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REVIEW ARTICLES

Oxidative Stress and Endoplasmic Reticulum Stress as Potential Therapeutic Targets in Non-Alcoholic Fatty Liver Disease

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ABSTRACT | Non-alcoholic fatty liver disease (NAFLD) is nowadays recognized as a common cause of chronic liver disease and aminotransferase elevation. Its incidence has been increasing through the last few years, raising a global prevalence of approximately 25%. The etiopathogenic mechanisms of this disease are not fully understood, but it has been related with various pathologies that compound the metabolic syndrome. Oxidative stress and endoplasmic reticulum (ER) stress have been recognized as key mechanisms in NAFLD pathogenesis. In this review, an updated overview of the role of oxidative stress and ER stress in the progression of NAFLD is provided. Besides, some current treatments focused on the above mechanisms are presented, with the objective to discuss new therapeutic strategies that could help physicians on their daily clinical practice.

KEYWORDS | Endoplasmic reticulum stress; Hepatocellular carcinoma; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Oxidative stress; Reactive oxygen species

ABBREVIATIONS | ALT, alanine transferase; ER, endoplasmic reticulum; HCC, hepatocellular carcinoma; JNK, c-Jun N-terminal kinases; MS, metabolic syndrome; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PJ, pomegranate juice; RNS, reactive nitrogen species; ROS, reactive oxygen species; T2DM, type 2 diabetes mellitus; UPR, unfolded protein response; XBP-1, X-box binding protein 1

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1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has been recognized as a major cause of liver disease worldwide. Recently, the development of cirrhosis in the absence of alcohol exposure has been identified as a frequent and important cause of elevation of aminotransferases [1–3]. The prevalence of NAFLD worldwide has been increasing through the last years and it is thought to be on the rise, being nowadays reported, just in the United States, a prevalence between 10% and 30%, with similar rates reported from Europe and Asia [3].

Although the etiopathogenic mechanisms of this disease are not fully understood, individuals with components of metabolic syndrome (MS), such as obesity, insulin resistance, and hyperlipidemia, have an increased risk of developing non-alcoholic fatty liver (NAFL) [4]. The pathogenesis of this disease must be understood as a multifactorial process, with oxidative stress being one of the key mechanisms involved in the progression from NAFL to non-alcoholic steatohepatitis (NASH), and finally to cirrhosis and hepatocellular carcinoma (HCC).

Oxidative stress is derived from the loss in the balance of the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and the consumption of these reactive species by antioxidants produced inside the hepatocyte. Disruption of this balance that favors the formation and accumulation of ROS and RNS finally leads to hepatotoxicity. This occurs as a result of the direct attack by these species to the essential biomolecules in the hepatocytes, producing an impairment in their biological functions and compromising the cell viability [3, 5, 6].

The aim of this review is to present an updated overview about the pathogenesis and novel therapies of NAFLD based on the discussion that oxidative stress and reticulum stress act as key mechanisms involved in the progression of NAFLD. Accordingly, the identification of the oxidant and antioxidant factors that are pathologically disrupted in NAFLD could help improve not only the current therapeutic strategies, but also give rise to new prophylactic approaches that could be used by physicians in a near future for the effective management of this disease. In addition, novel therapeutic approaches based on the pathophysiology presented in this review will be discussed.

2. GENERAL OVERVIEW OF NAFLD

NAFLD was firstly described in the 1980s in subjects with an alcohol intake between or lower than 10 and 40 g/day or, as used in some studies, no more than 40 g of alcohol per week. Nowadays, this is the most common type of chronic liver disease in the western countries and it includes the whole spectrum of static steatosis to NASH that could progress to cirrhosis and HCC [1, 2, 7, 8].

NAFLD is usually asymptomatic, although some patients might consult for weakness with or without hepatomegaly in the physical exam. The laboratory tests could show elevation of the aminotransferases, an increase in the alkaline phosphatases, and an elevation of the gamma-glutamyl transpeptidase. Moreover, some studies have suggested that NASH patients tend to be older than the patients with NAFL and to have a higher homeostatic model assessment, indicating insulin resistance. On the other hand, NASH-HCC patients tend to be older than NASH patients and have the lowest platelet counts and alanine transferase (ALT) levels of the spectrum [1, 8]. In addition, these patients show an increase in the levels of triglycerides (TG), cholesterol, and free fatty acids in comparison with patients without NASH [9, 10].

Histologically, NAFLD is categorized into NAFL, in which it can be found steatosis without hepatocellular injury. NASH is characterized by the presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning), which is a cardinal histologic feature of lipotoxic hepatic injury, and has been also correlated with the disease severity. MS is also correlated with the histological severity [1, 7, 11, 12].

Some studies have recognized NASH as a more advanced stage of NAFLD. This is based, among others, on the higher probabilities to develop more serious diseases, and the higher cardiovascular risk of the patients and NASH mortality rate (25.6 per 1.000 person-year). Its progression rate to cirrhosis is 20–30% in 10 years versus the 1% found in static steatosis [1, 8]. Recently, there have been studies indicating that both NAFL and NASH are likely to have different genetic backgrounds and lipid contents, thus suggesting that the pathogenesis of steatosis in simple fatty liver and NASH is different, and disease-specific treatments are therefore required [8].

3. PATHOPHYSIOLOGY OF NAFLD

Several factors are involved in the pathogenesis and progression of NAFLD, such as lipotoxicity, oxidative stress, mitochondrial dysfunction, and immune dysregulation [3]. All of these factors act concomitantly in the hepatocyte to generate the liver damage seen in more advanced stages of this disease, with lipotoxicity-induced oxidative stress and the endoplasmic reticulum (ER) stress being the ones that appear to be the central drivers in this progression [3, 7]. Actually, the pathogenesis of NAFLD is based in a modified "two hits" hypothesis [13]. According to this hypothesis, the "first hit" refers to the generation of an steatotic liver, which is produced by the pathologic accumulation of TG as a result of an increased free fatty acid (FFA) influx. The "second hit" is produced later with the action of many other pathologic factors, such as cytokines, adipokines, oxidative stress, and mitochondrial dysfunction, which together cause the progression to NASH and fibrosis [14]. Nowadays, some genetic factors have been identified as key mechanisms in the pathogenesis of NAFLD, such as a patatin-like phospholipase 3 (PNPLA3) gene polymorphism, which has been proposed to have a key role in the development of NASH [15].

A "third hit" has also been described, which is related to the repairing response induced when the adaptive mechanisms that protect hepatocytes from the fatty acid-mediated lipotoxicity are overwhelmed. The repairing response involves the activation of hepatic stellate cells to myofibroblasts, which differentiate to replace dead hepatocytes. These cells also produce factors that attract various kinds of inflammatory cells to the liver, finally leading to NASH and fibrosis [16]. In addition, a "multifactorial hit" has been proposed, which adds the interaction of the inflammasome within the hepatocyte with the gut microbiota dysbiosis [17]. All of these mechanisms and factors have been considered crucial in the NAFLD pathogenesis (**Figure 1**).

3.1. Role of Oxidative Stress

Normally, ROS are physiologically generated as an intrinsic property of any kind of aerobic organism, with the mitochondria being the most important source of their production. About 5% of the oxygen used in the metabolism produces some of the most relevant ROS affecting the cell homeostasis. The rest of oxygen is metabolized directly to water [18]. Within the hepatocytes, ROS are generated by the free fatty acid metabolism of organelles, such as peroxisome and ER (besides the mitochondria), with this process being proposed to be responsible for the initiating necroinflammation [8]. Hydroxyl radicals, singlet oxygen molecules, superoxide anions, and hydrogen peroxide are the most relevant ROS that have been associated with NAFLD pathogenesis, with hydroxyl radical being considered one of the strongest oxidants in nature [19, 20].

The prooxidant-favored dysregulation in NAFLD leads the above ROS to attack essential hepatocyte biomolecules, such as lipids, proteins, and DNA. In fact, a clinical trial showed that patients with type 2 diabetes mellitus (T2DM) and NASH have increased levels of oxidative stress markers. It was found that protein oxidation and lipid peroxidation, malondialdehyde (MDA), and 8-isoprostane are higher in T2DM than controls [5]. Another clinical trial showed that patients with NASH-HCC have significantly lower levels of antioxidant molecules, such as reduced glutathione and superoxide dismutase (SOD), compared with both patients only with NAFL and controls [8].

3.2. Role of Endoplasmic Reticulum Stress

Oxidative stress affects the ER, a major organelle that controls the production of cholesterol and lipid membrane biosynthesis, and also participates in the calcium homeostasis [21]. In fact, free cholesterol can cause ER stress in NASH liver by inhibition of the sarco-endoplasmic reticulum Ca^{2+}-ATPase [22]. ER stress is elevated by accumulation of fatty acids and is involved in the pathogenesis of NASH via activation of the fibrotic and inflammatory responses [22, 23]. The initiation of ER stress is thought to be due to conditions associated with protein overload or an increased amount of unfolded proteins. These changes have both physiological and pathological roles causing, in severe cases of stress, an accumulation of these unfolded proteins [7, 24]. Based on the organelle's role, another activator of ER stress is the high-fat diet [25].

The evidence demonstrating that ER stress is a common feature in NAFLD is increasing [26]. Some

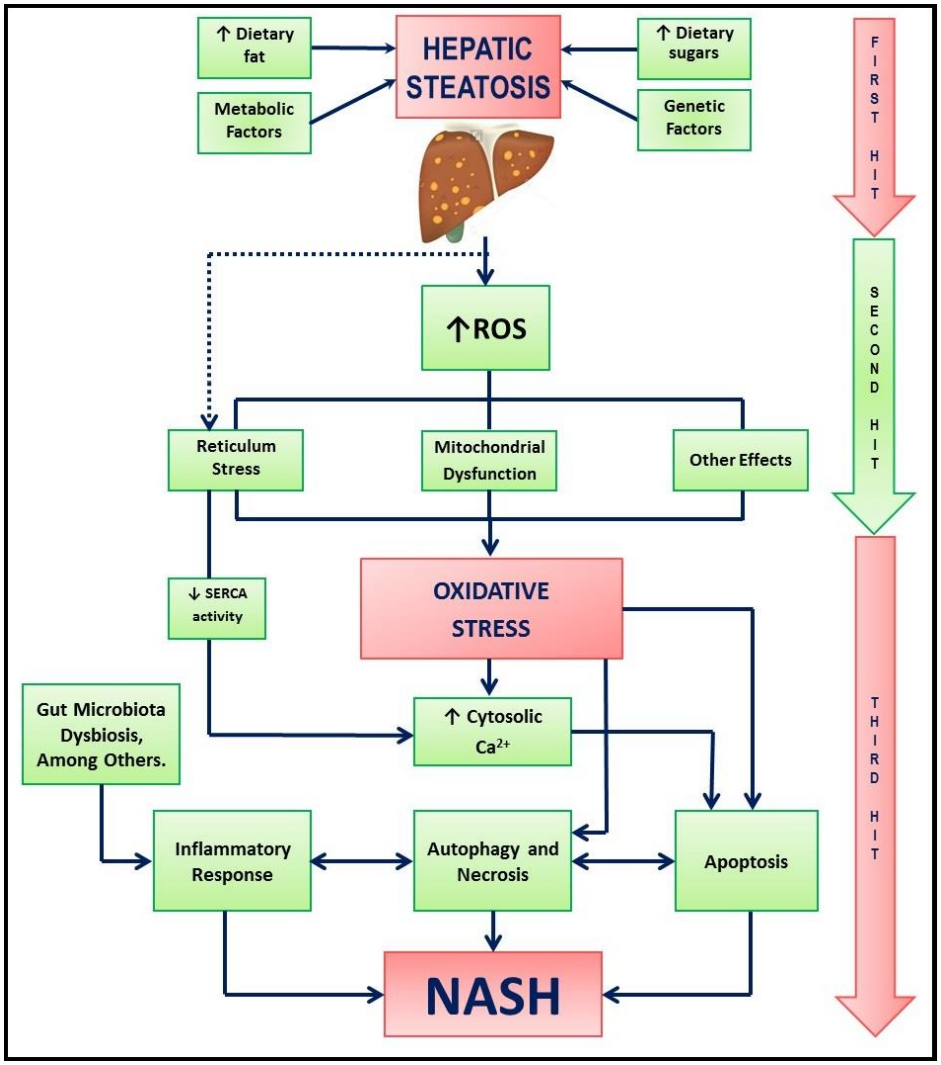

FIGURE 1. Pathophysiology of NAFLD based on the modified "two hits" hypothesis. The pathologic accumulation of FFA in the liver leads to steatosis (first hit). The metabolism of the lipids within the hepatocytes produces a disturbance in the prooxidant/antioxidant balance, favoring the accumulation of ROS. This imbalance causes endoplasmic reticulum stress and mitochondrial dysfunction, among other outcomes, leading to oxidative stress (second hit). The oxidative stress produces necrosis and an increase of the cytoplasmic Ca^{2+}, thereby activating the apoptotic pathways, leading to inflammation and NASH (third hit). NAFLD, non-alcoholic fatty liver disease; FFA, free fatty acids; ROS, reactive oxygen species; NASH, non-alcoholic steatohepatitis; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase.

studies in mice have demonstrated that the treatment with pharmacological ER stress inducers, such as thapsigargin and tunicamycin, leads to lipid accumulation in the liver [21], and while a moderately induced ER stress causes adaptation and recovery of homeostasis, a severe or prolonged ER stress can ultimately lead to inflammation and apoptosis [7].

It has been suggested that activation of ER stress may trigger various inflammatory pathways, such as c-Jun N-terminal kinases (JNK) and nuclear

factor-kappa B signaling pathways, further enhancing NASH progression [26, 27]. Moreover, an adaptive machinery—the unfolded protein response (UPR)—organized by ER transmembrane receptors, is kept inactive under unstressed conditions. The enhancement in the release of GRP78 from the transmembrane receptors leads to their activation with the subsequent use of single mechanism that induces transcription factors and upregulates UPR target genes [21, 28].

The activation of the inositol requiring kinase 1 (which is part of UPR) splices X-box binding protein 1 (XBP-1) mRNA and activates UPR pathway, all of which can cause an inflammatory response and apoptosis [29, 30]. The deletion of XBP-1 inhibits lipid accumulation in mice [31], and there is evidence showing a correlation between deficiency of hepatic XBP-1 and NASH development in humans [32]. XBP-1 also increases JNK phosphorylation, which is followed by apoptosis. This event is correlated with a decrease of XBP-1 and NASH which, according to the authors, proves the role of ER stress in the pathogenesis of the disease [21, 32]. It has been proposed that other molecules, and post-transcriptional and post-translational modifications could contribute to explaining the role of ER stress in NAFLD and its progression, although further investigations are still lacking [21].

Reducing inflammation ameliorates ER stress-induced liver injury. For example, IL-1β-deficient mice show reduced inflammation, hepatocyte death, and liver damage in an ER stress-induced steatohepatitis model [24]. The fibrotic process, which is responsible for the progression of NAFLD, has been proposed as an effect of the activation of the inflammasome within the hepatocytes. Although it is not well understood how this happens [21], the fibrotic process could be due to the upregulation of profibrotic gene SMAD-2 causing the fibrosis, which is likely to contribute to the progression of NAFLD [23, 33].

4. CURRENT THERAPEUTIC STRATEGIES

Nowadays, the management of NAFLD is based on the treatment of the liver disease as well as the associated metabolic comorbidities, such as obesity, hyperlipidemia, insulin resistance and T2DM [11]. Several studies have demonstrated that lifestyle modification may reduce aminotransferases and improve hepatic steatosis when measured either by ultrasound [34] or through magnetic resonance imaging and spectroscopy [35]. Some pharmacological-dietary combined treatments have also been proposed. For example, one study reported that a therapy with orlistat (an enteric lipase inhibitor) in conjunction with lifestyle modification improved ALT levels and steatosis [36]. Nevertheless, taking into account that patients with NAFLD without steatohepatitis have excellent prognosis from liver standpoint, treatments aimed at improving liver disease should be limited to those with NASH [11].

5. NOVEL THERAPEUTIC APPROACHES

Many natural compounds, such as vitamin E and polyphenols, represent potential therapeutic candidates, essentially due to their antioxidant, anti-inflammatory, and antifibrotic properties [37]. For example, there is a study showing that mice with a high-fat and sugar diet in association with pomegranate juice (PJ) consumption, had a decrease of body weight gain, food intake and serum levels of lipids, leptin, and glucose compared with high-fat and high sugar diet model. The outcomes, according to the authors, were a consequence of an up-regulation of the hepatic mRNA of different factors, such as hormone-sensitive lipase, pyruvate kinase, fatty acid synthetase, and adiponectin, among others. The results also indicate that PJ reduces the gene expression of hepatic proinflammatory and profibrotic cytokines. The changes presented above are likely to "restore" the metabolic imbalance and favor an anti-inflammatory context with a lower progression of NAFLD. Furthermore, these results could also justify the reduction of TG and hepatic steatosis, generating a reduction in the score of the disease in mice with PJ intake [38].

During the last several years, different natural and pharmacological therapies related to oxidative stress and ER stress have been proposed, after being tested in mice and/or humans. Some of the studied compounds/modalities were: PJ, vitamins E, C, D, and A, toyocamycin, curcumin, and *Meretrix meretrix* oligopeptides, among others. The hepatic outcomes of the different experiments usually include promising results, such as a decrease of apoptosis, steatosis,

TABLE 1. Novel therapeutic approaches targeting NAFLD oxidative stress and endoplasmic reticulum stress

Ref	Compound	Dose	Duration	Model	Specie/n	Major Outcomes	Mechanisms
[39]	Vitamin E + symbiotic supplement	400 IU/d of vitamin E + 2 capsules of symbiotic supplement per day	8 weeks	Randomized double-blind controlled clinical trial	Human/60	↓Serum levels of ALT, AST, ALP, TG, LDL-c ↓Steatosis, ↓Fibrosis ↓Necroinflammation	↓ROS ↓TNFα ↓TGFβ
[38]	Polyphenols (Pomegranate juice)	60 ± 5 ml/day	7 weeks	Randomized HFD Induced NAFLD	Mice/10	↓Steatosis, ↓Ballooning ↓Lobular inflammation ↓Portal inflammation ↓Proinflammatory and profibrotic gene expression ↓Serum levels of ALT, AST, TG	↓Expression of TNFα, IL-1β, IL-6 ↓TGF-β
[40]	Toyocamycin	0.25 mg/kg/day	2 weeks	Palmitic acid diet. Induced NAFLD	Mice/36	↓Apoptosis, ↓steatosis ↓Mitochondrial dysfunction ↓ER stress ↓Serum levels of ALT, AST, TG, cholesterol	↓XBP-1 expression ↓Bim protein ↓Bax activation.
[41]	Polyphenols (Curcumin)	2 g/kg/day	24 weeks	Humans: had NAFLD before the study Mice: HFD-induced NAFLD	Humans/120 Mice/32	↓Serum levels of cholesterol ↓ weight gain ↓steatosis ↓Inflammation ↓ballooning	↓Leptin-induced TNFα and IFNγ production ↓Linoleic acid-induced ROS generation
[42]	*Meretix meretrix* oligopeptides	10 mg/ml 20 mg/ml	24 hours	In vitro human cells	Liver cells/96-well plates	↓Apoptosis, ↓ROS ↑SOD ↓Mitochondrial dysfunction	↓JNK pathway activation ↓TNFα
[43]	Losartan	10 μM 10 mg/kg/day	24 hours 2 days 3 days	Tunicamycin-induced ER stress TGF-β-, AII-, high glucose-, and albumin-induced ER stress Tunicamycin-induced ER stress	Human HK-2 cells Rats/6	↓Tunicamycin-, AII-, high glucose-, and albumin-induced ER stress.	↓BiP expression ↓p-eIF2α expression ↑SIRT1 expression ↑HO-1 and thioredoxin expression
[44]	Polyphenols (Naringenin/ Hesperetin)	15 mg/kg/day	4 weeks	24-month-old Wistar rats	Rats/30	↑AOE activity ↑GSH levels ↑MUFAs content ↑n-3 PUFA content ↓n-6 PUFA content	↑CAT, GPx, SOD2, and GR gene expression

Note: AII, angiotensin II; ALP, alkaline phosphatase; ALT, alanine transferase; AOE, antioxidant enzymes; AST, aspartate transferase; BiP, binding immunoglobulin protein; CAT, catalase; ER, endoplasmic reticulum; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; HFD, high fat diet; HO-1, heme oxygenase 1; IFNγ, interferon γ; IL-1β, interleukin 1β; IL-6, interleukin 6; JNK, c-Jun N-terminal kinases; LDL-c, low-density lipoprotein cholesterol; MUFAs, monounsaturated fatty acids; n-3 PUFA, n-3 polyunsaturated fatty acids; n-6 PUFA, n-6 polyunsaturated fatty acids; NAFLD, non-alcoholic fatty liver disease; p-eIF2α, phosphorylated eukaryotic initiation factor 2; ROS, reactive oxygen species; SIRT1, silent mating type information regulation 2 homolog 1; SOD, superoxide dismutase; SOD2, superoxide dismutase 2; TG, triglycerides; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; XBP-1, X-box binding protein 1.

proinflammatory and profibrotic gene expression, and an improvement of the redox imbalance [38–42]. All these outcomes were associated with mechanisms that, in general, include changes in the gene expression (**Table 1**).

6. CONCLUDING REMARKS

It should be noted that NAFLD remains as an unsolved problem in the clinical practice. Nevertheless, oxidative stress and ER stress are two key factors. Accordingly, evidence suggests that novel therapies, such as the use antioxidants, might be beneficial to slow down the progression of the disease. Therefore, it should be expected that targeting the pathophysiology of these factors could contribute to improving the clinical outcome of NAFLD patients. The administration of antioxidant vitamins and PJ could provide a low risk and economic alternative in the near future. It is important to investigate the unknown steps in the genesis and progression of the disease, expanding the knowledge that will not only be beneficial to patients with NAFLD/NASH, but also to those with MS and cardiovascular risk. Thus, it is necessary to enhance investigation about the pharmacokinetic and pharmacodynamic properties of the compounds used as novel therapies. It is necessary to know their therapeutic margins and possible adverse effects to run new clinical trials that may involve combined therapies with antioxidants and drugs, looking forward not only to treating the disease, but also to preventing it.

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