Hepatology Research and Clinical Developments

Hepatic Insulin Resistance and Nonalcoholic Fatty Liver Disease

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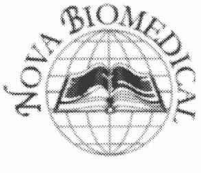

Nova Science Publishers, Inc.
New York

Library of Congress Cataloging-in-Publication Data

Hepatic insulin resistance and nonalcoholic fatty liver disease / Marcia Barbosa Aguila ... [et al.].
p. ; cm.
Includes index.
ISBN 978-1-61209-528-8 (softcover)
1. Fatty liver. 2. Insulin resistance. I. Aguila, Marcia Barbosa.
[DNLM: 1. Fatty Liver--etiology. 2. Fatty Liver--physiopathology. 3. Insulin Resistance--physiology. WI 700]
RC848.F3H47 2011
616.3'62--dc23

2011020036

Published by Nova Science Publishers, Inc. † New York

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Preface

Insulin resistance (IR) and metabolic syndrome are associated with the pathogenesis and progression of non-alcoholic fatty liver disease (NAFLD). It encompasses a spectrum of hepatic pathology, ranging from simple steatosis in its most benign form (fatty liver), going through nonalcoholic steatohepatitis (NASH) in its intermediate form, until cirrhosis and hepatocellular carcinoma, its most advanced forms. The metabolic pathways leading to the development of hepatic steatosis are multiple, including enhanced non-esterified fatty acid release from adipose tissue (lipolysis), increased *de novo* fatty acids synthesis (lipogenesis) and decreased beta-oxidation. Steatosis is characterized by intracellular triglyceride (TG) accumulation, which can be either macrovesicular or microvesicular, depending on the underline cause. TG *per se* is not hepatotoxic, but under chronic exposure the adaptive mechanisms protecting hepatocytes from fatty acid-mediated lipotoxicity become overwhelmed and rates of hepatocyte death begin to outstrip mechanisms that normally regenerate dead hepatocytes. This triggers repair responses involving activation of hepatic stellate cells to myofibroblasts, leading to excessive matrix synthesis, and excessive production of chemokines as well, which in turn attract various kinds of inflammatory cells to the liver. The intensity of this repair response generally parallel the degree of hepatocyte death, resulting in variable distortion of the hepatic architecture with fibrosis, infiltrating immune cells, and regenerating epithelial nodules. Thus, this chapter will approach: background of fatty acid oxidation; hepatic steatosis and its progression to NASH and cirrhosis; fibrosis and myofibroblastic cells differentiation; methods of diagnosis; clinical features and laboratory tests and, finally, rodent NAFLD and NASH models.

Contents

Introduction

Liver steatosis or fatty liver is histologically common in liver biopsies, referring to a histopathological condition characterized by excessive accumulation of lipid droplets within hepatocytes. *Nonalcoholic fatty liver disease* (NAFLD) is a clinical pathological term for a spectrum of structural findings ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) with progressive fibrosis, hepatocellular carcinoma and liver failure [1]. As it is considered as the hepatic manifestation of the metabolic syndrome [2], recent research reveals the fatty liver is essential for insulin resistance pathogenesis [3]. The term metabolic syndrome refers to health problems including obesity, glucose intolerance or diabetes, dyslipidemia and hypertension. Around 90% of NAFLD patients have at least one feature of metabolic syndrome and about 33% having the full diagnosis [4].

The NAFLD risk factors have been extensively investigated, however gender association is controversial: since earlier studies showed NAFLD was more frequent in women, but the contrary has been found in recent studies [5]. The most common symptoms include vague upper abdominal discomfort or right upper quadrant fullness, while the most common physical exams determine obesity and hepatomegaly. NAFLD is most common in the asymptomatic, nondrinking patient with mildly elevated transaminases (alanine aminotransferase [ALT] usually greater than aspartic aminotransferase [AST]). Besides obesity, numerous widespread ailments are linked to liver steatosis, such as alcohol consumption, diabetes, hyperlipidemia, insulin resistance, hepatitis C genotype 3, abetalipoproteinemia and medicine use [6-7]. NAFLD is the main cause of liver dysfunction and cirrhosis, with a 20-30% prevalence in the West, reaching

levels as high as 75-100% in obese (body mass index - BMI $\geq$ 30) and morbidly obese (BMI $\geq$ 40) patients [8-9]. Notably, this problem is not limited to adults, since nearly 10% of obese children have NAFLD [10].

The hepatocyte lipid content is regulated by the integrated activity of cellular enzymes catalyzing lipid uptake, synthesis, oxidation and export. Hepatic steatosis occurs when the "input" of fat into this system (either because of increased fatty acid delivery, hepatic fatty acid uptake, or fatty acid synthesis) exceeds the capacity for fatty acid oxidation or export (i.e., "output"). Hepatic fat storage is an important physiological function, but abnormal fat partitioning inside the liver causes hepatic steatosis, where normal lipid metabolic equilibrium is disrupted in the hepatocytes. Whereas NAFLD is characterized by macro- and micro-vesicular steatosis, NASH encloses macro-vesicular or a mix between micro- and macro-vesicular steatosis with mild lobular inflammation [4]. Evidence of progressive steatohepatitis is apparent with degenerative hepatocyte ballooning, formation of Mallory bodies, lobular neutrophilic inflammation and eventual perisinusoidal fibrosis [11].

Factors contributing to hepatocyte triglyceride accumulation and disrupting fatty acid oxidation are dealt with in this chapter. Also, the main mechanisms causing NAFLD, NASH and cirrhosis, including steatosis development, inflammation and myofibroblastic cell differentiation and fibrosis are discussed. Finally, current methodological strategies, as well as some experimental models, for determining these pathologies are surveyed.

Chapter I

Triglyceride Storage Regulation in Hepatocytes

The liver's central metabolic function is maintaining plasma glucose levels despite the nutritional state. Glucose is converted to fatty acid, being used for triglyceride synthesis — the primary source of energy storage and transport. In healthy hepatocytes, fatty acids are oxidized by enzymes in peroxisomes, mitochondria, and the endoplasmatic reticulum (microsomes) [12]. Considered the source of fatty acids, triglycerides are normally packaged into lipoproteins in the endoplasmatic reticulum, and then exported to adipose tissue for storage [13]. Molecular and physiological alterations in insulin resistance result in an excessive accumulation of triglycerides in the hepatocyte. Excessive liver fat accumulation results from increased fat delivery, increased fat synthesis, reduced fat oxidation and/or reduced fat export in the form of VLDL [14], with all these pathways being implicated in hepatic steatosis pathogenesis. Humans [15] and mice [16] with hepatic steatosis accumulate excessive oleic acid, the end-product of de novo fatty acid synthesis, suggesting increased fatty acid synthetic rates in the insulin-resistant liver.

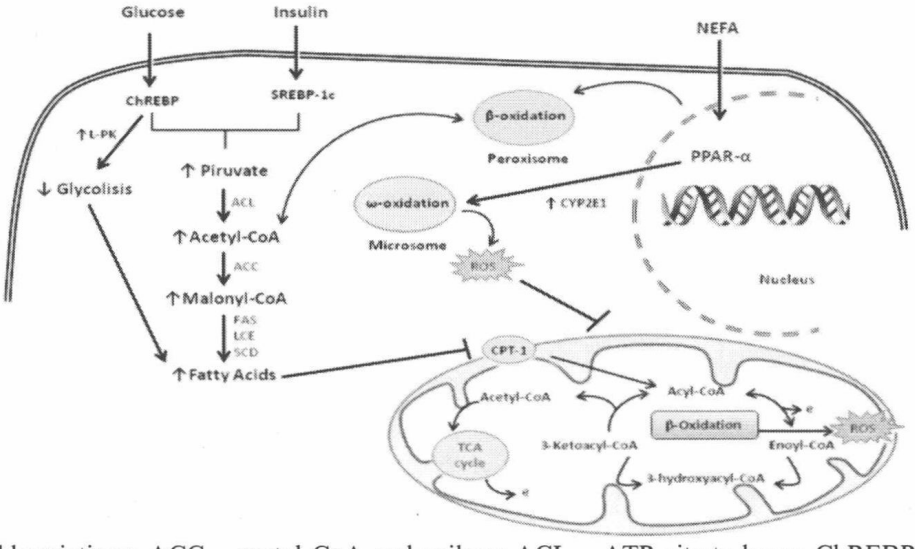
Hepatocyte accumulation of triglycerides is indicative of hepatic steatosis in NAFLD, although triglycerides are natural end products of fatty acid metabolism, they are not hepatotoxic and do not cause steatohepatitis [17]. Hepatocytes normally increase their triglyceride synthesis rates when energy intake exceeds use, and this energy excess greatly stimulates hepatocyte triglyceride synthesis in NAFLD [13]. However, storage of fatty acids, such as triglycerides, protect hepatocytes from the potentially noxious consequences

of fatty acid accumulation [18]. Dietary fatty acids are an important source which can be used to generate triglycerides, as well as fatty acids derived from lipolysis of adipose tissue triglyceride depots are delivered to the liver, taken up by hepatocytes, and also converted into triglycerides [19]. Figure 1 shows the events in the hepatocyte. Malonyl-CoA is synthesized in the liver during the first step of *de novo* fatty acid synthesis and malonyl-CoA levels demonstrate the organism's metabolic status reflecting the increases in circulating blood glucose and insulin due to feeding which promotes hepatic lipogenesis and fat storage via increased hepatic malonyl-CoA levels and inhibition of carnitin-palmitoylacyltransferase-1 (CPT-1) activity [20].

De novo lipogenesis is another factor contributing to the development of steatosis in NAFLD. This process is regulated by transcription factors activated by insulin, especially sterol regulatory element binding protein-1c (SREBP- 1c) [21]. In addition, glucose activates the nuclear transcription factor carbohydrate response element-binding protein (ChREBP), by upregulating the conversion of glucose into piruvate by increasing the expression of L-pyruvate (L-PK), a glycolysis rate limiting enzyme. ChREBP also increases transcription of the lipogenic enzyme acetyl CoA carboxylase (ACC) and fatty acid synthase genes (FAS) [22]. The transcription of these genes is also increased by the transcription factor SREBP-1c, which is upregulated by high insulin levels [23]. Therefore, both glucose and insulin, via ChREBP and SREBP-1c, respectively, synergistically promote the conversion of pyruvate into fatty acid. Ob/ob mice are characterized by hyperglycemia and hyperinsulinemia having a marked increase in ChREBP and SREBP-1c gene expression and nuclear localization. Indeed, liver-specific ChREBP inhibition in ob/ob mice diminishes hyperglycemia and hyperinsulinemia, by not only decreasing free fatty acid plasma concentration, but also the level of triglycerides [22]. In obese patients, the high insulin levels increase the SREBP-1c expression, which in turn increases the expression of all lipogenic enzymes, consequently increasing hepatic FFA synthesis [24]. In patients who are both obese and diabetic, high blood glucose levels further enhance hepatic lipogenesis by ChREBP activation, hence increasing the hepatic expression of all hepatic lipogenic genes, so further enhancing hepatic FFA synthesis [25].

Another transcription factor implicated in *de novo* fatty acid synthesis is peroxisome proliferators-activated receptor gamma (PPAR-gamma). PPAR-gamma is normally expressed in high levels in adipose tissue and very low levels in liver. Besides PPAR-gamma activity increases insulin sensitivity and fatty acid uptake, by promoting adipocyte differentiation and the expression of

proteins involved in fatty acid synthesis in adipose tissue, it also limits the fatty acid influx into the liver. Mice deficient in liver-specific PPAR-gamma are protected against the steatosis development, suggesting the hepatic PPAR-gamma role in liver fat accumulation [26].



Abbreviations: ACC – acetyl-CoA carboxilase; ACL – ATP citrate lyase; ChREBP - carbohydrate response element binding protein; CPT-1 – carnitine palmitoyl transferase; CYP2E1 - cytochrome P450 2E1; FAS – fatty acid synthase; LCE – long-chain fatty acid acyl elongase; L-PK - liver-type piruvate kinase; PPAR-α - peroxisome proliferator activated receptor-alpha; ROS – reactive oxygen species; SCD - stearoyl CoA desaturase; SREBP-1c - sterol regulatory element binding protein 1c; TCA cycle - tricarboxylic acid cycle.

Figure 1. Hepatic lipid impairment. *De novo* lipogenesis is induced by insulin and glucose via the transcription factors SREBP-1c and ChREBP, respectively. SREBP-1c leads to a transcriptional activation in all lipogenic genes. ChREBP activates L-PK, a glycolysis rate limiting enzyme and lipogenic genes. Therefore, both glucose and insulin promote the conversion of pyruvate into fatty acids. A consequence of increased fatty acids synthesis is the increased production of malonyl-CoA, which inhibit CPT-1, the protein responsible for fatty acids transportation into the mitochondria. Fatty acids not incorporated into triglyceride are degraded by oxidation being catalyzed by enzymes located within three cellular compartments: mitochondria, peroxisomes, and microsomes. Transcription of enzymes catalyzing fatty acid beta-oxidation in peroxisomes and omega-oxidation microsomes is regulated by the fatty acid-sensitive PPAR-α. Since mitochondrial damage occurs in NAFLD, the capacity for fatty acid oxidation in this organelle is limited, leading to increased peroxisomal (and microsomal) oxidation of fatty acids, which in turn generates ROS, a potential source of oxidant stress. Oversupply of fatty acids or impaired fatty acid oxidative capacity leads to accumulation of fatty acyl-CoAs in hepatocytes.

Chapter II

Impairment of Fatty Acid Oxidation

Fatty acids not incorporated into triglyceride are degraded by oxidation being catalyzed by enzymes located within three cellular compartments: mitochondria, peroxisomes, and microsomes. Transcription of enzymes catalyzing fatty acid beta-oxidation in peroxisomes and mitochondria is regulated by the fatty acid-sensitive peroxisome proliferator activated receptor-alpha (PPAR-α) [27]. Mitochondrial oxidation of fatty acids generates superoxide, adenosine triphosphate (ATP), ketone bodies, and acetyl CoA. Since mitochondrial damage occurs in NAFLD, the capacity for fatty acid oxidation in this organelle is limited, leading to increased peroxisomal (and microsomal) oxidation of fatty acids, which in turn generates hydrogen peroxide, a potential source of oxidant stress [28].

Reactive oxygen species (ROS) are also produced when fatty acids undergo omega-oxidation by cytochrome P450 enzyme within microsomes [29-30]. In addition, microsomial omega-oxidation of fatty acids generates dicarboxylic acids (DCA) uncoupling mitochondrial oxidative phosphorylation, thereby reducing the mitochondrial membrane potential [31]. This decreases the efficiency of mitochondrial ATP production, enhancing vulnerability to other dangerous molecules capable of promoting depolarization of mitochondrial membranes, including tumor necrosis factor-alpha (TNF-α), together with other proapoptotic signals [32]. DCA are also PPAR-alpha ligands amplifying expression of fatty acid oxidizing enzymes, thus reinforcing expression of microsomal fatty acid oxidizing enzymes, such

as cytochrome P450 2E1 (CYP2E1), and hence explaining why CYP2E1expression and other microsomal enzymes are increased in NAFLD.

Decreased lipid disposal by either beta-oxidation or very low-density lipoprotein (VLDL) export is partly responsible for a moderate fatty acid accumulation in the liver. The determining rate of mitochondrial beta-oxidation is the translocation of fatty acids into the mitochondria, regulated by carnitin-palmitoylacyltransferase-1 (CPT-1) [33]. CPT-1 catalyses the conversion of fatty acyl-CoAs into fatty acyl-carnitines enabling them to enter the mitochondrial matrix, giving the CPT-1 status of the main regulatory enzyme in mitochondrial fatty acid oxidation [34]. Increased the CPT-1 hepatic expression partly results from the PPAR-alpha activation by free fatty acids (FFA). In addition, PPAR-alpha modulates the expression of enzyme of mitochondrial and peroxisomal beta-oxidation [33]. As FFAs are esterified into triglycerides and then exported as VLDL, their failure in synthesizing and secreting VLDL further contributes to triglyceride accumulation in the liver [35].

Chapter III

NAFLD and Its Progression to NASH and Cirrhosis

The "two-hits" model of NASH pathogenesis has been proposed by Day and James [36], whereby a "first hit" (steatosis) increases the liver sensitivity to secondary insults and "second hits" such as oxidative stress, lipid peroxidation, mitochondrial dysfunction and increased cytokine production causing NASH.

Lipid metabolism by hepatocytes generates reactive oxygen species (ROS) within several intracellular compartments such as mitochondria, peroxisomes, and the endoplasmatic reticulum [29]. ROS generates in an environment enriched in lipids, in turn, inducing lipid peroxidation by releasing highly reactive aldehydic derivatives (e.g. malondialdehyde) and 4-hydroxynoneal (HNE). When they bind to hepatocyte proteins there is potentially harmful immune response from neoantigen formation, by cross-linking cytokeratins to form Mallory hyaline bodies or activating hepatic stellate cells to promote collagen synthesis, and neutrophil chemotaxis stimulation [37]. ROS produce hepatocellular injury through several mechanisms, including direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehydes-3-phosphate dehydrogenase, inhibition of membrane Na^+/K^+ adenosine triphosphatase activity, inactivation of membrane Na^+ channels, and other oxidative modifications in cellular proteins. ROS are potent triggers of DNA strand breakage, resulting in activation of the nuclear enzyme poly-adenosine 5'-diphosphate ribosyl synthetase, eventually bringing about severe energy depletion [38].

There are multiple potential sources for ROS generation; the hepatic CYP2E1, hepatic mitochondria, adipose tissue and iron overload being mentioned as some possibilities. Moreover, FFA can activate CYP2E1 and CPY4A isoforms, increasing both ROS production and uncoupling mitochondrial respiration. Insulin down-regulates CYP21 expression, and insulin resistance potentially explains its regulation during NASH [39]. Increased CYP2E1 activation occurs with obesity, but when they are significantly increased in the morbidly obese they are linked to NASH [40]. Obesity, *per se*, is a state of increased oxidative stress measured by waist circumference correlated with urinary isoprostane levels [41]. Immunohistochemical staining for 3-nitrotyrosine – another marker for oxidative stress – is elevated in liver biopsies from NAFLD patients , as well as those with NASH, not only when compared to normal controls, but also NAFLD patients [42]. Thioredoxin, an oxidative, stress-inducible thiol-containing protein, which has major antioxidant properties, is also significantly elevated in the NASH patients' serum, compared to those with simple steatosis or healthy people [43].

Mitochondrial dysfunction is mainly the result of insulin resistance with excess FFA (lipotoxicity) being the hallmark of NAFLD. Mitochondrial impairment enhances ROS production initiating lipid peroxidation, which together with damage of mitochondrial DNA (mtDNA) impairs its function by perpetuating ROS generation [44]. UCP-2 is a mitochondrial inner membrane protein assisting proton leak across the inner membrane by coupling substrate oxidation (such as fatty acids) by ATP synthesis, whereby the main function of uncoupling protein-2 (UCP-2) is to control mitochondria-derived ROS. Fatty acids and TNF-α induce mitochondrial UCP-2 expression in primary hepatocytes, with an UCP-2 increase in livers of NASH patients, ob/ob mice and in rats fed with a methioninine-choline-deficient diet. Induced UCP-2-lack in ob/ob mice improves serum ALT levels, hepatocellular necrosis and liver ATP levels after ischemia [45].

The mechanisms related to the cytokine–adipokine interaction in NASH are being intensely investigated. Insulin resistance is possibly regulated by proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and some adipokines, e.g., adiponectin and leptin [46]. Lipid peroxidation generate malodialdehyde and HNE, serving as chemoattractants for neutrophils (necroinflammation), stimulating hepatic stellate cell activation (fibrosis), and up-regulating transforming growth factor-beta1 expression in macrophages (fibrosis) [47]. Also, ROS mediates release of TNF-α by Kupffer cells, adipose tissue, and hepatocytes in NASH [48]. TNF-α increases mitochondrial