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Chapter 3

Role of Lipid Droplet Proteins in the Development of NAFLD and Hepatic Insulin Resistance

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Additional information is available at the end of the chapter

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Abstract

NAFLD is diagnosed, when the liver fat exceeds more than 5% of liver weight. Inside of hepatocytes, these fats are stored in cytosolic lipid droplets. The lipid droplets can be formed from a bud, vesicles of the lipid bilayer, which lines at a vicinity of the endoplasmic reticulum (ER). On the surface of droplets, there are several structural/functional proteins such as lipid droplet proteins, lipogenic enzymes, and lipases. Interestingly, the lipid droplet proteins seem to have great impact on a development of NAFLD. Some proteins can interact with transcriptional factors such as SREBP1c and PPAR-alpha/gamma, and some proteins strongly impact a mitochondrial structure. As a result, the lipid droplet proteins highly influence lipid handling and fatty acid oxidation in hepatocytes. This chapter will elucidate our recent understanding of the role of each lipid droplet protein in fatty liver formation and in hepatic insulin resistance. Existing information on genetically modified animals as well as on human NAFLD was reviewed on Perilipin families, CIDE proteins, Seipin, and PNPLAs. Finally, the chapter will discuss how the lipid droplet proteins could potentially lead/protect from hepatic insulin resistance via abnormal accumulation of ceramides and diacylglycerols, autophagy, ER stress, and oxidative stress.

Keywords: perilipin, CIDE proteins, insulin sensitivity, mitochondrial oxidation, autophagy, ER stress

1. Introduction

Liver can store a certain amount of excess glucose as a form of glycogen. A part of the stored glycogen can then be retransformed into glucose (so-called gluconeogenesis) during a fasting condition to leave the liver. Contrary to the glucose, an excess lipid is not normally stored in the liver. However, in a pathological case, excess lipid storage can be observed

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in hepatocytes, which is called hepatic steatosis. Hepatic steatosis could be diagnosed in different grades according to histological observation (Grade 1: 5–33% lipid invasion in hepatocytes, Grade 2: 33–66%, and Grade 3: >66%). Up to 90% of obese patients could have the hepatic steatosis, and the presence is linked to several metabolic dysfunctions such as insulin resistance, oxidative stress, endoplasmic reticulum (ER) stress, and mitochondrial dysfunctions [1–3]. As a result, hepatic steatosis often leads to abnormal gluconeogenesis, which is a typical phenotype of type 2 diabetes. To better prevent such metabolic dysfunctions, scientists have been actively investigating mechanisms by which hepatic lipids impact metabolic functions.

2. Lipid droplet structure

When excess fat is present in a liver, these fats are stored intracellularly in cytosolic lipid droplet compartment. Today, an origin of a lipid droplet biosynthesis has not been understood completely. Walther and Farese suggested different models of the lipid droplet biosynthesis [4]. Most accepted lipid droplet biosynthesis model is that triglyceride (TG) accumulated at the ER membranes forms a bud, a vesicle of the lipid bilayer [5]. On ER membranes, phospholipids are added onto the surface of growing lipid droplets. Secondary, lipid droplets could increase their size via lipid droplet fusion. Finally, a matured lipid droplet is formed consisting TG and cholesterol esters in a core, coated by a membrane monolayer of phospholipids and sphingomyelin (**Figure 1A**). On the surface and/or vicinity of droplets, there are several structural/functional proteins such as lipid

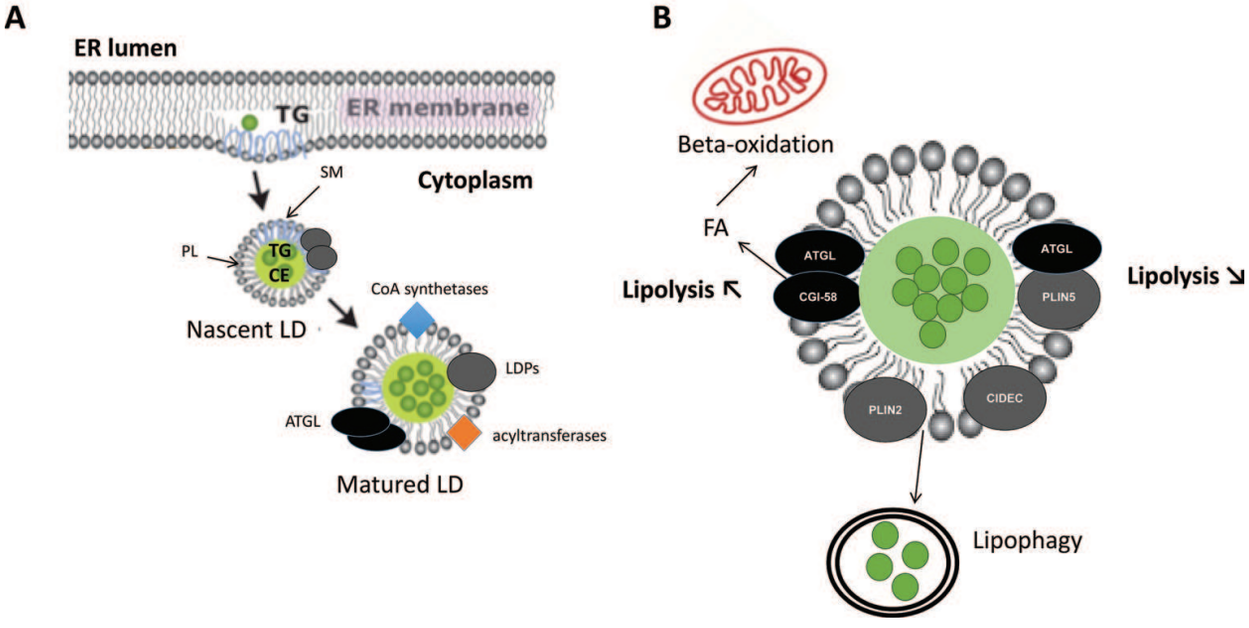


Figure 1. Lipid droplet formation. (A) Formation of nascent lipid droplet (LD) with lipid droplet proteins (LDPs), (B) lipid droplet biology.

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droplet proteins, lipogenic enzymes, and lipases (**Figure 1B**). Enzymes required for lipid droplet synthesis are also located in the ER. This strategic enzyme location helps to form, stabilize, and degrade lipid droplets when necessary. Hydrolysis of lipid is highly regulated by different enzymes such as adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL). ATGL is responsible for the hydrolysis of triglyceride, followed by HSL, which cleaves one molecule of fatty acid from diacylglycerol, and finally hydrolysis is completed by MGL. In hepatocytes, ATGL interacts both lipid droplet protein, perilipin 5 (PLIN5) and comparative gene identification-58 (CGI-58). When ATGL interacts with PLIN5, this decreases lipolysis; however, if ATGL interacts with CGI-58, this increases lipolysis [6]. Therefore, lipid droplet proteins play a key role regulating a fate of cellular lipid storage (**Table 1**).

LD protein	Expression cites	Observed functions	Reported interactions with other genes/proteins
PLIN1 (perilipin A)	WAT, cardiac muscle liposarcoma, BAT	LD stability, control of hormone-induced lipolysis	ATGL, CGI58, [97, 98], SREBP1c [19], CIDEC [15]
PLIN2 (ADRP, adipophilin)	Liver, WAT, mammary gland, macrophages, sebocytes, ubiquitous expression	LD stability, adipocytes differentiation, VLDL lipidation	PPAR alpha, gamma [21, 99, 100], delta [101] hepatic von Hippel-Lindau protein [102]
PLIN3 (TIP47)	Ubiquitous expression, skeletal muscle, neutrophils, sebocytes	LD stability, PGE_2 production, intracellular trafficking	Mannose-6-phosphate receptor [9]
PLIN4 (S3-12)	WAT, skeletal muscle	LD stability, adipocytes differentiation	PLIN5 [32]
PLIN5 (OXPAT, LSDP5, MLDP)	Skeletal muscle, BAT, heart, liver, beta-cells	LD stability, fat oxidation, mitochondrial recruitment	PPARalpha [60], delta [103], CIG58, ATGL [6], ABHD5 [104]
PLPNA3 (Adiponutrin)	WAT, liver, skeletal muscle, pancreas	Triglyceride and retinyl palmitate esterase activity	SREBP1c [105]
PLPNA2 (ATGL)	Adipose tissue	Lipolysis	SIRT1 [106], PLIN5 [6], PLIN1 [33, 97]
CIDEA	Adipose tissue, liver	Lipogenesis	SREBP1c [41, 42]
CIDEB	Adipose tissue	Contributes to lipogenesis, lipidation of VLDL, hepatitis virus assembly	HCV NS58 protein [107]
CIDEC (FSP27)	Adipose tissue, liver	LD stability, LF fusion, lipid transfer	PPAR alpha, gamma [42, 45]
SEIPIN (BCSL)	Adipose tissue, liver, brain, testis	Maturation of LD, lipolysis	

Table 1. Lipid droplet proteins.

3. Lipid droplet protein families

Lipid droplet proteins were discovered in the 1990s in phospholipid monolayer of lipid droplet [7–9]. At that time, each lipid droplet proteins had a different nomenclature. In 2010, it was suggested to uniform their names as the "perilipin family protein: PAT protein" [10]. PAT was named after the three proteins: PLIN1 (Perilipin), PLIN2 (Adipose differentiation-related protein; ADRP), and PLIN3 (Tail-interacting protein of 47 kDa; TIP47). All PLIN families contain a conserved domain called PAT domain [11] with an exception of PLIN4 that only contains long 11-mer repeat motifs [12]. The expression of these proteins and their functions are slightly different, and their exact roles for each cell type have not been yet completely understood. Interestingly, their expression depends on a size of lipid droplets (small lipid droplets: PLIN3, PLIN4, PLIN5; medium lipid droplets: PLIN2; and large lipid droplet: PLIN1).

3.1. PLIN1

PLIN1 was one of the first lipid droplet protein identified in adipocytes [13, 14], and its expression is mainly observed in matured adipocytes. During a differentiation of premature adipocytes, PLIN2 plays a major role to lipidate small lipid droplets. Once lipid droplet gains enough size, PLIN1 replaces PLIN2 to stabilize large lipid-rich lipid droplets and helps to mature adipocytes. PLIN1 also interacts with cell-death-inducing DNA-fragmentation-factor 45-like effector (CIDE)-C for a lipid droplets fusion process [15]. Among different reported functions of PLIN1 in adipocytes, the most well-characterized role of PLIN1 is a control of lipolysis. PLIN1 co-localizes with ATGL and CGI-58 on a surface of lipid droplets at a basal condition. Upon a lipolytic stimulation, PLIN1 is phosphorylated and CGI-58 is released and activates ATGL for a lipolysis. In hepatocytes, PLIN1 is not expressed in normal healthy liver, but its expression is observed in steatotic hepatocytes [16–18]. During a formation of hepatic steatosis, the expression of PLIN1 is synchronized with sterol regulatory element-binding protein (SREBP)-1c, a key regulator of *de novo* lipogenesis [19]. As a result, both genes could strongly contribute to accelerate the pathogenesis of hepatic steatosis [20].

3.2. PLIN2

PLIN2 was originally named as adipose differentiation-related protein due to its high expression during an adipocyte differentiation [8]. PLIN2 is ubiquitously expressed and its expression in the liver is high among other lipid droplet proteins [21]. Chronic alcohol consumption stimulates *de novo* lipogenesis and induces hepatic steatosis together with an upregulation of PLIN2 [22]. Hepatocellular ballooning and oxidative injury were also observed under such condition [18]. Magne et al. observed in human NAFLD patients that PLIN2 polymorphism (ser1Pro) was linked to a decreased VLDL levels [23].

A recent study demonstrated that the PLIN2 and PLIN3 double-knockout in hepatocytes induced insulin resistance [24]. In addition, the overexpression of PLIN2 in rat skeletal muscle resulted in an accumulation of TG in muscle without insulin resistance [25]. However, general deletion of PLIN2 in mice also showed a protective role against hepatic steatosis and insulin resistance (discussed in a later paragraph, **Table 2**). The exact impacts of PLIN2 modification on insulin resistance have not yet been fully elucidated.

LD proteins	Up or downregulation	Outcomes
PLIN1	**Downregulation** KO mice [61] KO mice [62]	Body fat↓, fat oxidation↑ Lipolysis↑, cardiac steatosis↑, cardiac hypertrophy
PLIN2	**Downregulation** ASO [63, 64] KO—alcohol diet [66] KO—high fat diet [65] Liver specific KO—methionine-choline deficient diet [95] Lep (ob/ob)/Plin2 double KO mice [108] KO–high fat diet [109]	Hepatic steatosis↓ (ceramide-, DAG↓), IR ↓ Hepatic steatosis↓ (ceramide↓), IR ↓, glucose tolerance- Hepatic steatosis↓, body fat↓, adipose inflammation↓ Hepatic steatosis↓, hepatic inflammation↓, fibrosis↓ VLDL secretion ↑ Hepatic steatosis ↓, IR↓ (liver, muscle) Hepatic steatosis ↓VLDL -
PLIN3	**Downregulation** ASO [67]	Hepatic steatosis ↓ IR↓
PLIN4	**Downregulation** KO [32]	Cardiac steatosis↓
PLIN5	**Downregulation** KO [38] KO [40] **Upregulation** Adenovirus [68]	Lack of lipid droplet in heart, ROS↑, heart mal function, Hepatic steatosis ↓, mitochondrial oxidative capacity↑, lipotoxic injury Hepatic steatosis ↑ IR-
PLPNA3	**Downregulation** KO [55, 56] **Upregulation** G allele knock-in Ref. [57]	Hepatic steatosis - Hepatic steatosis ↑
PLPNA2	**Downregulation** KO [69] Liver-specific KO [70]	Steatosis in different organs↑, IR↓ Hepatic steatosis ↑ (DAG↓), IR -
CIDEC	**Downregulation** KO [47, 110] KO in ob/ob by shRNA [46] ASO [111] **Upregulation** Adenovirus [46]	Hepatic steatosis ↓, IR↓ Hepatic steatosis ↓, IR↓ Hepatic steatosis ↓, body fat↓, IR↓ Hepatic steatosis↑
SEIPIN	**Downregulation** Adipose-specific KO [112] KO [49] **Upregulation** Transgenic mice overexpressing a short isoform of human BSCL2 in adipose [113]	Hepatic steatosis↑, IR↑, adipocyte hypertrophy and progressive lipodystrophy Hepatic steatosis↑, IR↑ Hepatic steatosis↑, IR↑, white adipose tissue ↓, lipolysis ↑

Abbreviations: KO; knockout, ASO; antisense oligonucleotide, IR; insulin resistance.

Table 2. Experimental modification of lipid droplet protein and the effect on steatosis.

3.3. PLIN3

PLIN3 was originally named tail-interacting protein of 47 kDa and ubiquitously expressed among tissues. PLIN3 is localized at the cytosol and lipid droplet [9, 26]. It is also implicated in intracellular trafficking of lysosomal enzymes [9]. Four-helix bundle in PLIN3 has been

suggested to contribute to fatty acid binding and lipid droplet recruitment [9]. PLIN2 and PLIN3 share similar functions, and both proteins cannot bind CGI-58 and, therefore, influence lipolysis [27]. Co-expression of PLIN2 and PLIN3 has been reported in many tissues; however, a distinct role of PLIN3 has not been clearly identified. Interestingly, PLIN3 expression was also observed in stellate cells in the liver [17]. Lipopolysaccharide treatment also predominantly stimulated PLIN3 expression in HL-60-derived neutrophils [28]. Knockdown of PLIN3 via siRNA decreased a lipid droplet formation as well as PGE_2 secretion. This observation was unique to PLIN3, and PLIN2 was not detected under such conditions. This implies that PLIN3 might be implicated in a lipid droplet formation related to conditions with cellular stresses.

Another unique observation to PLIN3 was that mice lacking mTORC2 (mammalian target of rapamycin complex) activity in skeletal muscle showed increased fat mass and PLIN3 [29]. This was due to an increased AMPK activity. mTORC plays an important role in insulin signaling. However, the implication of PLIN3 in insulin resistance has not yet been addressed.

3.4. PLIN4

PLIN4 was originally called S3–12 and is the only PAT protein that does not contain the PAT domain. Its molecular weight is three times higher than other PAT proteins. The protein has been shown to present at cytosol and lipid droplets [30]. Its expression was induced during adipogenesis [31]. PLIN4-KO mice present no phenotypic changes in adipose tissues, whereas TG content in heart tissues was significantly reduced [32]. PLIN5 expression was also decreased under such condition. Given close location of PLIN4 and PLIN5 in chromosome 19 in human, it was suggested a potential transcriptional interference between two genes. PLIN4 remains the least studied PAT protein, and further investigations are required to understand PLIN4 roles in lipid droplet physiology.

3.5. PLIN5

PLIN5 was discovered by different researchers simultaneously and named as myocardial lipid droplet protein (MDLP), OXPAT, or lipid storage droplet protein 5 (LSDP5) [33, 34]. This was due to a high expression of PLIN5 in heart and other oxidative tissues such as skeletal muscle and liver. PLIN5 expression is also reported in pancreatic beta cells and hepatic stellate cells [35, 36]. A unique feature with PLIN5 is that mitochondria are physically recruited to lipid droplets expressing high PLIN5 (**Figure 2**) [37, 38]. Its expression is regulated by PPAR-alpha, and most importantly, PLIN5 plays roles in regulating cellular fat oxidation. PLIN5 stabilizes lipid droplets by sequestrating fatty acids, and because PLIN5 can recruit mitochondria to lipid droplet surface, it facilitates to release fatty acids to mitochondria for the oxidation [39]. Given its gatekeeper roles on the lipid oxidation, it has been suggested that PLIN5 could protect cardiac myocytes and hepatocytes from oxidative stress [38, 40]. PLIN5 leads several modifications on the lipid metabolism as well as insulin sensitivity, and details are discussed in a separate paragraph.

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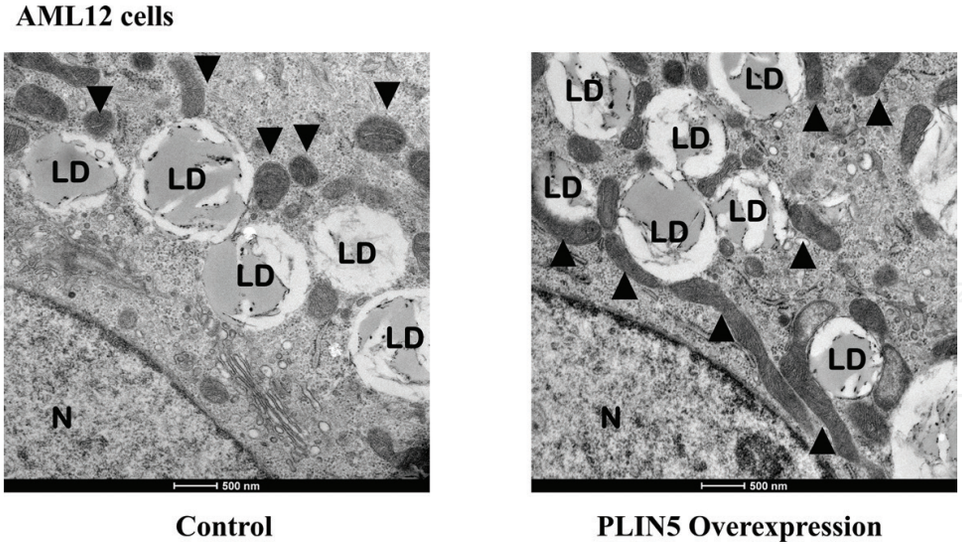


Figure 2. Electron microscopy image of hepatocyte overexpressing PLIN5. When PLIN5 is overexpressed, mitochondria (closed triangle) is highly recruited toward lipid droplets (LD). N: nucleus.

3.6. CIDE proteins

Cell-death-inducing DNA fragmentation-factor 45-like effector (CIDE) proteins have also been found on a lipid droplet surface. CIDEA expression is controlled by SREBP-1c and found in a fatty liver [41, 42]. CIDEB is constitutively expressed in a liver and plays a role in VLDL production [43, 44]. Interestingly, it has been reported that CIDEB and PLIN2 exert opposite functions for a control of VLDL lipidation [44]. CIDEC, is also named as fat-specific protein 27 (FSP27), found as a cofactor of PLIN1 for lipid droplet fusion in adipocytes [15]. CIDEC is regulated by PPAR-alpha/gamma [42, 45]. Like CIDEA, hepatic CIDEC was induced in leptin-deficient *ob*/*ob* mice. It has also been demonstrated that CIDEC overexpression induces steatosis, whereas knockdown of CIDEC alleviates hepatic fat accumulation in *ob*/*ob* mice lacking hepatic PPARγ [46]. Effect of CIDEC on mitochondrial activity and insulin sensitivity is an active research field of today [15, 47, 48]. Toy et al. demonstrated that white adipocytes from CIDEC KO mice had accelerated mitochondrial activities and increased proteins and size, leading to brown adipocyte characteristics.

3.7. SEIPIN

SEIPIN is highly expressed in brain, testis, and adipose tissue. Mutations in SEIPIN are known as Berardinelli-Seip congenital lipodystrophy (BSCL), a rare recessive disorder characterized by near absence of adipose tissue accompanied by a severe insulin resistance [49]. Several studies convincingly demonstrated that SEIPIN plays a crucial role in adipogenesis, lipid droplet homeostasis and lipolysis. It is also well known that human BSCL patients and mice lacking SEIPIN develop diabetes and severe hepatic steatosis. Interestingly, our previous studies demonstrated that SEIPIN expression seems to be dependent on lipid droplet size (**Figure 3**), and the low SEIPIN expresser had an impaired gluconeogenesis in NAFLD patients (personal observation).

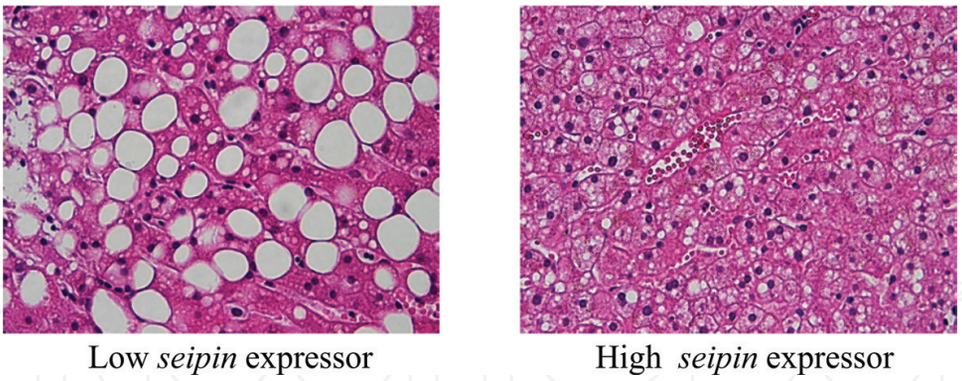


Figure 3. Liver histology from NAFLD patients.

3.8. PNPLA3 and PNPLA2

Patatin-like phospholipase domain-containing protein 3 (PNPLA3), also called adiponutrin, was consistently associated with NAFLD in GWAS observations [50, 51]. The single nucleotide polymorphism (SNP) in PNPLA3 was identified as a major determinant of hepatic fat content from exome-wide association studies. This is the rs738409 C > G SNP encoding for the isoleucine to methionine substitution [50]. PNPLA3 is expressed in the retina, hepatic stellate cells, and hepatocytes and localized in the endoplasmic reticulum and at a surface of lipid droplets. A mechanism by which PNPLA3 leads the hepatic steatosis phenotype has not been clearly understood. PNPLA3 has a triglyceride and retinylpalmitate esterase activity [52–54], suggesting a possible link to hepatic fat accumulation. However, PNPLA3 knockout mice did not develop hepatic steatosis [55, 56]. Mice having Pnpla3i148m knock-in recently showed increased hepatic steatosis [57]. The role of PNPLA3 in the development of NAFLD still remains elusive.

PNPLA2 is known as adipose triglyceride transfer protein (ATGL) and is expressed on lipid droplet surface at a basal condition. Its activity is strongly influenced by PLINs [25]. As shown in **Figure 1**, ATGL is the first enzyme hydrolyzing neutral lipids.

4. Expression of lipid droplet proteins in human NAFLD

Lipid droplet proteins are highly expressed in human NAFLD. PLIN1, PLIN2, and PLIN3 were upregulated in NAFLD [18, 58, 59]. And the distribution of PLINs seems to depend on the lipid droplet size [17, 18]. PLIN2 was also observed in stellate cells [18, 59]. We have compared the expression of different lipid droplet proteins in human NAFLD. The steatosis was judged by a histological assessment showing four different grades (S0 < S1 < S2 < S3). Despite a similar BMI among different groups, the gene expression of lipid droplet proteins increased depending on the degree of steatosis (**Figure 4A**). When compared the livers from patients with or without type 2 diabetes who had a similar degree of hepatic steatosis (S3), the expressions of lipid droplet proteins were significantly lower in diabetic patients than in nondiabetic individuals (**Figure 4B**). This result implies possible link between lipid droplet proteins and hepatic insulin signaling.

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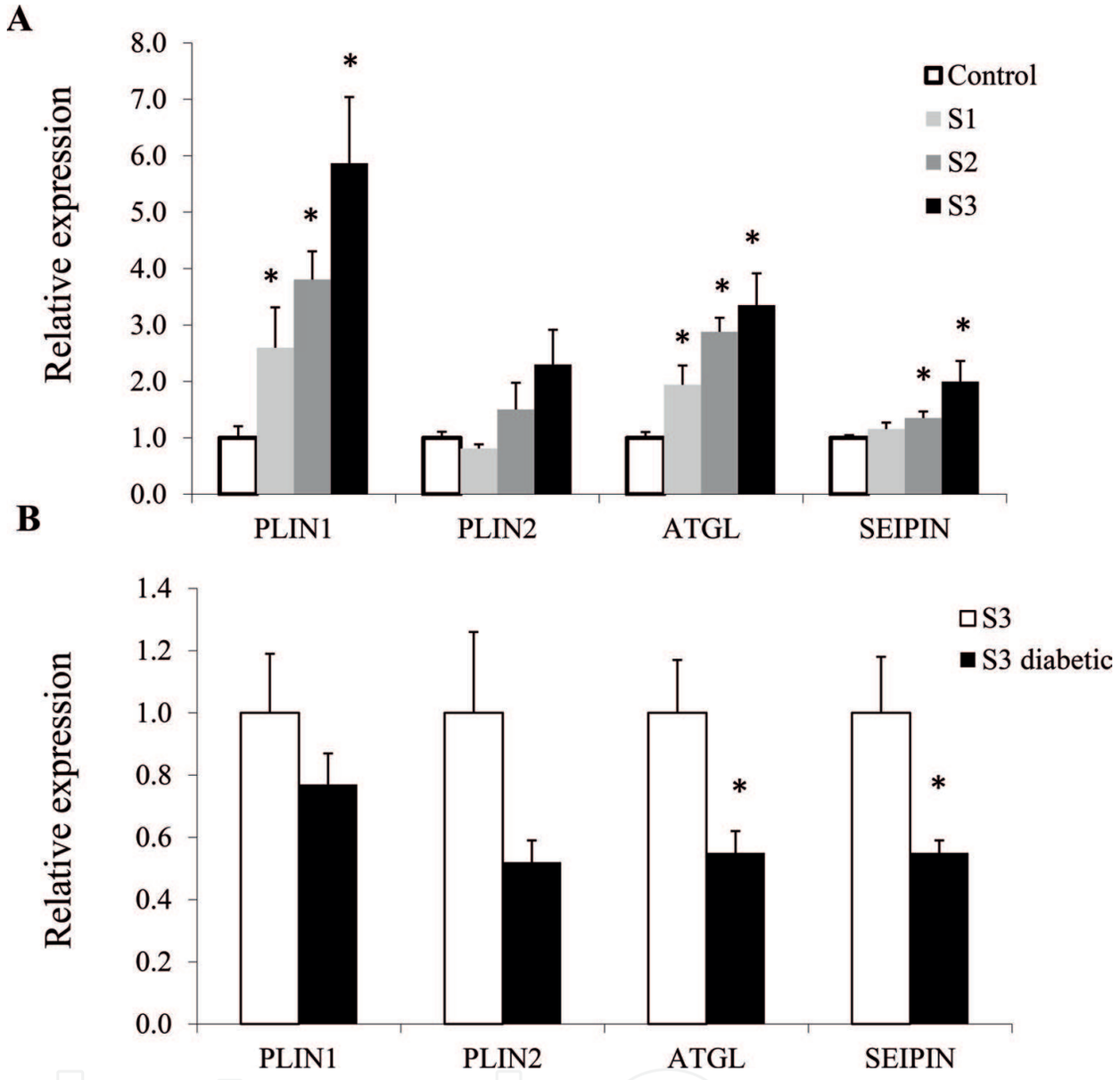


Figure 4. Expression of lipid droplet proteins in human NAFLD. NAFLD patients were separated into 5 groups depending on their degree of fatty liver. Gene expression of lipid droplets genes were analysed and compared among groups. (A) NALFD non diabetic, (B) Comparison of S3 NAFLD patients with or without type 2 diabetes.

5. Experimental modification of lipid droplet proteins and TG accumulation in hepatocytes

It has been shown that modifications on the lipid droplet proteins have striking impacts on lipid droplet biology. Hepatic cell line AML-12 is used to study the effect of downregulation of SEIPIN gene. Downregulation of the gene markedly altered lipid droplets size distributions and increased smaller droplets size, suggesting a default in lipid droplet maturation (**Figure 5**). When PLIN5 was knocked down in hepatocytes, the TG content dramatically

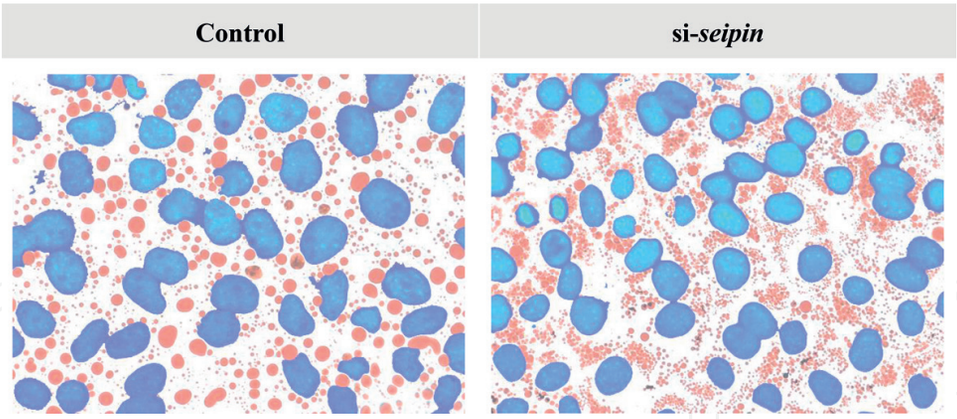


Figure 5. Lipid droplet morphology when SEIPIN was downregulated. Downregulation of SEIPIN by siRNA resulted in a fractionation of lipid droplets.

decreased due to accelerated lipolysis and beta-oxidation [60]. PPAR-alpha was required for the PLIN5-induced beta-oxidation. On the contrary, the overexpression of PLIN5 leads a significant increase in cellular TG. Therefore, modifications on lipid droplets proteins govern intracellular TG content in hepatocytes, although the mechanisms and phenotypes (lipid droplet size and/or localization) might be depending on a type of lipid droplet proteins involved.

6. Experimental modification of lipid droplet proteins in mice

Table 2 displays animals with genetic modifications in lipid droplet proteins and their effect on steatosis and metabolism (**Table 2**). PLIN1 KO mice present reduced body fat as well as fat oxidation judged by respiratory quotient [61]. Another research group also studied the PLIN1 KO mice and found increased lipolysis and cardiac hypertrophy [62]. PLIN2 null mice studied by different scientific groups consistently demonstrated a protection against diet-induced obesity, fatty liver, and alcohol-induced fatty accompanied by an improved insulin sensitivity [63–66]. Similar results were obtained in mice treated by PLIN2 antisense oligonucleotide, demonstrating improved insulin sensitivity [64]. PLIN3 downregulation was studied by using antisense oligonucleotide (ASO) in C57BL/6 J mice fed high fat diet. The reduction in PLIN3 significantly decreased hepatic fat content and improved glucose tolerance as well as insulin sensitivity in liver, adipose, and skeletal muscle [67]. Chen et al. generated PLIN4 KO mice [32], which showed no major modification in body weight and fat mass. Interestingly, only cardiac TG content was significantly reduced. The KO mice did not alter any gene expression involved in glucose and lipid metabolism. PLIN5 KO mice also developed cardiac dysfunction. The PLIN5 KO animal displayed reduced hepatic steatosis with increased mitochondrial proliferation, lipotoxic injury in the hepatocytes [40]. Interestingly, overexpression of PLIN5 by use of adenovirus technology demonstrated a development of severe hepatic steatosis without a sign of hepatic insulin resistance [68].

Although a strong link between PNPLA3 and hepatic steatosis has been demonstrated in GWAS studies [50], absence of PNPLA3 gene did not influence TG hydrolysis, nor did

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hepatic steatosis [55, 56]. One of a pioneer study on the lipid droplet biology and insulin resistance was the PNPLA2/ATGL KO mice published in 2006 [69]. Mice with global ATGL deletion induced TG accumulation in all tissues [69]. Surprisingly, despite the severe steatosis, the mice exhibit enhanced glucose tolerance and insulin sensitivity. Later on, Wu et al. studied the effect of liver-specific deletion of ATGL [70]. The liver-specific KO mice progressively developed a severe form of hepatic steatosis; however, the hepatic DAG content was 50-fold lower and had comparable plasma glucose, TG, and cholesterol levels to those of controls.

CIDEC/FSP27 downregulation was studied in Fsp27−/− and ob/ob × Fsp27−/− mice [46, 47], demonstrating decreased hepatic steatosis and insulin resistance. These animals are resistant to diet-induced obesity, dyslipidemia. Deletion of SEIPIN, as seen in BSCL patients, leads to severe form of hepatic steatosis accompanied by insulin resistance [49].

7. NAFLD and insulin resistance, implication of lipid droplet proteins

It has been widely accepted that increased TG content in ectopic organs, especially in the liver and skeletal muscle, induces insulin resistance [71–73]. Despite strong evidences demonstrating the link, there are also a few studies to show that hepatic steatosis can be dissociated from

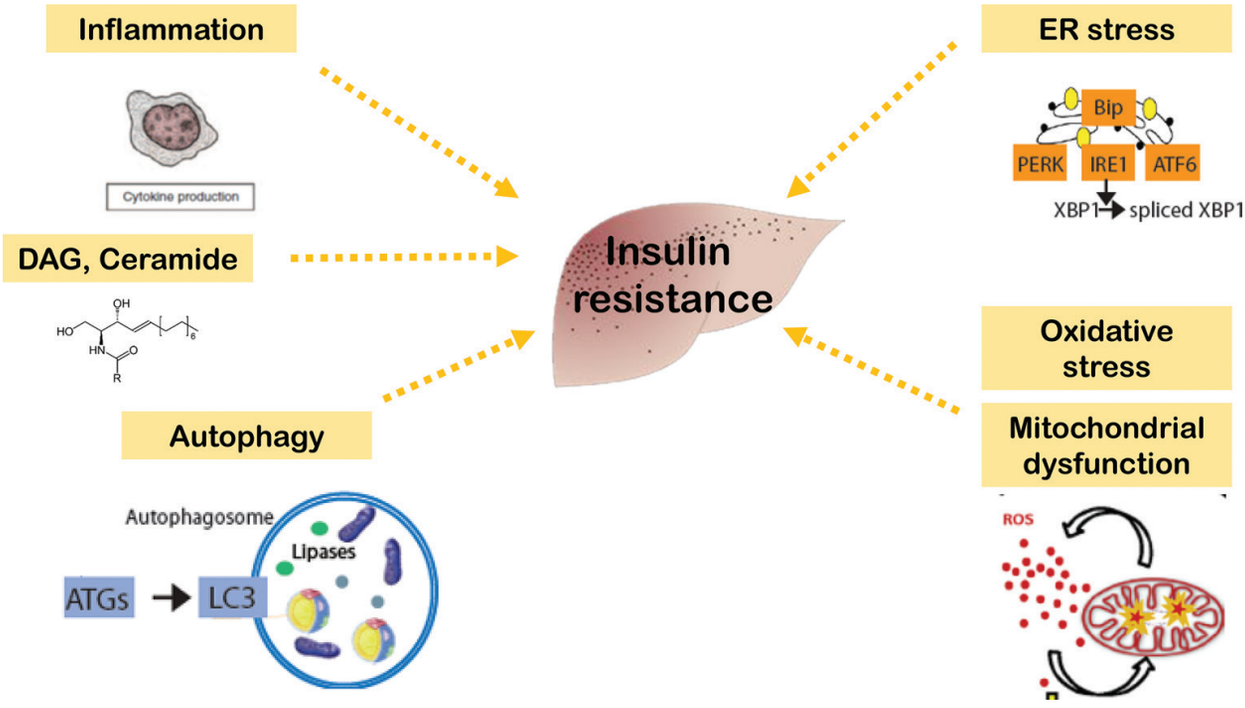


Figure 6. Different events leading hepatic insulin resistance in NAFLD. In human NAFLD, increased diacylglycerols (DAG) and ceramides are often observed. Under such conditions, autophagy is frequently decreased, leading to a disturbed lipid handling as well as increased ER stress and oxidative stress. These factors are known to induce insulin resistance.

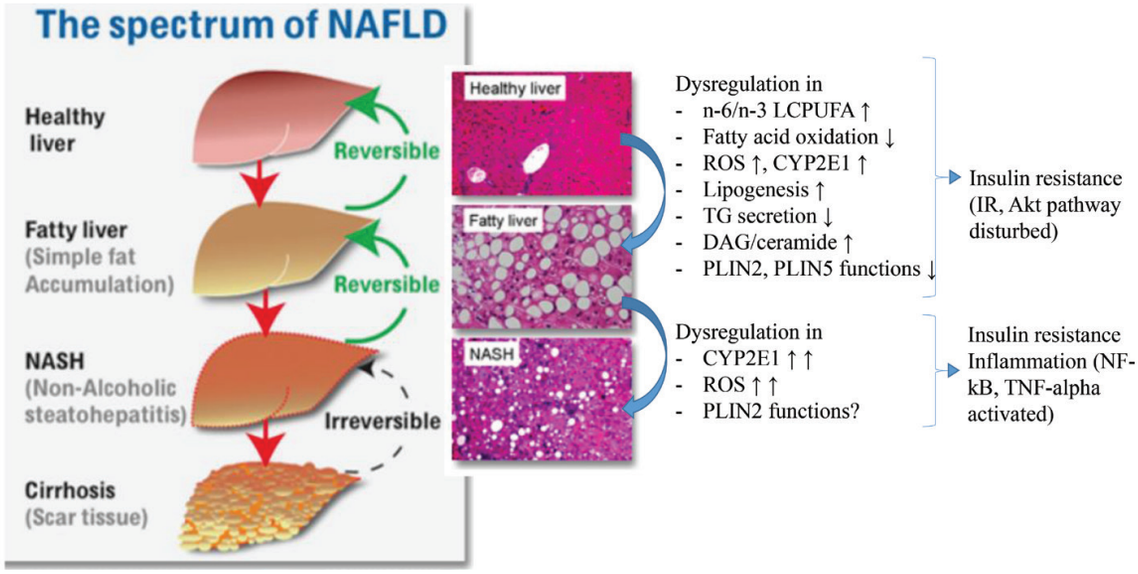


Figure 7. Dysregulated factors during steatosis and NASH development. Hepatic steatosis has many dysregulation in lipid metabolism, leading to insulin resistance. As a result of accelerated cellular damages due to oxidative stress and ER stress, inflammation is installed and NASH development starts.

insulin resistance such as liver-specific microsomal TG transfer protein (MTP) knockout mice [74]. While it is not physiological context at all, it may help to understand the contribution of each gene to the development of hepatic steatosis and insulin resistance at a molecular level. In a steatotic liver from the MTP knockout mice, we had identified upregulations of different lipid droplet proteins such as PLIN5, PLIN2, and SEIPIN. We then hypothesized that the upregulation of these genes was not deleterious in terms of hepatic insulin sensitivity. Indeed, the overexpression of these lipid droplet proteins always induced TG accumulation in hepatocytes; however, none of these *in vitro* models developed apparent insulin resistance. If true, what mechanisms possibly explain the development of hepatic insulin resistance in NAFLD? Different theories are briefly introduced below (**Figures 6** and **7**).

8. Ceramide/Diacylglycerol theory

In a steatotic liver, different lipid species are accumulated such as diacylglycerol (DAG) and ceramide. It was postulated that the accumulation of DAG and/or ceramide induced insulin resistance [75]. However, some of the models of NAFLD present increased hepatic DAG and ceramide without insulin resistance. Interestingly, a recent study demonstrated that the abnormal compartmentalization of these lipid intermediates, rather than total lipid content, is what might truly interfere with the insulin sensitivity [76]. This might support some inconsistent results from various mouse models studied by modulating PLINs (**Table 2**). DAG induces PKC epsilon translocation to the plasma membrane, inhibiting the intracellular kinase domain of the insulin receptor [77, 78]. In the case of PLIN5 overexpressed liver that was dissociated from hepatic insulin resistance, we did not observe the PKC epsilon translocation to the plasma membrane despite significant increase in DAG (personal observation). Accumulation of ceramides in the plasma membrane has also been demonstrated to disturb insulin signaling [79], and we have only observed significant increase in some of ceramides. As the PLIN-induced hepatic steatosis might depend on the type

of PLIN proteins, each phenotype of hepatic steatosis needs to be finely studied. Therefore, the link between DAG/ceramide and hepatic insulin resistance in NAFLD requires more scientific investigations on each specific PLIN protein modification.

9. Autophagy theory

Selective autophagy process for a degradation of intracellular lipids is called lipophagy. The lipophagy is an additional mechanism that contributes to lipid droplet breakdown, and PLIN2 has been shown to play a role in this process [80, 81]. Autophagy is also involved in delivering fatty acids to lipid droplets for a lipolysis and mitochondrial oxidation [82, 83]. Inhibition of autophagy has been shown to accelerate lipid accumulation and impair beta-oxidation in the liver [84, 85]. Interestingly, *atg7* inhibition via shRNA in a mouse liver resulted in an impairment of autophagy system together with an impaired insulin signaling and an induced ER stress [85]. These effects were completely reversed when a downstream target *atg5* was blocked. This strongly supports an idea that autophagy plays one of a key role in insulin signaling pathway. How the lipophagy and lipid droplet proteins are involved in the insulin resistance are still open questions.

10. ER stress theory

Hepatic ER deals with redox regulation, glucose deprivation, protein synthesis, VLDL assembly and secretion, and cholesterol biosynthesis. It has been suggested that ER stress is implicated in insulin resistance, inflammation, and lipotoxicity, which are frequently observed in NAFLD patients [86]. Recently, Akoumi et al. tested a hypothesis that palmitate might induce ER stress by disturbing lipid droplet formation in cytosol and lead to an abnormal accumulation of lipid in an ER compartment. They found in cardiomyoblast cell line that palmitate-induced ER stress was associated with an abnormal storage of DAGs located in the ER. Concomitantly, significant degradation of PLIN2 but not PLIN3 or PLIN5 was observed, suggesting a potential scenario of PLIN2 as a protector/regulator of ER stress. Pharmacological ER stress also demonstrated a role of PLIN2 in ER stress-induced lipogenesis [87]. Author suggested that the presence (or induction) of PLIN2 during the ER stress might protect hepatocytes by storing lipids in a cytosolic compartment. It was a very tentative hypothesis; however, it was not supported at least in *Saccharomyces cerevisiae* that an absence of lipid droplet formation did not affect cell viability during ER stress [88]. There are still many remaining questions to be answered in the field of ER stress and the role of lipid droplet proteins. Their implication on metabolic disease such as NAFLD is one of a key aspect to be further investigated.

11. Oxidative stress theory

Reactive oxygen species (ROS) is produced in a highly regulated manner in multiple organelles such as the ER and mitochondria. ROS is produced as a result of oxidative protein

folding and mitochondrial respiration. NAFLD, especially NASH (nonalcoholic steatohepatitis), has been strongly linked with the biomarkers of oxidative stress [89, 90]. In a case of NAFLD development, beta-oxidation can be abnormally stimulated due to an excess fat accumulation, which surcharges mitochondrial system for the oxidation. As a result, abnormal ROS production at complex I of the mitochondrial electron-transport chain is induced, leading to the mitochondrial oxidative damage. Under such condition, the redox imbalance, lower antioxidant potential, and an enhanced free-radical activity lead to a significant reduction in systemic antioxidant capacity of plasma [91]. These conditions are indeed considered as a trigger of "second hit," which then induces NASH. Cytochrome P450 E12 (CYP2E1), a member of the cytochrome P450 mixed-function oxidase system, has been found as a marker of NASH, which distinguishes NASH from hepatic steatosis [92]. The induction of liver microsomal CYP2E1 contributes as a major free-radical source that aggravates oxidative stress in NASH. In addition to ROS production, cytokine is produced progressing a fatty liver to NASH. A depletion of *n*-3 long-chain polyunsaturated fatty acids (LCPUFA) was also found in NAFLD/NASH. *n*-3 LCPUFA has been shown to highly influence signaling pathway in a liver and contributes to NAFLD development [93, 94]. The *n*-3 LCPUFA downregulates sterol regulatory element-binding protein-1 (SRBBP-1), therefore inhibiting *de novo* lipogenesis. It could also act as a ligand activators of PPAR-alpha, therefore stimulating fatty acid oxidation. The decreased n-3 LCPUFA and increased ratio of n-6/n-3 LCPUFA were reported in NAFLD/NASH patients. These conditions accelerate high oxidative stress and hepatocellular injury. Interestingly, Fujii et al. demonstrated in NAFLD/NASH patients that PLIN2 seemed preferentially expressed in droplets of ballooned hepatocytes. The presence of PLIN2-positive ballooned hepatocytes was indeed correlated with inflammation [18]. Indeed, liver-specific knockout of PLIN2 in mice had reduced hepatic inflammation [95]. The role of PLIN2 on hepatic inflammation in NAFLD/NASH needs further investigation.

As indicated previously, PLIN5 has strong interactions with mitochondrial functions. Zheng et al. studied myocardium from PLIN5-deficient mice and found that the ROS production and malondialdehyde levels, a marker for oxidative stress, were significantly increased [96]. In this model, the phosphorylation of PI3K and Akt, which was induced by ischemia/reperfusion injury, was greatly reduced by PLIN5 deletion in the myocardium. It remains an open question whether the PLIN5 may have an impact on NASH development by interfering with the ROS production.

12. Conclusion

Research on lipid droplet proteins and lipid droplet biology has gained strong insights during the last decades. Most of the lipid droplet proteins are induced in NAFLD and required for a normal adipogenesis. Given specific expression patterns and roles of lipid droplet proteins, the phenotypes lead by experimental modifications of the lipid droplets proteins displayed diverse patterns. Further research is required to clarify their roles in NAFLD, specially by focusing on interactions of different lipid droplet proteins and other functional

proteins, lipid droplet localization, interaction with mitochondria as well as fatty acid compositions in the droplets. Their roles in mitochondrial physiology are a particular importance to understand how lipid droplet protein could influence hepatic energy metabolism and insulin signaling.

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