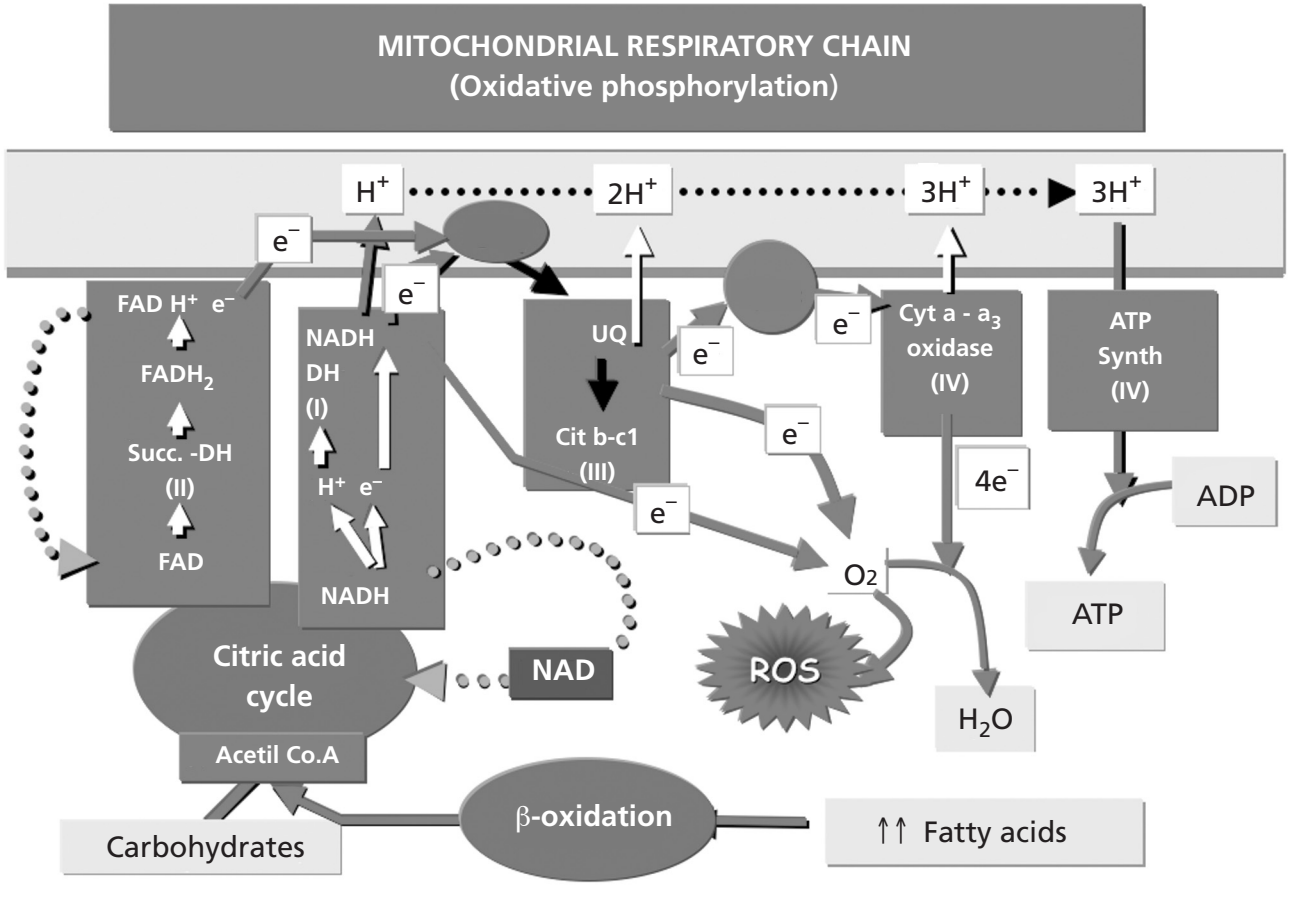

Fig. 4.- Mitochondrial respiratory chain and oxidative phosphorylation. Succ-DH: succinate dehydrogenase; NADH-DH: NADH-dehydrogenase; UQ: ubiquinone: Cyt: cytochrome; Synth: synthase; ROS: reactive oxygen species.
Cadena respiratoria mitocondrial y fosforilación oxidativa. Succ-DH: succinato deshidrogenasa; NADH-DH: NADH-deshidrogenasa; UQ: ubiquinona; Cyt: citocromo; Synth: sintetasa; ROS: sustancias reactivas derivadas del oxígeno.

In order to gain a deeper insight in the study of factors potentially responsible for MRC hypofunction, we used an animal NAFLD model that reproduces many of the disturbances commonly seen in humans. Mice of the *ob/ob* ($Lep^{-/-}$) type have their leptin gene neutralized, and thus lack this hormone; as a result they experience polyfagia and weight gain, and develop insulin resistance, hyperglycemia, and hyperlipemia (136). Histological, the liver of animals studied by us had steatosis in 42% of hepatocytes, as well as hydropic degeneration, Mallory hyaline, and inflammatory infiltrates. That is, these animals met histological criteria for NASH. The study of MRC activity in these mice showed the presence of a defect similar to that found in patients with NASH. MRC enzyme activity was reduced by 40 to 60% *versus* healthy animals (137). Hence, these *ob/ob* mice seem to represent a fine experimental model to research the etiopathogenesis of mitochondrial dysfunction as found in patients with NAFLD.

MRC dysfunction as found in these mice allows to predict that both electron escape and ROS formation are likely increased in them (138). Indeed, the measurement of substances reacting with thiobarbituric acid (TBARS), a marker of oxidative stress, showed highly elevated levels. These findings are consistent with those already mentioned suggesting the presence of oxidative stress in the liver of patients with NASH (79,96,128,139-141).

Mitochondrial dysfunction mechanisms

Mechanisms potentially involved in mitochondrial dysfunction either in patients with NAFLD or *ob/ob* mice are varied. One may well be *oxidative stress* itself. MDA and 4-HNE, two products resulting from cell lipid peroxidation, are known to inhibit cytochrome c oxidase (MRC complex IV) activity after making up a number of conjugates with this complex's peptics (142,143). Furthermore, ROS may damage both mitochondrial DNA (mtDNA) (144,145) and mitochondrial iron-sulfur cluster enzymes (138), thus leading to MRC hypofunction (146). Such mitochondrial DNA (mtDNA) lesions, which are difficult to repair in mitochondria (147), should impact the expression of complexes I, III, IV, and V in this chain, as mtDNA codes for 13 polypeptides making up these complexes. In accordance with this, Haqué et al. (135) found that patients with NASH had mtDNA depletion. The presence of oxidative stress in cells may initiate a series of vicious circles contributing to increase mtDNA damage, and to induce a greater mitochondrial disturbance (132,148).

Despite such evidence, findings in our studies with *ob/ob* mice do not support the role of oxidative stress as the causal factor for mitochondrial dysfunction. In effect, treating these animals with N-acetyl-cysteine (NAC) via the peritoneal route for 3 months markedly reduced liver TBARS concentration, but could not improve MRC complex activity or liver histological lesions (95). NAC inability to improve NAFLD histology has been reported also by other authors (96). These results, together with the fact that MRC complex II activity –with components not encoded in mtDNA– is also diminished in NAFLD and *ob/ob* mice, render the role of oxidative stress in the pathogenesis of this mitochondrial defect uncertain. Nevertheless, it is essential that experiments are repeated using antioxidants preferentially acting on mitochondria –e.g., superoxide dismutase analogues– before definitely excluding the role of oxidative stress (149).

Another important factor to consider in the pathogenesis of mitochondrial dysfunction is TNFα. There is strong evidence available advocating for the role of this cytokine in the pathogenesis of NASH (150,151). High blood TNFα levels have been found in patients with NASH (124,152-155), and we found that reduced MRC activity correlated with increased blood TNFα (124). In *ob/ob* mice we saw that TNFα concentrations in liver tissue were some 20-fold higher than in normal mice (137). In a previous study we demonstrated that treating cells with TNFα increases ROS, decreases messenger RNA for some ATPase components, and reduces the number of peptides making up ATPase and cytochrome c oxidase (156). The source of this hepatic TNFα is likely not one, since fat tissue, as well as hepatocytes and Kupffer cells may produce TNFα (150,157,158). Abdominal fat tissue may be a significant source for liver TNFα, as its passage through the liver is mandatory. In obese subjects fat tissue is infiltrated by macrophages (159,160), which may release TNFα besides adipocytes themselves (161). Preadipocytes exhibit some antimicrobial and phagocytic properties, just as macrophages do, and may also potentially transdifferentiate themselves into macrophages (162). Potential stimuli for TNFα release are varied (adipocyte cytokines, lipoperoxide phagocytosis, endotoxins). Furthermore, FFAs released during abdominal fat lipolysis may themselves induce TNFα expression both in the adipose tissue (163) and hepatocytes (164). This effect would occur via NFκB activation. FFAs would give rise to Bax translocation into lysosomes, and facilitate cathepsin B release to the cytoplasm, which would –via IKKβ– activate NFκB (165).

Increased TNFα production would be a part of the *chronic liver inflammation* status that is present in liver steatosis. As a result of oxidative stress and FFA liver uptake Kupffer cells, IKK-β, and NFκB would become activated (165, 166). This transcription factor increases gene expression for TNFα, TGFβ, IL-8, IL-6, and IL-1β, among other factors. These may reproduce many of the histological changes usually found in NAFLD. For example, IL-8 induces neutrophil chemotaxis, TNFα, hepatocyte necrosis/apoptosis, TGFβ, stellate cell activation, liver fibrosis, and Mallory body formation.

Biological effects by TNFα may be antagonized by *adiponectin* (167). This is an adipocyte-produced hormone with antilipogenic effects that inhibits fat from

building up in the liver and other non-fat tissues, and hence prevents NAFLD, NASH, and liver inflammation and fibrosis development (168-171). The administration of recombinant adiponectin to *ob/ob* mice reduces hepatomegaly, fatty acid synthesis, and inflammation, while increasing fatty acid oxidation at the same time (168). Mice producing no adiponectin develop severe fibrosis following exposure to Cl_4C, but this effect may be avoided if mice are infected with an adiponectin-expressing adenovirus (169). The antiinflammatory effect is probably exerted through a number of mechanisms, including reduced TNFα production by fat-tissue macrophages (167,172), NFκB pathway inhibition via AMPc (173), and inhibited macrophage activation (174). Decreased steatosis results from PPARα and cAMP-dependent kinase activation. This increases fatty acid oxidation and lipid export, and decreases lipogenesis (175). Via this same pathway, adiponectin enhances insulin sensitivity (176-179), and may revert many NAFLD-related disturbances. Decreased fibrosis is mediated by its antiproliferating and apoptotic effects on liver stellate cells (171). In metabolic syndrome, including NAFLD, blood adiponectin levels are decreased (153,180-182), which relates to central fat extent, increased liver steatosis, and liver insulin resistance (183,184). In contrast with other adipokines, circulating adiponectin levels are lower in obese subjects, particularly in visceral obesity. When visceral fat is diminished by losing weight, circulating adiponectin –and adiponectin mRNA– levels significantly increase in fat tissue (162). These changes are concurrent with and opposed to those experienced by TNFα and IL-6. These two cytokines inhibit adiponectin mRNA expression.

In a previous study, we found proof of TNFα's negative effect on mitochondria. This cytokine induced relevant morphologic and functional changes on mitochondria. After incubating cells with TNFα for 8 hours, mitochondria swelled, became rounded, lost their septa, lightened their matrix, and broke their external membrane (185). In addition, our study revealed that TNFα may interfere with electron flow in MRC complexes I and III (185,186). This cytokine determines electron retention in cytochrome *b,* so the latter may donate such retained electrons to oxygen so that superoxide anions are formed (185). In fact, many NAFLD-related disorders may be explained by TNFα's biological effects, as this factor not only induces MRC dysfunction, but also increases cell resistance to insulin, induces the expression of several proinflammatory cytokines and enzymes (including iNOS *[Inducible Nitric Oxide Synthase],* and brings about cell death by apoptosis or necrosis, among other things.

The critical role of TNFα in the pathogenesis of NAFLD and mitochondrial dysfunction is supported by results obtained in *ob/ob* mice treated for 3 months with anti-TNFα (infliximab) through the peritoneal route. While this therapy was insufficient to fully normalize TNFα levels in liver tissue, it did suffice to strikingly normalize or improve complex I, II, III, and V activity, to decrease β-oxidation activity, and to regress liver histology almost to normal (95). The effect we observed on β-oxidation has also been seen by Li et al. (128), and may be attributed to TNFα actions on insulin sensitivity (99, 187), oxidative stress (99), and stearoyl-CoA desaturase (128), an enzyme involved in fatty acid synthesis (188).

Simultaneous improvement of mitochondrial dysfunction and histological lesions after therapy with anti-TNFα antibodies allows to advocate for a role of TNFα in the pathogenesis of both disorders, and suggest that mitochondrial defects may well participate in lesion development.

The multiple biological effects of TNFα include iNOS expression induction (189), particularly when its activity is combined with that of IL-1β, IFNγ, and endotoxin (190). This enzyme catalyzes L-arginine oxidation in the presence of oxygen to give nitric oxide (NO). A normal liver expresses only endothelial NOS. However, under given circumstances –for example, under the effects of TNFα– liver iNOS expression strikingly increases, and the liver generates great amounts of NO (191). This TNFα effect is mediated by transcription factor NFκB (192), whose activity is greatly increased in o*b/ob* mice (128). In our study we found that the liver of these mice had, in addition to significantly increased levels of TNFα, also a marked induction of mitochondrial iNOS. Such enzymatic induction is no doubt dependent upon TNFα, as its expression dramatically decreased in obese mice treated with anti-TNFα, and approached control levels. The study by Laurent et al. also supports a non-activation of the iNOS pathway in *ob/ob* mice (128), as very high nitrite, nitrate, and 3-tyrosine nitrated protein concentrations were found in the liver.

These findings may have pathogenic implications, since NO and other nitrogen-derived reagents may alter both mitochondrial and MRC function (193). Indeed, NO reacts with cytochrome c oxidase *(complex IV),* interrupts electron passage, and blocks their binding of oxygen (194). On the other hand, peroxynitrite ($ONOO^-$), a product resulting from NO reaction with O_2^-, is an activity inhibitor for various proteins, including some MRC components (195,196). *In vitro* studies have shown that peroxynitrite may inactivate complexes I, II, V, cytochrome *c* (196-198), and also complex III under selected circumstances (199). Mechanisms through which peroxynitrite exerts these effects are varied and include its oxidative potential (200), and its ability to damage DNA (201), nitrate protein tyrosine residues, and generate 3-tyrosine nitrated proteins (202). The presence of 3-tyrosine nitrated proteins in tissues is a marker of tissue aggression by peroxynitrite radicals (203). Hence, we searched for such proteins in the liver of *ob/ob* mice.

Using immunofluorescence techniques we found that 3-tyrosine nitrated proteins was largely increased in obese mice when compared to control mice. These find-

ings suggest that liver proteins in obese mice have been damaged by peroxynitrite radicals or derivatives. To gain a deeper insight on the origin of 3-tyrosine nitrated proteins, we looked for these proteins in a mitochondrial protein extract. Also in this case we saw that proteins in these organelles had been damaged by peroxynitrite. Moreover, after immunoprecipitating these proteins with anti-3-nitrotyrosine, we observed that a number of MRC components, at least cytochrome c and protein ND4, a component of complex I, had been 3-tyrosine nitrated.

Considering that MRC enzyme nitration is associated with a decrease in their catalytic activity (204), such nitration is likely to have been responsible for their low enzyme activity. In order to assess the role of peroxynitrite and reactive derivatives (205) in the pathogenesis of this disorder, we treated *ob/ob* mice with uric acid through the intraperitoneal route for three months. This acid rapidly reacts with peroxynitrite to form inactive nitrogenous urates (205,206). Therefore, uric acid is considered a natural neutralizer for peroxynitrite (205,207) and reactive derivatives (205,206). Treating mice with uric acid has been shown to reduce 3-tyrosine nitrated protein formation (205,206), and to prevent neurologic lesion progression in multiple sclerosis experimental model (203,208). Uric acid therapy effects in *ob/ob* mice were dramatic, as liver lipoperoxide and 3-tyrosine nitrated protein levels decreased, specifically decreasing cytochrome *c* and MRC ND4 peptide 3-tyrosine nitration. These effects were associated with a normalization of MRC complexes I and V activity, and a marked improvement of complexes II and III. Finally, this therapy led to the regression of liver lesions, and a recovery of the liver structure's normal appearance. Uric acid effects on liver lesions support the role of peroxynitrite not only in MRC dysfunction, but also in the pathogenesis of lesions.

Results from our studies prompt us to suggest that liver TNFα –probably from abdominal fat tissue or enhanced expression in hepatocytes by FFAs– induces iNOS, and hence a greater formation of NO. This radical, when in the presence of O_2^-, originates peroxynitrite radicals, which would bind MRC proteins and determine a decrease in their activity. Similar effects to those reported with uric acid have been seen when *ob/ob* mice were treated with MnTBAP *(Manganese [III] 5,10,15,20 Benzoic Acid Porphyrin),* an analogue of Mn superoxide dismutase (MnSOD) that turns O_2^- into H_2O_2 (96). Besides a reduction in oxidative phosphorylation and ATP formation, decreased MRC enzyme activity increases the number of electrons escaping the system; these electrons bind oxygen and then give rise to ROS formation. Such electron leak would be particularly high in situations where FFA provision to the liver for mitochondrial oxidation –as is the case with NAFLD– is elevated. This would be the origin of higher lipoperoxide levels as found in the liver of these obese mice.

Other sources of oxidative stress

While mitochondrial dysfunction plays a predominant role in ROS generation, ROS may also come from FFA oxidation in peroxysomes and microsomes, and from Kupffer cell activation. In the obese and in patients with NASH, *CYP2E1* activity is increased (209-213), likely induced by FFAs or ketones (214). This microsomal enzyme, besides taking part in the degradation of xenobiotics, induces FFA ω-oxidation (215), during which ROS are generated (216,217). The real significance of ROS from this source in the pathogenesis of human NASH remains to be demonstrated. *Kupffer cells* may also generate ROS via the NADPH-oxidase system (218). In experimental NASH models these cells have been shown to be activated, and to possess a high number of endotoxin receptors (166,219,220). Various factors may play a role in these cells' activation. One would be lipoperoxide phagocytosis; another, the phagocytosis of endotoxins from the intestine (219).

Consequences of oxidative stress

ROS may induce *lipid peroxidation,* particularly for unsaturated fatty acids in cell membranes. The impact of such aggression is manifold.

1. On the one hand it has an impact on membrane *physico-chemical properties,* which in turn has an impact on membrane receptor and enzyme activity, antigen expression, intercellular interactions (221-223), and *membrane permeability.* Changes may occur that compromise cell viability (passage of calcium into cells) (224) and condition cell death through *necrosis* as a result of the latter.

2. A lesion characteristic of NASH is *liver fibrosis.* It initially has a pericellular and pericentral distribution in Rapapport's lobule area 3, but in advanced stages alters lobule architecture and takes on the pattern of *micronodular cirrhosis.* Cells primarily involved in the production of such fibrosis include *liver stellate cells* (LSCs). In a normal liver LSCs are in a latent state, and cannot produce extracellular matrix components. When the liver is damaged these cells become activated, change their morphology and function, and synthesize the various components of extracellular matrix (225-227). In human and experimental NASH, these cells have been shown to be activated and in greatly increased numbers (228-230). Mechanisms conducing to liver fibrosis in NASH are probably multiple.

α) *Oxidative stress* may induce LSC activation, and hence help stimulate liver fibrogenesis. This effect may occur following the activation of transcriptional factors NFκB and c-Myb. ROS may lead to IκB degradation in the cytoplasm, which conditions the release of transcription factor NFκB and its nuclear translocation (231). This effect would follow IKK (IκB Kinase) activation and IκB

phosphorylation. In activated LSCs NFκB activity is increased and NFκB p50/p65 heterodimer may be found in the nucleus. Similarly, oxidative stress may induce gene expression of factor c-Myb and its binding to DNA (232). This transcription factor may play a role in the expression of smooth muscle actin, and in LSC contractility, differentiation, and proliferation (233). These cells may become activated by the phagocytosis of *apoptotic bodies* resulting from hepatocyte death (234).

β) In addition, lipid peroxidation-derived *reactive aldehydes,* including MDA and 4-HNE, may take part in liver fibrogenesis. Indeed, Chojkier et al. (235) showed that MDA significantly increased the expression of messenger RNA (mRNA) for collagen α1(I) in human fibroblast cultures. Maher et al. (236,237) found that collagen synthesis doubled up when fibroblasts were cultured with MDA. Findings consistent with these were reported by other investigators (238-241). While mechanisms of this effect are not unique, conjugates made up of reactive aldehydes with protein amino acids or sulphydryl radicals are likely to play a role (242). Such conjugate formation has been demonstrated in animal models with lipid peroxidation induction, and in a number of clinical circumstances with active fibrogenesis (241,243-247). On the other hand, antioxidant therapies decrease the formation of such conjugates, and prevent fibrogenesis (241,246,248). In a previous study we found evidence that aldehyde conjugates are involved in increased collagen expression (110,249.), since treating cells with p-hydroximercuribenzoate (pHMB) or pyridoxal-5´-phosphato (P5P) abolished the effects of both MDA and an oxidizing combination (ferrous chloride, ascorbic acid, cytric acid) on collagen expression. In these studies we determined that these aldehydes exert their effects through elements located between sequences -116 and -110 pb in the collagen α_1 (I) promoter, and that transcription factors Sp1 and Sp3 act as mediators for this stimulus. These factors recognize G+C-rich sequences (250), and act as expression stimulating factors for a wide variety of genes, including the collagen α1(I) gene (250-254).

χ) In patients with insulin resistance and NASH, blood *leptin* levels are usually increased (255). Leptin is a 16 kDa peptide expressed by gene *obese* (Ob) (256) that is released by adipocytes and has varied metabolic effects, with the most significant of these being related to body weight and energy expenditure (257). TNFα is a major inducer (258). Together with metabolic effects, it has been seen to exert a powerful fibrogenic effect (259). Leptin-lacking *ob/ob* mice are particularly resistant to liver fibrosis development (260,261), but lose such resistance when exogenous leptin is administered (260). On the other hand, leptin administration enhances fibrosis as induced by other aggressions (262). High circulating leptin levels, which relate to fibrosis severity (266), have been found in patients with chronic hepatitis C, alcoholic liver disease, or NASH (255,263-265).

The mechanisms through which leptin exerts these fibrogenic effects require further study, but several have been mentioned. Some have found that leptin directly stimulates LSCs (259,267), and others that this effect would be indirectly exerted after inducing TGFβ *(Transforming Growth Factor-β)* release from Kupffer, stellate, and endothelial cells (259-261,268). Some have shown evidence that it may delay extracellular matrix degradation after increasing TIMP-1 expression (269), and others that it stimulates LSC proliferation (270) and inhibits LSC apoptosis (271). Finally, leptin may induce oxidative stress by acting on MRC (272,273). It might well activate the aforementioned fibrogenic mechanisms through such oxidative stress.

δ) Other fat tissue hormones may also behave in a fibrogenic manner. *Angiotensin II* and *norepinephrine* may act directly on LSCs and induce their activation (274, 275). *Osteopontin* may lead to this through its proinflammatory effects (276). Mice lacking osteopontin have been seen to be protected against liver inflammation and fibrosis when on a choline-methionine-deficient diet.

ε) *Steatosis* itself may be a fibrogenesis-stimulating factor. NAFLD may exhibit fibrosis in the absence of necroinflammatory activity (228-277), fibrosis extent as experimentally induced is influenced by dietary fat types (278).

ϕ) Another factor closely linked to obesity, liver steatosis, and the progression of liver disease –including chronic hepatitis C and NASH– is type-2 diabetes mellitus and insulin resistance (279,280). Type-2 diabetes includes peripheral insulin resistance, and high blood insulin levels, hence insulin may likely play some role in fibrosis progression. In fact, Hickman et al. found a significant association between blood insulin levels and increased fibrosis (281). Similarly, other authors have confirmed such association of insulin resistance with fibrosis severity in chronic hepatitis C (282,283). In this regard LSCs have been shown to possess insulin receptors, and thus this hormone may contribute to these cells' proliferation (284). This proliferative effect of insulin may take place by stimulating the MAPK *(MAP kinase)* pathway (285), which is closely related to cell growth. In addition, insulin increases TGFβ (286) and CTGF production (287).

γ) Finally, LSC and hence fibrogenesis activation may be an indirect consequence of hepatocyte *apoptosis*. This type of cell death yields apoptotic body formation –these bodies are phagocyted by macrophages or LSCs themselves, determine TGFβ release, and the latter activates LSCs (288,289).

3. NFκB activation, which brings about oxidative stress, may explain the *chronic inflammatory status* of the liver in NAFLD (165,166), as it induces the expression of genes for numerous proinflammatory factors, including TNFα (290), interleukins 2, 6 and 8, ICAM-1 (291), MCP-1 (292), MIP-2 (293), CINC *(Cytokine-Induced Neutrophil Chemoattractant)* (294), and several

proinflammatory enzymes (lipoxygenase, cyclooxygenase, iNOS) (231). At this point, TNFα, on activating NFκB, starts a new vicious circle that helps increase inflammation. Our studies (95), as those by Li et al. (128), show that treating *ob/ob* mice with anti-TNFα reverts or deletes liver infiltrates. On the other hand, the binding of reactive aldehydes (MDA, 4-HNE) to hepatocyte surface proteins may modify these proteins' antigenic structure and initiate an immune response contributing to the inflammatory response seen in patients with NASH (295).

4. While mechanisms leading to *Mallory hyaline* formation are little understood, reactive aldehydes resulting from oxidative stress and TGFβ are also likely involved. In effect, TGFβ may activate transglutaminase, and the latter may result in the formation of cytokeratin polymers by establishing transversal links between lysine molecules in some cytokeratin chains and glutamine molecules in other cytokeratin chains (296).

5. In NASH hepatocyte death results not only from necrosis but also from *apoptosis* (297-300). Several pathways and factors may lead to this programmed death, including oxidative stress itself, TNFα, and FFAs. (α) ROS increase the expression of Fas receptors in the surface of hepatocytes, and thus may induce death by apoptosis (297). This effects has been attributed to NFκB activation, as this factor may increase cell death receptor expression (301,302); however, NFκB mainly behaves as a cell survival factor by inducing the expression of various enzymes (Mn-SOD, iNOS) or multiple antiapoptotic factors (Mcl-1, cFLIP, IAPs, Bcl-XL, A1) (192,303). The binding of *Fas ligand* to *Fas receptor* initiates a cascade of events in which the binding of adapting protein FADD (Fas-Associated Death Domain) to Fas, caspase 8 activation with eventually caspase 3 activation (304), Bid *(BH3 interacting domain death)* cleavage (305), fragment tBid translocation to the outer mitochondrial membrane, and binding of Bak *(Bcl-2 antagonist/killer)* and Bax *(Bcl-2-associated X protein)* by this fragment –which induces these proteins' activation and increased mitochondrial membrane permeability– all play a role. In this way cytochrome *c* and other proapoptotic proteins leave the mitochondrial intermembrane space [Smac/DIABLO *(Second mitochondrial-derived activator of caspase/Direct IAP-binding protein with low pI);* AIF *(Apoptosis inducing factor),* endonuclease G] (306-309) and enter the cytoplasm. In the cytoplasm cytochrome c forms a complex with cytosol factor Apaf-1 *(Apoptotic protease activating factor-1),* ATP, and procaspase 9 (apoptosome), which leads to the latter's activation, and then to procaspase 3 activation (310-312). Caspase 3 starts cell degradation and death by apoptosis (313). This latter process includes DNA degradation, nuclear and cellular fragmentation, and *apoptotic body* formation. These bodies may undergo phagocytosis by macrophages and other neighboring cells, and become fully degradated in their lysosomes. Another consequence of cytochrome *c* leaving mitochondria is that it interferes with electron flow through MRC. As a result, ROS formation increases (314), and a new vicious cycle begins, which will worsen the disturbance.

Oxidative stress through NFκB activation may induce *TNFα* formation, and this factor may in turn induce apoptosis in hepatocytes (315,316). In fact, this cytokine may originate cell death both by apoptosis and necrosis, depending upon the cell's energy status, as apoptosis is an active process using up huge energy amounts (317). *TNFα* action follows a pathway partly similar to that of FasL, as on binding its receptor (TNFR-1) forms a complex (complex I) made up with TRADD (*TNF Receptor-Associated Protein with Death Domain),* RIP *(Receptor-Interacting Protein),* and TRAF-2 *(TNF Receptor-Associated Factor-2).* This complex initiates a survival pathway where NFκB, Bcl_{XL}, Mcl-1, Gadd45β, c-FLIP, IAPs, and A1 play a role (303,318,319). On the other hand, this molecular complex undergoes a number of changes and gives rise to another complex known as DISC *(Death-inducing signaling complex)* (complex II), which is bound by FADD. This leads to the activation of caspase 8, and the latter cleaves Bid to its truncated form, tBid, which permeabilizes mitochondria as was mentioned above (316). In addition, after the binding of TNFα to its receptor, sphingomyelinase becomes activated and generates ceramide from cell membrane-derived sphingomyelin (316). Ceramide induces cell apoptosis through various pathways, including its direct action on mitochondrial membrane pores, and inducing glutathione depletion (320,321). Furthermore, in previous studies in our laboratory (156) we showed that, at least partly, TNFα-related cytotoxicity is mediated by ROS.

To conclude, *fatty acids* may also play a relevant role in cell death. Evidence suggests that fatty acid accumulation in non-adipose cells is associated with cell dysfunction and death (322,323). This phenomenon has been designated *lipotoxicity*. This toxicity may contribute to the pathogenesis of various conditions. For instance, long-chain fatty acid deposition in pancreatic β cells or cardiomyocytes of diabetic rats induces death in these cells (324,325). The severity of myocardiopathy in diabetic patients has been seen to be related to the extent of myocardial triglyceride deposition (326). The mechanism through which triglyceride or FFA deposition results in these lesions or dysfunction is unknown. Fibroblasts and endothelial cells exposed to high long-chain saturated fatty acid concentrations reduce their proliferation and die (327). Death has been suggested to occur by apoptosis, which has been at least demonstrated in cardiomyocytes, pancreas β cells, and hematopoietic cells exposed to palmitic or stearic acid (328, 329). These proapoptotic effects would develop through at least two different mechanisms: a) by increasing lysosomal permeability, thus facilitating cathepsin B release, and favoring TNFα expression (164); b) by increasing mitochondrial permeability through JNK, thus facilitating cytochrome *c* release (330). Some authors have implicated ceramide as a second messenger for cell death. This mediator derives

from sphingomyelin hydrolysis in cell membranes, and is used by TNFα to induce cell apoptosis (331). As was mentioned above, ceramide favors mitochondrial pore aperture, but other cell targets such as increased nitric oxide (332,333), CAPK (Ceramide-Activated Protein Kinase), PKCζ (Protein Kinase Cζ), "CAPP (Ceramide-Activaded Protein Phosphatase), MAPK (Mitogen Activated Protein Kinase), JNK (c-Jun N-terminal Kinase), and NFκB (334,335) have been considered as well.

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