

Molecular Mechanisms of Nonalcoholic Fatty Liver Disease: Potential Role for 12-Lipoxygenase

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of pathologies associated with fat accumulation in the liver. NAFLD is the most common cause of liver disease in the United States, affecting up to a third of the general population. It is commonly associated with features of metabolic syndrome, particularly insulin resistance. NAFLD shares the basic pathogenic mechanisms with obesity and insulin resistance, such as mitochondrial, oxidative and endoplasmic reticulum stress. Lipoxygenases catalyze the conversion of poly-unsaturated fatty acids in the plasma membrane—mainly arachidonic acid and linoleic acid—to produce oxidized pro-inflammatory lipid intermediates. 12-Lipoxygenase (12-LOX) has been studied extensively in setting of inflammation and insulin resistance. As insulin resistance is closely associated with development of NAFLD, the role of 12-LOX in pathogenesis of NAFLD has received increasing attention in recent years. In this review we discuss the role of 12-LOX in NAFLD pathogenesis and its potential role in emerging new therapeutics.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a clinicopathologic spectrum of liver pathologies associated with excessive accumulation of fat in the liver (Browning et al., 2004; Neuschwander-Tetri & Caldwell, 2003). This spectrum is continuous but can be graded based on pathological features; in increasing severity, these are: bland steatosis, steatohepatitis, fibrosis and cirrhosis. (Matteoni et al., 1999). NAFLD affects 31% of the US population, and is strongly correlated with the high incidence of obesity in Western cultures (Browning et al.).

Simple hepatic steatosis or non-alcoholic fatty liver (NAFL) is a largely benign and reversible condition defined by an excess accumulation of lipid droplets in the liver (Burt, Mutton, & Day, 1998). However, when non-alcoholic hepatic fat accumulation is associated with a significant inflammatory reaction—seen as lobular inflammation and cellular ballooning injury on histopathology—the pathology is considered nonalcoholic steatohepatitis (NASH) (Ludwig, McGill, & Lindor, 1997). An estimated 20-33% of individuals with NAFL patients show evidence of NASH on histopathology (Williams et al., 2011). Further progression of the disease in the setting of ongoing inflammation results in fibrosis (Ludwig et al., 1997) and eventually occurs in 20% of individuals with NASH. (Matteoni et al., 1999) Individuals with NASH progress to fibrosis and cirrhosis at a rate of 7-10% annually. (Argo, Northup, Al-Osaimi, & Caldwell, 2009; Harrison, 2003; Mishra & Younossi, 2012). Annual incidence of hepatocellular cancer and liver related death in patients with NASH related cirrhosis is around 2.6% and 1.4-3% respectively. (Sanyal et al., 2006)

The prevalence of NAFL reaches up to 90% in the obese population, and more than half of these show evidence of NASH based on histopathology. (Spaulding, Trainer, & Janiec, 2003). Furthermore, NAFLD is commonly associated with features of type 2 diabetes and metabolic syndrome. For instance, among individuals with type 2 diabetes mellitus (T2D) up to 70% have NAFL, and NASH is evident in ~67% of those biopsied (Matteoni et al., 1999). Reciprocally, T2D is seen in 30% of patients with NAFLD (Loomba et al., 2012). This strong

association of NAFLD with metabolic syndrome, suggests that mechanisms may be shared between these pathologies, in particular the conditions of maladaptive inflammation and insulin resistance observed in both. One biochemical pathway that is likely to be relevant but has not yet been extensively studied in NAFLD, is the eicosanoid generating lipoxygenases pathway. In this review, we summarize the current understanding of the pathogenesis of NAFLD, introduce the pertinent mechanisms by which 12-LOX could play a part in NAFLD pathogenesis, and discuss current and potential new therapeutic approaches.

NAFLD Pathogenesis

NAFLD is a complex disease, and accordingly its etiology involves multiple interacting factors, such as nutrient excess, obesity and metabolic syndrome (Assay et al., 2000; Beymer, 2003; Leite, Salles, Araujo, Villela-Nogueira, & Cardoso, 2009; Prashanth et al., 2009). In such “overfed” states, free fatty acids (FFAs) are directed to adipose tissue where they are converted into triglycerides under the control of the insulin signaling pathway. However, with chronic over-nutrition and obesity the presence of low grade inflammation in adipose tissues drives the development of peripheral insulin resistance, creating a state of relative insulin deficiency (Hirosumi et al., 2002). Under these conditions, lipolysis is no longer inhibited in adipocytes by insulin, leading to an increase in circulating FFAs (Samuel & Shulman, 2012); these FFAs in turn are sequestered by the liver for lipogenesis. Furthermore, in states of insulin resistance gluconeogenesis is uninhibited, while enhancing *de novo* lipogenesis. This is referred to as selective insulin resistance, as in normal conditions insulin inhibits gluconeogenesis while promoting *de novo* lipogenesis (Figure 1). Moreover, locally generated lipid products from cells in the liver (hepatocytes, invading immune cells) may also contribute substrate for lipogenesis. Together, the above mentioned dysfunctions drive the accumulation of triglycerides as lipid droplets in the liver, which upon exceeding 5% of the hepatocytes on histopathology is clinically defined as nonalcoholic fatty liver (NAFL), or bland steatosis (Burt et al., 1998). A considerable

percentage of these patients (20-30%) develop hepatic inflammation and progress to nonalcoholic steatohepatitis (NASH) (Ludwig et al., 1997). Although the transition from NAFL to NASH generally occurs in the setting of obesity and insulin resistance, the triggering events and downstream mechanism of progression are not yet completely understood; however it is likely that progression requires two hits that lead to the disruption of distinct molecular pathways (Day & James, 1998). Traditionally it has been hypothesized that a first hit results in development of simple steatosis, while a second hit results in progression from simple steatosis to steatohepatitis (Day & James, 1998). In recent years, a consensus has been emerging that the first of these hits encompasses insulin resistance, continued nutrient excess, and impaired autophagy that lead to steatosis, and that the second of these hits encompasses oxidative stress, ER stress, impaired autophagy, altered intestinal microbiome and intestinal translocation that allow progression to steatohepatitis (Buzzetti, Pinzani, & Tsochatzis, 2016). The hepatocyte alone is not responsible for the spectrum of molecular disorders leading to steatohepatitis, and other cells such as adipocytes and hepatic dendritic cells, NK-T cells, CD4 and CD8 T cells likely contribute (He et al., 2017; Heier et al., 2017; Walker & Lemon, 2016). In the rest of this section, we review three interconnected molecular pathways in hepatocytes—autophagy, ER stress and oxidative stress—that have been implicated in NAFLD progression and introduce 12-LOX pathway which we believe plays an important role in the pathogenesis of NAFLD. Figure 1 provides an overview of the pathogenesis of NAFLD described in this paper.

Autophagy

Autophagy is a critical cellular mechanism that regulates intracellular recycling and energy homeostasis through the orderly degradation of cellular components. The three pathways of autophagy (macroautophagy, microautophagy, and chaperone-mediated autophagy) have all been described in the liver. In macroautophagy, large cytosolic regions are sequestered within double membrane autophagosome vesicles. ATG7 is an important protein

for autophagosome formation and when this pathway is blocked, as in *Atg7*^{-/-} mice, damaged organelles and altered proteins accumulate even under basal conditions in hepatocytes (Komatsu et al., 2005). In microautophagy, direct lysosomal engulfment of the cytoplasmic cargo occurs through membrane invagination. Chaperone-mediated autophagy is a more selective pathway that relies on the recognition of specific amino acid motifs by the chaperone Hsp70, that directs the delivery of specific proteins to lysosome. Upregulation of chaperone-mediated autophagy occurs as a response to cellular stress including nutrient deprivation and oxidative stress (Kiffin, Christian, Knecht, & Cuervo, 2004).

Autophagy has been shown to regulate lipid metabolism. Under conditions of starvation, autophagy is induced leading to lipolysis and free fatty acid production, which provides an additional source of energy (Singh et al., 2009). Notably, this process of utilizing lipids as a source of energy is hindered during nutrient excess as shown in the RALA255-10G hepatocyte cell line treated with the fatty acid oleate. Knockdown of *Atg5* (a gene important in autophagy) caused fat accumulation after treatment with fatty acid oleate showing the importance of autophagy during nutrient excess (Singh et al., 2009). Nutrient excess has also been shown to inhibit autophagy in the liver of high fat diet (HFD)-fed mice. Conversely, treatment of the LO2 hepatocyte cell line with ω -3 fatty acids decreased cellular lipid accumulation partly by increasing autophagic flux and downregulation of lipogenesis genes (Chen et al., 2015). This finding suggests that in the setting of excess fat accumulation in the liver, autophagy is down regulated, leading to an additional increase of lipid accumulation in liver. Furthermore, these data indicate that not only over nutrition *per se*, but also diet composition, is crucial to driving NAFLD pathogenesis.

Autophagy also plays a role in controlling inflammation through regulatory interactions with inflammatory signaling pathways by removing endogenous inflammasome activators and through effects on the release of cytokines and immune mediators (Deretic, Saito, & Akira, 2013). *Atg5*^{-/-} mice infected with mycobacterium resulted in macrophages that hypersecrete IL-

1 α and IL-17, resulting in a pro-inflammatory state (Castillo et al., 2012). Greater cell death was seen in *Atg5*^{-/-} macrophages compared to wild type under conditions of oxidative and ER stress in setting of atherosclerosis (Liao et al., 2012).

Endoplasmic Reticulum Stress

Nutrient overabundance places increased demand on the endoplasmic reticulum (ER) to synthesize the additional proteins that are required to process excess fat and to package it with lipoproteins for transport throughout the body. When increased protein synthesis demands exceed the capacity of the ER, unfolded proteins accumulate in the lumen, triggering the unfolded protein response (UPR). The protein folding chaperone BiP (binding immunoglobulin protein) normally binds to and suppresses the activation of ER stress sensors in a steady state. However, as unfolded proteins mount, BiP migrates from these "folding" sensors to facilitate protein folding. This dissociation permits activation of each of the sensor pathways—PERK (double stranded RNA-dependent protein kinase-like ER kinase), IRE- α (Inositol requiring element-1 α), and ATF6 (activating transcription factor 6)—initiating the UPR. Chronic activation of the UPR generates a maladaptive state of ER stress. In fact, saturated fats such as palmitate have been shown to alter ER membrane integrity by saturating the phosphatidyl choline and triacylglycerol content in ER membranes. (Borradaile et al., 2006)

ER stress is closely tied to the induction of inflammatory pathways. For example, ER stress activates the transcription factor NF- κ B through PERK and IRE α pathways by overcoming the constitutively expressed I κ B (inhibitor of NF- κ B). This frees NF- κ B to translocate to the nucleus, thereby activating transcription of pro-inflammatory genes. *Ob/ob* mice, which develop steatosis due to a mutation in leptin, have been used to study fatty liver. However, these mice do not develop NASH until a second insult occurs, such as treatment with lipopolysaccharide (LPS). LPS-treated *ob/ob* mice develop hepatic inflammation through increased expression of pro-inflammatory IFN- γ and decreased expression of anti-inflammatory

IL-10 (Yang, Lin, Lane, Clemens, & Diehl, 1997). Hepatic activation of ER stress pathway proteins, such as XBP1s, p-eIF2 α and ATF4, and the downstream mediator CHOP are also increased in LPS-treated *ob/ob* mice. Furthermore, these mice showed increased Bcl-2 and Bcl-XL protein expression, consistent with the promotion of apoptosis with an increased CHOP expression. Lastly, ER stress in hepatocytes has been shown to activate inflammasomes—proinflammatory multiprotein complexes of the innate immune system that regulate activation of caspase-1 in response to infectious microbes or host proteins. This was demonstrated in *ob/ob* mice, which showed elevated mRNA levels of many inflammasome components upon LPS treatment (Lebeaupin et al., 2015).

Oxidative Stress

Oxidative stress is believed to play an important role in progression from steatosis to steatohepatitis. The metabolism of excess nutrients in hepatocytes places a high demand on the electron transport chain in the mitochondria, resulting in free radical generation, damage to cellular proteins, and increased oxidative stress. Furthermore, the increased demand for electron transport chain proteins feeds ER stress. ER stress feeds back to increase oxidative stress as free oxygen radicals are generated at the time of disulfide bond formation during protein folding (Zhang & Kaufman, 2008).

Levels of fatty acid oxidation have been shown to be elevated in the liver of obese individuals while fatty acid uptake and esterification remain similar to lean subjects (Iozzo et al., 2010). This increase correlated with insulin resistance. Interestingly this increase in fatty acid oxidation was not accompanied by mitochondrial respiratory chain (MRC) activity. When liver biopsy specimens of patients with NASH were compared to healthy controls, long chain acyl carnitine/carnitine ratio was increased, while MRC complexes I through IV were lower, suggesting that mitochondrial respiratory chain lags behind fatty acid oxidation, thereby increasing oxidative stress in the hepatocytes (Pérez-Carreras et al., 2003). Numerous studies

explored and found evidence of oxidative stress in patients with NAFLD. Lipid peroxidation products such as malondialdehyde (MDA), hydroxynonenol (HNE), oxidized LDL (ox-LDL), thiobarbituric acid-reacting substances (TBARS) were found to be higher in plasma of patients with NASH compared to patients with steatosis alone, suggesting that oxidative stress could have contributed to progression from steatosis to NASH (Chalasani, Deeg, & Crabb, 2004). Also, HNE and 8-hydroxy deoxyguanosine staining is significantly higher in liver tissue of patients with NASH compared to those with steatosis alone (Seki et al., 2002). Intensity of staining in the livers with 3-Nitrotyrosine, a lipid peroxidation product was also found to be highest in subjects with NASH, high in those with steatosis alone compared to healthy subjects (Sanyal et al., 2001). Studies have also explored using serum thioredoxin as a non-invasive marker of NASH as it was significantly elevated in patients with NASH compared to steatosis or healthy controls (Sumida, Niki, Naito, & Yoshikawa, 2013).

Lipoxygenases

A potential new pathway linking the three molecular mechanisms described with NAFLD is the lipoxygenase (LOX) pathway. Lipidomics analysis compared two mouse models of NAFLD, wild-type mice on a high fat diet (HFD) and ob/ob mice on a HFD. The study demonstrated that the enrichment of triacyl glycerol and 18:1 fatty acids are the most prominent difference compared with wild-type on regular chow (Hall et al., 2017). This finding suggests that abundance of lipid species, and perhaps their metabolism, may be important in the development of NAFLD. LOX enzymes catalyze the conversion of polyunsaturated fatty acids in the plasma membrane—mainly arachidonic acid and linoleic acid—to produce oxidized pro-inflammatory intermediates (Powell & Rokach, 2015; Tersey et al., 2015). LOXs are classified based on the carbon atom (5, 12 or 15) on arachidonic acid that is the target for oxygenation and stereo-selectivity (R or S enantiomer). Humans and mice have three homologues: 5-LOX, 12-LOX and 15-LOX, which produce 5-(S)-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE,

and 15-HETE from arachidonic acid, respectively (Tersey et al., 2015). However, since the mouse 15-LOX enzyme produces predominantly 12-HETE (in a 6:1 ratio over 15-HETE), this orthologue is also commonly known as 12/15-LOX. Henceforth in this review, we will use the term 12-LOX to refer to the mouse 12/15-LOX enzyme, as it is functionally equivalent to human 12-LOX and produces the majority of 12-HETE in mice. Listed in Table 1 are the major lipoxygenases, the gene encoding them, and their major lipid products.

12-LOX in the pancreatic islet. Whereas 12-LOX has been studied extensively during the inflammatory response in tissues such as islets, adipocytes and macrophages, its function in the liver is not as well understood. Mice harboring deletion of the gene encoding 12-LOX (*Alox15*) appear phenotypically normal, however they exhibit resistance to glucose intolerance when challenged with a high fat diet (HFD) (Nunemaker et al., 2008). HFD-fed *Alox15*^{-/-} mice also exhibit reduced insulin resistance and reduced macrophage infiltration within the adipocytes compared to control mice (Nunemaker et al., 2008). Whereas these studies showed the global importance of 12-LOX in the stress response to HFD-feeding, several different tissue-specific knockout models have been studied to differentiate the role of 12-LOX in various tissues/organs. 12-LOX is detected at low levels in human and mouse islets and treatment of human islets with pro-inflammatory cytokines results in an increase in both 12-LOX activity and protein levels (Chen, Yang, Smith, Carter, & Nadler, 2005). Inhibition of 12-LOX, either genetically or chemically, reverses islet β -cell dysfunction as seen in the presence of pro-inflammatory cytokines and restores normal insulin secretion, alluding to the crucial role played by 12-LOX in preserving insulin secretion during stress (Ma et al., 2017; Tersey et al., 2014). Treatment of human islets with 12-HETE shows a similar reduction in insulin secretion and β -cell dysfunction as seen with pro-inflammatory cytokines, further supporting the role of 12-HETE in islet stress. HFD-fed mice exhibited ER stress (shown by activation of CHOP) and oxidative stress (shown by activation of 4-HNE) in their islets and genetic deletion of *Alox15* in the

pancreas of mice results in decreased ER and oxidative stress as well as improved metabolic health (Tersey et al., 2014, 2015). This effect of 12-LOX appears to be mediated by p38MAPK, as islets treated with 12-HETE show increased phosphorylation of p38MAPK and *Alox15* knockdown in mouse islets demonstrated decreased phosphorylated p38MAK (Ma et al., 2010).

12-LOX in macrophages and adipose tissue: Recruitment of CD11b⁺, F4/80⁺ macrophages and elevated protein levels of inflammatory markers such as IL-1 β , IL-6, IL-10, IFN- γ , Cxcl1 and TNF- α were seen in adipose tissue of control mice but not in *Alox15*^{-/-} mice fed a HFD. This observation suggests a crucial role of 12-LOX in obesity and related inflammatory states (Sears et al., 2009). 3T3-L1 adipocytes treated with 12-HETE showed increased expression of inflammatory genes IL-6, TNF- α , MCP-1, IL-12p40 and reduced expression of anti-inflammatory adiponectin. These changes are mediated via janus kinase (JNK) phosphorylation, with subsequent phosphorylation of IRS-1(Ser) and impaired phosphorylation of IRS-1(Tyr) and protein kinase B phosphorylation (Chakrabarti, Cole, Wen, Keller, & Nadler, 2009). Likewise, 12-LOX expression in visceral adipose tissue of patients with T2D correlated with an increase in IL-6 and IL-12 cytokines (Lieb et al., 2014). Similarly, 12-HETE treatment of mouse macrophage cell lines (J773A.1) induced IL-6 and TNF- α production. Over-expression of 12-LOX also resulted in higher production of IL-6 and TNF- α . It appears that this action is at least partly mediated by p38MAPK and JNK (Wen et al., 2007). IL-12 production by macrophages upon IFN- γ stimulation is mediated through 12-LOX (Middleton, Rubinstein, & Pure, 2006). HFD-induced expression of TNF- α in adipose tissue was attenuated in adipocyte-specific *Alox15*^{-/-} mice. In addition, macrophage infiltration of adipose tissue was also reduced in adipocyte-specific *Alox15*^{-/-} mice fed HFD (Cole, Morris, Grzesik, Leone, & Nadler, 2012). It is of interest to note that in an adoptive transfer model of type 1 diabetes, splenocytes of *Alox15*^{-/-} mice congenic on the non-obese diabetic (NOD) background are not able to transfer disease, whereas splenocytes from control NOD mice transfer disease at 100% within two months

(Green-Mitchell et al., 2013). This role of 12-LOX in macrophages also has implications in atherosclerosis, as mice reconstituted with bone-marrow from *Alox15/Apoe* double-knockout mice displayed reduced atherosclerotic plaque size compared to bone-marrow from control *Apoe*^{-/-} mice (Huo et al., 2004). 12-LOX deficiency has also been shown to decrease HFD-induced atherosclerotic plaques in aorta of *Ldlr*^{-/-} mice (an atherosclerotic mouse model) without changing the composition of lipids in the plaque (George et al., 2001). In fact, 12-LOX has been shown to be a major player in the onset of diabetic cardiomyopathy in the streptozotocin (STZ) model of diabetes (single high dose), where increased expression of 12-LOX in cardiomyocytes was seen after exposure to high plasma glucose levels followed by deterioration in cardiac function; this deterioration was mitigated in *Alox15*^{-/-} animals (Suzuki et al., 2015).

Lipoxygenases in liver. A few studies have begun to investigate the role of lipoxygenases in the liver. Metabolomics in patients with NASH demonstrated increase in products of lipoxygenase pathway, including 5-HETE, 8-HETE, 11-HETE and 15-HETE compared to healthy patients and those with steatosis alone. There was no increase in products of cyclooxygenase pathway (Puri et al., 2009). In a Methionine choline-deficient mouse model of non-alcoholic steatohepatitis, liquid chromatography and mass spectroscopy of serum demonstrated significant elevation of 12-HETE, linoleic and oleic acids along with bile acids, tauro-B muricholate and taurocholate compared to mice supplemented with methionine and choline (Tanaka, Matsubara, Krausz, Patterson, & Gonzalez, 2012). In addition, MCD fed mice showed increased gene expression of *Alox12*, an alternate gene whose product also produces 12-HETE. Recent study in HFD-fed wild-type mice show a significant increase in 12-HETE. These mice also demonstrated an increase in 15-HETE, 5-HETE and 11-HETE. Livers of patients with NASH who demonstrated higher histologic inflammation score had increased 15-HETE levels. (Hall et al., 2016) In clinical trials using pentoxifylline, a methyl xanthine derivative with anti-inflammatory properties partly

mediated by suppressing TNF- α gene transcription, subjects who responded to pentoxifylline with improvement in lobular inflammation on histology demonstrated a decrease in plasma 12-HETE levels (Zein et al., 2012). Both 12-LOX and 5-LOX are expressed in normal mouse hepatocytes and upon liver damage via acetaminophen the transcript and expression levels of both are increased (Suciu et al., 2016). Likewise, upon HFD-feeding, 12-HETE and 5-HETE is increased compared chow-fed control mice (Lazic et al., 2014a). 5-LOX has been shown to be elevated in the liver of *ob/ob* mice and 5-LOX inhibition downregulated genes involved in hepatic fatty acid uptake and acyl-CoA oxidase expression, restored hepatic microsomal triglyceride transfer protein activity and hepatic VLDL-triglyceride and Apo-B secretion, suggesting a steatogenic role of 5-LOX. (Lopez-Parra et al., 2008). Also, in the *ApoE*^{-/-} mouse, 5-LOX deficiency protected mice from macrophage infiltration in the liver with decreased hepatic expression of pro-inflammation cytokines (IL-18 and MCP-1) (Martínez-Clemente, Clària, & Titos, 2011). Along similar lines, whole-body genetic knockout of *Alox15* in HFD-fed mice resulted in decreased hepatic steatosis, decreased macrophage infiltration, decreased mRNA expression levels of proinflammatory cytokine genes in the liver (IFN- γ , TNF- α and IL-10), and decreased immune cell chemoattractants (Cxcl2/3) (Lazic et al., 2014b). The liver includes multiple different cell types such as hepatocytes, cholangiocytes, Kupffer cells and stellate cells. While these studies suggest that LOXs (and in particular 12-LOX) play an important role in the pathogenesis of NAFLD in mice, the specific cell types have yet to be clarified, as studies were performed in whole animal genetic deletions. Arachidonic acid metabolism appears to interact with cholesterol transport. Products of cholesterol metabolism, bile acids have also found to have a role in pathogenesis of NAFLD. Methionine choline deficient mouse model of NASH were found to have elevated taurocholate and tauro- β -cholate were found to be elevated in addition to 12-HETE compared to control mice. (Tanaka, Matsubara, Krausz, Patterson, & Gonzalez, 2012) Reverse cholesterol transport by acetyl salicylic acid is mediated by diverting arachidonic acid metabolism from cyclooxygenase enzyme pathway to 5 lipoxygenases thereby

generating leukotrienes and lipoxins from 15-HETE, which have been shown to induce Abcb11 at a post translational level. (Demetz et al., 2014)

Available treatment modalities for individuals with NAFLD

There are several treatment modalities currently used or in clinical trials for individuals with NAFLD. The most widely recommended treatment is a lifestyle modification plan. As little as 5% weight loss has been shown to improve NASH histology in a pilot study of 23 overweight/obese subjects with biopsy-proven NASH (Huang et al., 2005). In another study, 31 overweight/obese individuals with biopsy-proven NASH were randomized to intensive lifestyle therapy or structured education. The patients in the intensive arm lost significantly more weight which led to improvement in steatosis, necrosis and inflammation (Promrat et al., 2010). A more recent study documented improvement in all histologic features of NAFLD with weight loss, including fibrosis. (Vilar-Gomez et al., 2015) Several studies have examined weight loss via bariatric surgery and have found improvement in hepatic steatosis, inflammation, and fibrosis (Furuya et al., 2007; Popov, 2015). Additionally, exercise alone without any dietary intervention has been shown to decrease hepatic liver lipids and improve overall metabolic health (Golabi et al., 2016; St. George et al., 2009). Hepatic staining of malondialdehyde and Cyp2E1 protein content decreased with surgical weight loss in obese subjects with NAFLD (Bell et al., 2010). A major limitation of lifestyle modification in the treatment of NASH is patient adherence, which can be as low as 30% (Martin, Williams, Haskard, & DiMatteo, 2005).

The next most common treatment is insulin-sensitizing agents. Several different insulin-sensitizing agents have been used to treat steatohepatitis with varying degrees of success. Although initial proof-of-concept studies have shown that metformin may be associated with histologic and biochemical improvement in NASH, subsequent larger studies failed to demonstrate histological benefit for metformin in patients with NASH (Haukeland et al., 2009; Lavine et al., 2011; Loomba et al., 2009; Nair, Diehl, Wiseman, Farr, & Perrillo, 2004).

Pioglitazone, another insulin-sensitizing agent, resulted in decreased inflammation and resolution of steatohepatitis when administered for 12-24 months in non-diabetic subjects with NASH. Both PPAR- γ and PPAR- α agonistic effects of pioglitazone are believed to aid in its effect on NASH (Aithal et al., 2008). However, pioglitazone is associated with higher rates of congestive heart failure and this concern has limited its widespread use in treatment of NASH. Likewise, while vitamin E (α -tocopherol) at a dose of 800 IU/day has been shown to improve NASH histology in non-diabetic adults with biopsy-proven NASH (Sanyal et al., 2010), data regarding association of high-dose vitamin E with prostate cancer has to be cautiously considered and discussed with patients before long-term use (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2007; Klein et al., 2011; Miller et al., 2005). Analysis in vitro showed that α -tocopherol reduces lipoxygenase dependent peroxidation of pig liver phosphatidylcholine micelles (Hirofumi Arai, Akihiko Nagao, & Kozo Takama, 1995).

Several other agents are also being actively studied for NAFLD. A multicenter clinical trial showed that 6-ethylchenodeoxycholic acid (obeticholic acid), an activator of farsenoid X nuclear receptor (FXR), significantly improved steatohepatitis histopathology. Contrary to expectations, subjects treated with obeticholic acid also witnessed worsening HOMA-IR and an increase in mean total cholesterol and LDL fraction and a decrease in HDL fraction (Neuschwander-Tetri et al., 2015). The long-acting glucagon-like-peptide-1 agonist Liraglutide was shown to resolve NASH without progression of fibrosis in a significant number of subjects compared to placebo in a recently-published phase 2 trial (LEAN trial) (Armstrong et al., 2016). In addition, the Liraglutide group showed significant decreases in hemoglobin A1C, absolute weight, BMI and increase in HDL cholesterol fraction, thus aiding cardiovascular risk optimization in this patient cohort (Armstrong et al., 2016).

Based on both the limitations of the current available treatments and the molecular mechanisms of NAFLD, there are compelling reasons to study novel therapeutic interventions based on the 12-LOX/12-HETE signaling pathway. Protection of HFD-fed *Alox15*^{-/-} mice from

ER stress and inflammation described above suggests that interventions that inhibit 12-LOX with small molecule inhibitors, such as ML127, ML351 and ML355 (Kenyon et al., 2011; D. Luci et al., 2010; D. K. Luci et al., 2014; Ma et al., 2017) would serve to protect these animals from steatohepatitis. Further studies are needed to confirm the potential of these proposed interventions, particularly given the possibility of off-target effects and non-tissue-specific effects of small molecule drugs of this nature.

Animal Models of NAFLD

Human research has greatly shaped our understanding of non-alcoholic fatty liver disease, but several limitations exist in studying the disease processes in humans, such as variations in environmental exposure, pre-existing genetic risk factors, racial and ethnic differences in disease presentation, and need for multiple invasive procedures among others. Research in animals enables us to circumvent several of these issues. An ideal animal model for NAFLD must replicate human disease closely, by exhibiting fatty liver associated with inflammation in an environment of nutrient excess, preferably associated with features of metabolic syndrome such as obesity and insulin resistance. The animal model should be easy to breed and maintain in the lab environment in addition to achieving the desired disease phenotype in a reasonable timeframe. Several animal models are available to study NAFLD; each one presenting both advantages and limitations. Currently, both mice and pigs have been used in NAFLD research, though rodent studies are far more common. NAFLD occurs naturally in mice, but it can also be induced more reproducibly through genetic alterations/mutations. Alternatively, NAFLD can be induced in animals by feeding mice diets with high fat or carbohydrate content, such that 60% of caloric count is derived from fat alone and/or cholesterol or simple carbohydrates. Simple carbohydrates, such as glucose and fructose generate abundant levels of glycerol-3-phosphate, which can be used in triglyceride synthesis. Moreover, as fructokinase is not regulated by insulin, excessive fructose can lead to unregulated

production of acetyl-Co-A, which is in turn converted to triglycerides (Jegatheesan & De Bandt, 2017). Diets deficient in essential nutrients can also result in fatty liver. Listed in Table 1 are the various rodent models currently used to study NAFLD and their advantages and limitations.

Ossabaw pigs, a breed of pig originally found on an island off the state of Georgia in the United States, acquired a “thrifty gene” to adapt to seasonable variability of food availability. These pigs develop steatosis and steatohepatitis when fed a diet high in fats. However, they are not widely used in research due to cumbersome nature of breeding and maintaining these animals in laboratory setting (Lee et al., 2009).

In light of the limitations of rodent and pig models of NAFLD, there is a need for new animal models that address these shortcomings. Zebrafish is being explored as a potential animal model to study NAFLD. The similarity of zebrafish hepatopancreaticobiliary anatomy to humans and presence of orthologues to most human genes in zebrafish, including synteny make zebrafish an attractive animal model for hepatopancreaticobiliary disease. In addition, genetic tractability and transgenic feasibility allow for development of desired animal models with expression of interested study pathways. Rapid external development of transparent zebrafish embryo allows for study of effect of gene expression patterns on embryonic development in a time efficient manner. All the above features lend zebrafish to study of hepatopancreaticobiliary disease (Schlegel, 2012).

Conclusion

In this review, we summarize the putative role of oxidative stress and ER stress in the development of nonalcoholic steatohepatitis and identify the 12-LOX as an under-recognized, albeit important, contributor to the pathogenesis of NAFLD. It is as yet unclear whether current approaches to therapy (weight loss, thiazolidinediones, GLP-1 receptor agonists, FXR agonists) might operate in part through the alteration of 12-LOX activity. As such, recently developed inhibitors of 12-LOX (Kenyon et al., 2011; D. Luci et al., 2010; D. K. Luci et al., 2014; Ma et al.,

2017) may represent a novel approach to the treatment and/or prevention of NAFLD, and such inhibitors may augment current approaches to treatment.

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Table 1: Major lipoxygenases, their genes, and major products in humans and mice.

	Gene	Enzyme	Product
Mouse	<i>Alox15</i>	12/15-LOX (or commonly 12-LOX)	12-HETE:15-HETE (6:1)
	<i>Alox12</i>	12-LOX	12-HETE
Human	<i>Alox15</i>	15-LOX	15-HETE
	<i>Alox12</i>	12-LOX	12-HETE

Table 2: Animal Models of nonalcoholic fatty liver disease

Model	Mechanisms	Salient Features	References
Dietary Deficiency			
Methionine choline deficient	Impaired VLDL secretion from liver	Features of steatohepatitis in 10 weeks But in presence of weight loss and improved insulin sensitivity	(Caballero et al., 2010; Oz, Chen, & Neuman, 2008)
Conjugated Linoleic Acid (CLA)	Diet with transfat conjugated with linoleic acid	Features of steatohepatitis with mild peri-sinusoidal fibrosis with insulin resistance and near universal HCC development But in presence of weight loss and improved insulin sensitivity	(Fujita et al., 2010)
Choline-def L-AA (CDAA)	Diet deficient in choline; containing only L-amino acids	Develops fibrosing NASH, cirrhosis and HCC. But in presence of weight loss, improved insulin sensitivity and increasing adiponectin levels	(de Lima et al., 2008)
Dietary Excess			
High Fat Diet	> 60% fat calories	Steatosis with minimal and variable inflammation and fibrosis associated with obesity, insulin resistance and dyslipidemia.	(Tetri, Basaranoglu, Brunt, Yerian, & Neuschwander-Tetri, 2008)
Western Diet	45% saturated and trans fats High cholesterol	Steatohepatitis w/ballooning and variable fibrosis But takes up to 20 weeks	(Kohli et al., 2010)
Atherogenic diet	1.25% cholesterol and 0.5% cholate	Steatohepatitis with ballooning and fibrosis But occurs in setting of weight loss and improved insulin sensitivity and takes up to 24 weeks	(Charlton et al., 2011)
Genetic Models			
ob/ob	Mutation in leptin	Steatosis, Steatohepatitis develops after 2 nd hit in obese, hyperphagic, inactive animals that show insulin resistance and dyslipidemia But resistant to fibrosis	(Ingalls, Dickie, & Snell, 1950; Zhang et al., 1994)
db/db	Mutation in leptin receptor	Steatohepatitis after 2 nd hit in obese animals with insulin resistance	(Chen et al., 1996; Hummel, Dickie, & Coleman, 1966)
ApoE KO	Absence of ApoE protein, a ligand of the LDL receptor	Steatohepatitis after 2 nd hit in animals with increased LDL, total cholesterol and triglycerides and atherosclerosis Model of dyslipidemia	(Schierwagen et al., 2015)
aP2-nSREBP-1c transgenic	Over-exp of SREBP-1c in adipose tissue	Steatohepatitis with mild fibrosis in animals with increased Glu; decreased adiponectin Model of lipodystrophy	(Shimano et al., 1996)
MAT1A KO	Absence of methionine adenosyltransferase (impaired anti-oxidant defense)	Steatohepatitis without fibrosis with high susceptibility to tumors But no evidence of metabolic syndrome,	(Lu et al., 2001)

Figure 1: Overview of pathogenesis of nonalcoholic fatty liver disease. Nutrient excess leads to insulin resistance and low grade inflammation at the level of adipose tissue. This leads to the increased circulatory free fatty acids (FFA), that can be accessed by the liver. Insulin in steady state promotes de-novo lipogenesis and inhibits gluconeogenesis. But in setting of peripheral insulin resistance, insulin selectively dis-inhibits gluconeogenesis and continues to promote de novo lipogenesis, thus compounding lipid accumulation in the liver. Increased triglyceride accumulation downregulates autophagy and perpetuates triglyceride accumulation. Increased triglyceride accumulation in liver increases demand on electron transport chain in the mitochondria, generating free radical species and leading to oxidative stress. This eventually increases demand on protein folding in the endoplasmic reticulum with ensuing unfolded protein response, where transcription of inflammatory genes, which perpetuates inflammation in setting of nutrient excess. 12-Lipoxygenase (12-LOX) acts on membrane lipids (arachidonic acid) to produce oxidized lipid products such as, 12-hydroxyeicosatetranoic acid (12-HETE) which have chemokine affect, thus amplifying inflammation in the setting of fatty liver. 12LOX inhibitors (ML127, ML351, and ML355) aid in alleviating inflammatory response by reducing oxidative end product production.

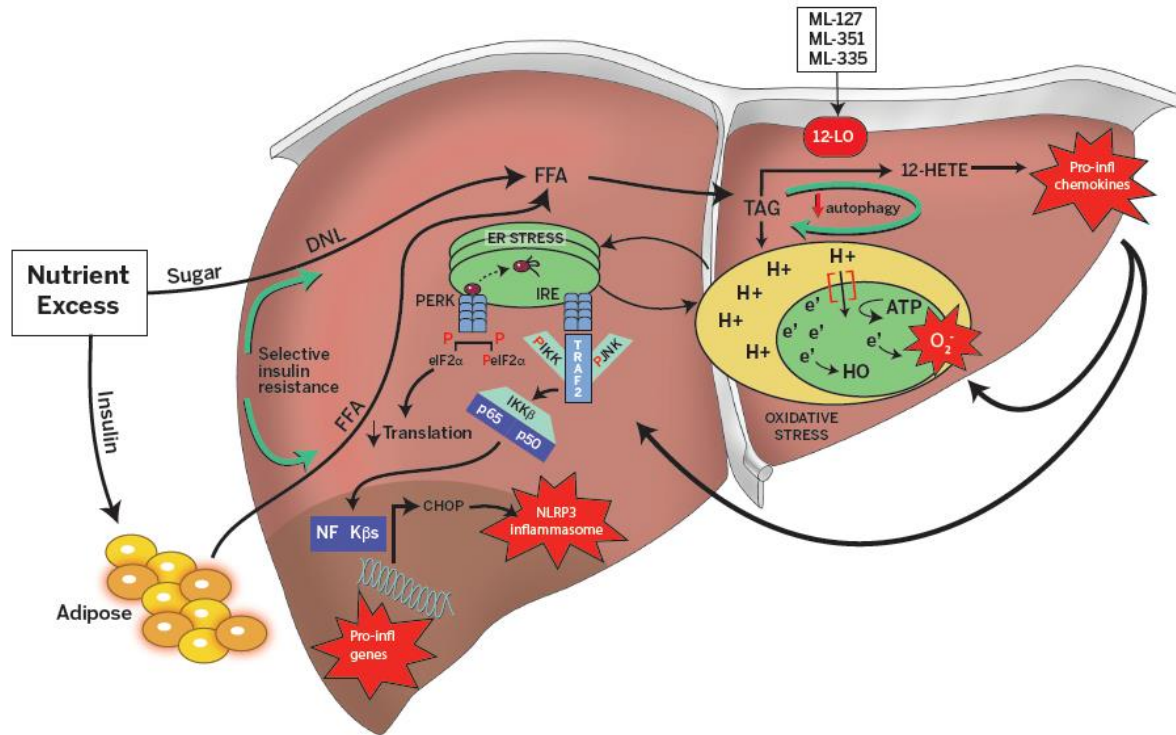


Figure 1

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Article Highlights

1. Nonalcoholic fatty liver disease is associated with obesity and affects more than 30% of the US population.
2. The molecular pathogenesis of nonalcoholic fatty liver disease involves endoplasmic reticulum stress, oxidative stress, and autophagy
3. 12-Lipoxygenase produces products that exacerbate the molecular stress pathways leading to nonalcoholic fatty liver disease
4. Multiple animal models of nonalcoholic fatty liver disease serve as preclinical models for testing of potential therapies for nonalcoholic liver disease

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