THE ROLE OF ADIPOSE TRIGLYCERIDE LIPASE IN HEPATIC LIPID METABOLISM, NON-ALCOHOLIC FATTY LIVER DISEASE AND INSULIN RESISTANCE

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Abstract

Hepatic triglyceride (TAG) accumulation leads to the development of non-alcoholic fatty liver disease (NAFLD), which is strongly correlated with other metabolic diseases including obesity, insulin resistance and type II diabetes. While the TAG synthetic pathway has been well-researched, our knowledge of the TAG hydrolysis pathway, especially in the liver, is scant. The research project is aimed at understanding the role and mechanisms of hepatic adipose triglyceride lipase (ATGL) and its downstream lipid metabolites in mediating the development of NAFLD and insulin resistance. To elucidate the metabolic functions of hepatic ATGL, we employed adenovirus-mediated knockdown and overexpression in primary hepatocyte cultures and mouse models. We have shown that ATGL is a key TAG hydrolase in the liver that preferentially channels fatty acids (FAs) to mitochondrial β-oxidation, but does not affect VLDL synthesis and secretion. Additionally, ATGL positively regulates PPAR-α and its target gene expression to influence β-oxidation transcriptionally. Liver FA binding protein (LFABP), a major intracellular FA carrier, is not necessary for ATGL-regulated changes in the expression of PPAR-α and its target genes or for the shuttling of hydrolyzed FA to the mitochondria. Moreover, the PPAR-α agonist fenofibrate is unable to normalize the expression of PPAR-α target genes in ATGL knockdown mice, suggesting that ATGL regulates PPAR-α target gene expression in a LFABP- and ligand-independent mechanism. Interestingly, despite enhanced TAG content, mice lacking hepatic ATGL are actually more

glucose tolerant without exhibiting impaired insulin signaling. ATGL knockdown also normalizes glucose intolerance in HF diet-induced obese mice. Hepatocytes isolated from mice receiving ATGL knockdown adenovirus display higher glucose oxidation and lower glucose production compared to control cells. Thus, hepatic ATGL knockdown enhances glucose tolerance by increasing hepatic glucose utilization, and uncouples impairments in insulin action from hepatic TAG accumulation. Taken together, hepatic ATGL is a major player in TAG catabolism and FA oxidation. Further investigation is warranted to understand the mechanisms through which ATGL mediates FA oxidation, PPAR-α activity and the uncoupling of hepatic TAG accumulation from impaired insulin signaling and insulin resistance.

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CHAPTER 1

The Role of Hepatic Lipolysis in the Development of Fatty Liver Disease and Insulin Resistance

Kuok Teong Ong wrote this chapter in its entirety.

Non-alcoholic Fatty Liver Disease

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease and is comprised of several stages including its most mild form, hepatic steatosis, which is frequently characterized by increased hepatic triglyceride (TAG) accumulation. In clinical terms, NAFLD is defined by hepatic fat content exceeding 5% to 10% by weight (1). NAFLD can advance to more severe stages of liver diseases such as nonalcoholic steatohepatitis (NASH), cirrhosis and fibrosis that are characterized by inflammation, hepatocyte injury and organ failure (2). NAFLD has been associated with numerous metabolic diseases including insulin resistance, diabetes and obesity, which along with other abnormalities such as increased blood pressure, elevated levels of blood TAG and decreased high-density lipoprotein cholesterol are classified as the metabolic syndrome (3).

It is estimated that approximately 20-30% of the general population suffers from NAFLD (4). However, the prevalence of NAFLD in obese patients (BMI ≥ 30) is about 75% while almost every morbidly obese individual (BMI ≥ 40) exhibits NAFLD with 25-70% of them having NASH (5–7). Up to 75% of diabetic patients are found to display hepatic triglyceride accumulation, suggesting that metabolic syndrome and NAFLD are tightly linked (8). In fact, scientific evidence also suggests that NAFLD is one of the pathological outcomes of metabolic diseases (9,10).

Although the mechanism that leads to fatty liver disease remains unclear, the etiology of NAFLD has been linked to insulin resistance (1). Nevertheless, it is still unknown whether insulin resistance or enhanced TAG accumulation develops first. Peripheral insulin resistance may contribute to the development of fatty liver disease by increasing plasma FA, glucose and insulin. In particular, adipocytes resistant to the actions of insulin contribute to the increased rate of triglyceride breakdown and fatty acid (FA) delivery to the liver. Simultaneously, hyperinsulinemic conditions stimulate hepatic TAG synthesis and decrease hepatic FA oxidation by mitochondria. Together, these effects result in FA accumulation in the liver which is counteracted by formation of TAG, leading to hepatic steatosis. In short, hepatic lipid accumulation can be attributed to four metabolic dysfunctions: increased hepatic FA content from adipose TAG, dietary lipids or de novo lipogenesis; enhanced TAG synthesis; decreased FA oxidation; and lack of TAG secretion in the form of VLDL (11).

Lipid Metabolism and Insulin Resistance

While hepatic TAG accumulation is frequently linked to hepatic insulin resistance, some animal models of NAFLD do not exhibit insulin resistance (12–15). These discrepancies may be attributed to the type of lipid intermediates accumulating, the cellular location and FA composition of the metabolites and the pathways leading to hepatic fat accumulation (11). Hepatic insulin resistance is linked to overexpression of hepatic lipoprotein lipase which upregulates FA flux into the liver and enhances hepatic TAG and acyl-CoA accumulation (16). The lipogenesis pathway has been implicated in the pathology of insulin resistance as depicted by protection from hepatic steatosis and insulin resistance in ob/ob mice lacking transcriptional regulators of de novo lipogenesis including sterol regulatory element binding protein 1c (SREBP-1c) or carbohydrate responsive element binding protein (ChREBP) (17,18). Furthermore, it has been shown that enhanced SREBP-1c activity in ob/ob mice stimulates upregulation of glycerol-3-phosphate acyltransferase 1 (GPAT1) mRNA expression and hepatic glycerolipid content (19). Findings from GPAT1 knockout mice and rats with hepatic overexpression of GPAT1 indicate that lipid accumulation from de novo glycerolipid synthesis plays a role in the etiology of hepatic insulin resistance without obesity (20,21). Interestingly, rat hepatocytes overexpressing mitochondrial GPAT1 also exhibit reduced β-oxidation rate suggesting that attenuated FA oxidation may contribute to the development of insulin resistance. Indeed, stimulating β-oxidation by activating peroxisome proliferator-activated receptor-alpha (PPAR-α) with its agonist, Wy-14,643 decreases hepatic TAG

content and enhances insulin sensitivity in ob/ob mice and lipoatrophic A-ZIP/F-1 mice (22,23). However, the relationship between FA oxidation and insulin resistance remains elusive because prolonged fasting or inhibition of a β-oxidation mediator, carnitine palmitoyl transferase I (CPT1) induces only fatty liver phenotype but not insulin resistance (13,24). Several studies reveal that PPAR-α knockout mice fed a HF-coconut oil diet, but not HF-palm oil or HF-lard diet, are protected from hepatic insulin resistance, suggesting that diet type influences the risk of developing insulin resistance (15,25). To that end, rats on HF-salfflower oil diet for only three days exhibit increased hepatic DAG and protein kinase C activity, which is postulated to cause hepatic insulin resistance leading to whole-body insulin resistance (26). Nevertheless, several animal models with elevated hepatic TAG, DAG and ceramide do not appear to be insulin resistant (12,14). A potential explanation for this discrepancy is the existence of divergent pools of lipid metabolites in various cellular locations that regulate differential pathways with insulin signaling being one of them. Specifically, liver specific overexpression of diacylglycerol acyltransferase 2 (DGAT2) induces hepatic steatosis characterized by enhanced hepatic TAG, DAG, and ceramides without affecting markers of glucose tolerance or insulin sensitivity (12). In addition, blocking VLDL secretion from the liver via deletion of microsomal triglyceride transfer protein increases hepatic TAG, DAG and ceramides levels but insulin and glucose action remains normal compared to control mice (14). More research on other pathways associated with lipid

metabolism will be beneficial in elucidating the mechanisms that regulate the development of insulin resistance.

Lipid Droplet Proteins

Lipid droplets (LDs) are intracellular and cytoplasmic organelles that function as a depot for neutral lipids in most types of cells (27). The major constituents of the core of most LDs are TAG and sterol ester, although other lipid forms including free cholesterol, retinol ester, DAG and FA can be found in the LD core (28,29). The core of LD is surrounded by a phospholipid monolayer. Once considered an inert storage space for neutral lipids, LDs have recently been intensely studied. New findings demonstrate that LDs are dynamic intracellular organelles involved in regulating lipid breakdown and numerous metabolic functions, including protein degradation and temporal protein sequestration (27). Our understanding of LD formation and breakdown was greatly advanced with the discovery of LD proteins, notably members of the PAT-domain family proteins. PAT-domain family proteins initially were comprised of perilipin, adipose differentiation-related protein or adipophilin/adipocyte differentiation-related protein (ADRP) and tail-interacting protein of 47 kDA (TIP47), which share a common conserved sequence of amino acids (30). Two additional LD proteins, S3-12 and OXPAT were later added to the PAT group (31–33). A new systematic nomenclature was recently introduced for the PAT proteins as follows: perilipin 1 (PLIN1) for perilipin; perilipin 2 (PLIN2) for adipophilin/ADRP; perilipin 3 (PLIN3) for PP17/TIP47; perilipin 4 (PLIN4) for S3-12, and perilipin 5 (PLIN5) for OXPAT (34).These LD proteins partake in many different activities not limited to lipid

metabolism including signaling, cytoskeletal organization and RNA metabolism, consistent with the notion of LD being a dynamic structure (35–37). Dynamic alteration in morphology of LDs depending on metabolic state may be partially accounted for by the observation of hetereogeneity in PAT proteins between LDs. The most well-studied family member of PAT is PLIN1 which is in expression limited to adipocytes and steroidogenic cells (38). Another important LD protein, PLIN5 is highly detectable in tissues with high rates of FA oxidation such as fasted liver and skeletal muscle (30). The other two founding members of the PAT LD protein group, PLIN2 and PLIN3 are expressed in most cell types although ADRP can only be found minimally in mature adipocytes (39). Taken together, differential localization of LD proteins suggests that each of them may have specialized functions in the regulation of LD metabolism.

It is now known that changes in the regulation of LD physiology and metabolism affect the pathogenesis of metabolic diseases such as diabetes (40). For example, PLIN1 null mice are lean but develop insulin resistance with aging (40). To that end, expressing mutated PLIN1 cells leads to unsuppressed TAG hydrolysis when compared to wild type PLIN1 (41). In agreement with increased lipolytic rate, patients with PLIN1 polymorphisms exhibit reduced body fat mass (40). Thus, further understanding of the mechanisms through which other LD proteins mediate the metabolism of LD may elucidate the pathophysiology of metabolic disorders.

TAG Hydrolysis

While most studies have focused on how TAG synthesis contributes to NAFLD, less is known regarding the role of TAG catabolism in the etiology of NAFLD. Lipolysis is a critical physiological process that provides FA as a substrate for energy via oxidation especially during fasting or famine. High lipolytic activity occurs in the white adipose tissue (WAT) where TAG synthesized to store excess calories is hydrolyzed by a cascade of lipases to yield three FA molecules and one glycerol molecule. Dysregulation of lipid hydrolysis is believed to contribute to the escalating rate of metabolic diseases including obesity, diabetes, insulin resistance, and NAFLD. For example, obese rodents and humans have been shown to exhibit increased basal lipolytic rate but decreased catecholamine-induced lipolytic rate (42). While most studies have focused on the anabolic pathway of lipid metabolism and its contribution to metabolic diseases including NAFLD, very little is known about the role of lipolysis in non-adipose tissue in metabolic syndrome (12,43,44). For that reason, determining the major lipases that facilitate the breakdown of TAG is of paramount importance to shed light into the contribution of lipolysis to the development of metabolic diseases.

The first lipase catalyzing hormone-induced lipolysis to be discovered in white adipose tissue was hormone sensitive lipase (HSL) (45). HSL has a broad substrate specificity towards TAG, DAG, monoacylglycerol (MG), cholesteryl esters and retinyl esters (46) although this enzyme exhibits the highest substrate

preference for DAG and the lowest for TAG. The three major domains of HSL are the C-terminal domain which contains the catalytic triad (serine 423, aspartate 703, and histidine 733), a regulatory domain encoding phosphorylation sites and the N-terminal domain which mediates protein-protein and protein-lipid interactions (47). Regulation of HSL enzyme activity is controlled by PKA-mediated phosphorylation of HSL (47). β-adrenergic stimulation which induces PKA-mediated phosphorylation of HSL enzyme only upregulates HSL enzyme activity by two-fold although it has been shown that β-adrenergic stimulation and PKA activation induces up to a 100-fold induction of FA and glycerol secretion (38). These findings suggest that there must be another regulatory mechanism which determines the enzymatic activity of HSL. Indeed, researchers discovered a protein called perilipin 1 (PLIN1), the first identified LD protein, which is highly expressed in WAT and steroidogenic tissues and interacts directly with HSL (48–50). Early research shows that translocation of HSL to LD and HSL enzyme activation requires dissociation of PLIN1 (51,52). However, researchers have shown that unphosphorylated PLIN1 mutants still enable translocation of HSL to lipid droplet while PLIN1 phosphorylation is needed for normal lipolytic activity of HSL (53). Many other tissues such as liver, cardiac and skeletal muscles do not express PLIN1, indicating that a different LD protein or mechanism is involved in the regulation of HSL activity. Until recently, HSL has been postulated to be the major lipase that catalyzes breakdown of TAG and DAG in most tissues including adipose tissue, skeletal muscles and liver (54). Generation of HSL-knockout mice changed our understanding of the role of HSL. HSL-knockout mice exhibit normal

lipid and energy metabolism and are not overweight or obese (55,56). These transgenic mice, instead, have lower WAT mass and are resistant to diet-induced obesity (57). Interestingly, HSL deficiency only causes DAG but not TAG accumulation which agrees with its high substrate specificity towards DAG (58). This finding implies that HSL is the rate-limiting enzyme for DAG catalysis and there is a separate enzyme that mainly promotes hydrolysis of TAG.

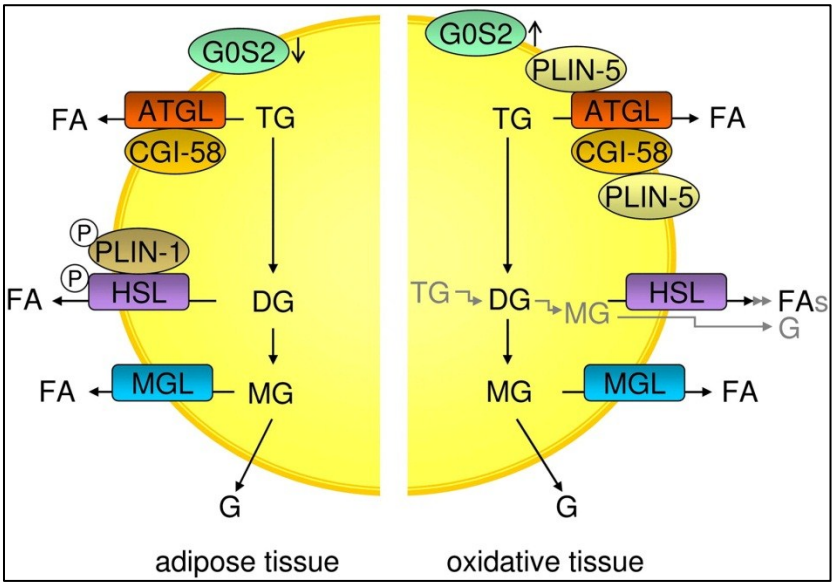

Figure 1. Lipolysis in adipose and oxidative tissues under fasting conditions. β-adrenergic stimulation of lipolysis leads to the hydrolysis of TAG where ATGL cleaves the first ester bond in TAGs, HSL hydrolyzes DAGs, and MG lipase (MGL) breaks down MGs to form both FAs and glycerol. For full hydrolytic activity, ATGL interacts with its coactivator protein CGI-58, whereas HSL translocates to the LD upon phosphorylation, and interacts with phosphorylated PLIN1. In oxidative tissues PLIN1 is not present on LDs. Instead, PLIN5 is expressed and interacts with both ATGL and CGI-58, facilitating LD localization of these proteins.

Figure courtesy of Cell Metabolism © 2012 (59)

Adipose Triglyceride Lipase

Three different groups in 2004 discovered adipose triglyceride lipase (ATGL), which catalyzes the breakdown of TAG (60–62). The nomenclature of this enzyme includes ATGL, desnutrin and calcium-independent phospholipase A2ζ (iPLA2ζ) (60–62). Mammalian ATGL belongs to a group of five phospholipases named patatin-like phospholipase domain-containing 1 to 5 (PNPLA1-5) (63,64). This family of enzymes contains a patatin-domain which encodes the major glycoprotein in potato with known DAG, MG and phospholipase but not TAG hydrolase activity (65,66). Characterization of human ATGL protein shows that the active site of the patatin-containing domain is a catalytic dyad comprising of serine 47 within a canonical GXSXG sequence, and aspartate 166 (67,68). Structural study of the enzyme also demonstrates that the catalytic dyad is embedded within a α/β hydrolase or esterase region (69). It has been postulated that a hydrophobic region mediates the binding of ATGL to LD (61). Similar to HSL enzyme, ATGL also possesses phosphorylation sites, namely serine 404 and serine 428 in the C-terminal region, suggesting that ATGL may possibly be regulated by post-translational modification via phosphorylation (35). Intriguingly, human and murine ATGL only share 84% sequence homology. These differences may partially be explained by the extra 19 amino acids in human ATGL compared to mouse ATGL and the high presence of a proline-rich sequence in the C-terminal region of human ATGL. Early study shows that ATGL has higher substrate specificity for TAG (approximately 10-fold over DAG) compared to HSL in adipose tissue (61). Additionally, ATGL has been shown to

exhibit a lower rate of phospholipase and transacylase activity compared to TAG hydrolase activity (60,70). Unlike HSL, ATGL does not catalyze hydrolysis of MG, CE or RE (61). The physiological and metabolic role of ATGL in lipid metabolism was further elucidated with the generation of ATGL-knockout mice (71). ATGL-knockout mice exhibit a marked decrease in TAG hydrolase activity in WAT and brown adipose tissue (BAT) (82% and 85%, respectively) (71). Furthermore, while basal release of FFAs and glycerol from gonadal white fat explants of ATGL-knockout mice remains similar to that of wild type, isoproterenol-stimulated lipolysis is decreased substantially in ATGL-knockout mice (71). Consistent with decreased TAG hydrolase activity, ATGL-knockout adipose explants incubated with isoproterenol for two hours releases 74% fewer FFAs and 78% less glycerol in comparison with wild type explants. In contrast to HSL-knockout mice, ATGL-knockout mice accumulate TAG in multiple tissues including WAT, BAT, cardiac muscle, kidney and liver, suggesting that ATGL also plays a major role in nonadipose tissues (71). Taken together with findings from previous studies, whole body ATGL deletion in mice pinpoints that ATGL is the rate-limiting enzyme that hydrolyzes TAG while HSL predominantly catalyzes degradation of DAG.

MG lipase (MGL) is known to be the rate-limiting enzyme for the catabolism of MGs derived from extracellular TAG hydrolysis, intracellular TAG hydrolysis and intracellular phospholipid hydrolysis (59). MGL has been shown to localize to cell membranes, cytoplasm and LDs (59). This enzyme is ubiquitously expressed but is found abundantly in adipose tissue. MGL contains

a consensus GXSXG motif within a catalytic triad and also shares homology with esterases, lysophospholipases and haloperoxides (59). In fact, its crystal structure was recently defined and now it is known that MGL exhibits the classic fold of the α/β hydrolases whereby an apolar helix-domain lid mediates the interaction of MGL with its substrate (72,73). Several mutant mouse models have shown that MGL suppression leads to impaired lipolysis and increased MG accumulation in both adipose and non-adipose tissues (74–76). Apart from lipid catabolism, MGL has also been implicated in the hydrolysis of 2-arachidonylglycerol, an abundant endocannabinoid (74,75). Taken together, the lipolytic cascade primarily consists of ATGL, HSL and MGL which sequentially hydrolyze TAG, DAG and MAG, respectively (Fig. 1).

Regulation of ATGL

Whereas regulation of HSL enzyme is well characterized, less is known about the regulatory mechanism of ATGL activity. Studies in WAT from HSL-knockout mice demonstrate that lipolytic rate can still be upregulated by β-adrenergic stimulation despite deletion of HSL in WAT, suggesting that ATGL enzymatic activity may be regulated hormonally (58,77). Nevertheless, unlike HSL, ATGL is not phosphorylated by PKA, although phosphorylation of ATGL is possible on two conserved serine residues (35,61).

Interestingly, the ATGL orthologue in *C. elegans,* ATGL-1 is phosphorylated at multiple sites by AMPK leading to the inactivation of enzyme activity and lifespan extension of *C. elegans* larvae (78). However, AMPK has recently been found to phosphorylate ATGL at serine 406 to activate lipolysis in adipocytes and *in vivo* (79). Nutritional status also influences ATGL mRNA expression whereby mRNA levels are upregulated during fasting but are decreased with feeding (62). Parallel with this observation, administration of insulin reduces ATGL mRNA expression in murine 3T3-L1 adipocytes (80,81). Interestingly, it has been discovered that the ATGL is regulated at the transcriptional level by several nuclear receptors including forkhead box protein O1 (FoxO1) and peroxisome proliferator-activated receptor-γ (PPAR-γ) (80,82). FoxO1 is a transcription factor which plays a role in energy metabolism including gluconeogenesis and its activity is inhibited by insulin and growth factors such as insulin-like growth factor 1 and epidermal growth factor (83). Chakrabarti and Kandror show that FoxO1 directly induces expression of ATGL while inhibition of

FoxO1 is the mechanism by which insulin suppresses ATGL mRNA levels (82). PPAR-γ is a known regulator of adipocyte differentiation and administration of a PPAR-γ agonist, rosiglitazone, upregulates ATGL mRNA expression and the rate of lipolysis in several adipose models (84,85).

A recent study has identified a protein, G_0/G_1 switch gene 2 (G0S2), that negatively regulates ATGL (86). Its function in cell-cycle regulation remains unknown, although it was first discovered to be associated with re-entry of cells from G_0 into G_1 phase (87). G0S2 is highly expressed in WAT, BAT, liver and heart, and plays a role in adipogenic differentiation of preadipocytes as suggested by its upregulation during this process (86,88). G0S2 is hormonally and metabolically regulated whereby its expression increases with insulin, glucose and ligands for the PPAR family and decreases in response to tumor necrosis factor alpha (TNF-α) and β-adrenergic agonist isoproterenol (88–91). Yang et al. demonstrated that overexpressing G0S2 in HeLa cells inhibits LD degradation by ATGL (86). Mutagenesis test and co-immunoprecipitation revealed that ATGL and G0S2 interact directly via the patatin domain of ATGL and hydrophobic domain of G0S2 (86). Interestingly, knockdown of ATGL leads to reduced G0S2 protein levels overall and absence of G0S2 in the LD fraction, implying that interaction with ATGL is required for G0S2 to bind to LD (86). G0S2 also has an inhibitory effect on lipolysis in adipocytes as demonstrated by enhanced basal and isoproterenol-stimulated FFA and glycerol release in G0S2 siRNA-treated 3T3-L1 adipocytes (86). A study has reported that ATGL is a receptor of pigment epithelium-derived factor (PEDF), a multifunctional

glycoprotein involved in neuronal survival and differentiation and possesses anti-angiogenesis and antitumor properties (70). Chung et al. discovered that PEDF not only binds to ATGL but also modulates its TAG hydrolase activity (92). PEDF-null mice and hepatocytes exhibit increased TAG accumulation compared to their controls, but restoration of PEDF expression in hepatocytes reverses steatosis, suggesting that PEDF is an important regulator of ATGL activity (92).

Comparative gene identification-58 (CGI-58), also known as α/β-hydrolase domain containing 5 (ABHD5), has been shown to enhance TAG hydrolase activity of mouse ATGL by 20-fold in COS-7 cells (93). Intriguingly, human ATGL is also activated by CGI-58, but only by approximately 5-fold (93). CGI-58 also contains a canonical esterase/lipase motif but the catalytic serine within GXSXG is replaced by asparagine, thus rendering it without lipase activity (94) . During basal (non-stimulated) state, CGI-58 is tightly bound to LD via interaction with PLIN1 in 3T3-L1 adipocytes (95). Upon β-adrenergic stimulation and phosphorylation of PLIN1 during starvation or fasting, CGI-58 dissociates from LD and disperses to the cytosol (95). Specifically, Miyoshi et al. have demonstrated that phosphorylation of serine-517 on PLIN1 is a required activity prior to hydrolytic activity of ATGL (96). In addition, fluorescence resonance energy transfer experiment demonstrates that CGI-58 colocalizes with ATGL once dissociated from PLIN1 (97). Current findings on CGI-58 suggest that binding of CGI-58 to PLIN1 prevents activation of ATGL while dissociation of CGI-58 allows interaction of CGI-58 to ATGL, thus activating the lipase. In vitro studies show that CGI-58 knockdown decreases PKA-activated FA and glycerol

release substantially (98,99). To that end, overexpression of CGI-58 and ATGL synergistically decreases TAG content in COS-7 cells (93). However, the exact mechanism of ATGL activation by CGI-58 remains unclear. While ATGL lacking its hydrophobic region fails to degrade LD, coexpression of CGI-58 and the mutant form of ATGL results in significant LD breakdown, suggesting that CGI-58 is able to activate the hydrolase activity of ATGL without having ATGL bound to LD surface (100). CGI-58 has also been shown to attenuate the inhibitory effects of G0S2 on ATGL (86). However, the presence of CGI-58 is not sufficient to prevent dose-dependent inhibition of ATGL by G0S2 (86). Moreover, co-immunoprecipitation experiment showed that the presence of CGI-58 does not affect interaction between G0S2 and ATGL (86,100). This observation suggests that CGI-58 and G0S2 do not directly compete with each other in the regulation of ATGL hydrolase activity despite having the N-terminal of ATGL as a common binding site.

Another LD protein, PLIN5 has been shown to facilitate the targeting of CGI-58 to the surface of LD and promotes interaction of CGI-58 with ATGL in cardiomyocytes (101). However, increased interaction between PLIN5 and ATGL decreases lipolysis (102). In agreement with this observation, overexpression of PLIN5 in hepatocytes increases cellular TAG content by decreasing lipolysis and FA oxidation (103). Cardiac-specific PLIN5 overexpression also enhances TAG amount by providing a lipolytic barrier to prevent from excessive release of free FAs (104). Both microscopy and immunoblotting results demonstrate that ADRP inhibits TAG hydrolysis by decreasing the presence of ATGL on the LD surface

(105). Notably, knockdown of both ADRP and TIP47 result in larger but fewer LDs which is attributed to relentless LD fusion and increased localization of ATGL on LDs (106)

Regulation of the localization of ATGL on LD is also an important factor in determining its metabolic role. Studies performed in *Drosophila (D.) melanogaster* L2 cells implicate the function of vesicular transport in ATGL delivery to the LD (107). Specifically, deletion of ADP-ribosylation factor 1, small GTP-binding protein 1, the guanine-nucleotide exchange factor Golgi-Brefeldin A resistance factor, or deficiency of the coatamer protein coat-complex I and II, translocation of ATGL from the ER to LDs is abolished (108,109). Recently, ubiquitin-like domain 8, a membrane-embedded recruitment factor associated with TAG synthesis in the ER has been shown to inhibit the activity of ATGL by recruiting p97/VCP (a hexameric, ring-shaped ATPase segregase) (110). Ubiquitin-like domain 8also directly binds to ATGL and dissociates its coactivator, CGI-58 (110).

Other TAG Hydrolases

Given that ATGL and HSL account for more than 90% of the TAG hydrolytic activity in white adipose tissue, other TAG hydrolases must exist especially in non-adipose tissues, which have low expression of these two lipases (77). In fact, several members of the carboxylesterase and PNPLA families have been shown to possess TAG hydrolase activity. Carboxyl esterase-3 (Ces3) or triglyceride hydrolase-1 (TGH) is highly expressed in the liver and adipose tissue while to a less extent in kidney heart and small intestine (111). Apart from its function in the assembly and secretion of hepatic VLDL (112), Ces3 also plays a role in LD maturation and formation (113). Two members of the PNPLA family, PNPLA4 and PNPLA5 possess TAG hydrolase, DAG transacylase and retinyl ester hydrolase activity in vitro (114). PNPLA3 (also called adiponutrin) shares the highest homology to ATGL (over 50% amino acid homology) and was first discovered as a nutritionally regulated adipose-specific protein (115). PNPLA3 is highly expressed in the adipose tissue (116). PNPLA3 mutation (I148M) in humans is strongly correlated with NAFLD, hepatic fibrosis and liver cirrhosis, indicating that PNPLA3 may be an important TAG lipase (117–119). In addition, PNPLA3 also contains the α−β−α sandwich structure and the GXSXG motif within a catalytic dyad. Consistent with its structure, several studies have shown that PNPLA3 exhibits TAG hydrolase and DAG transacylase activity (60,120,121). However, mice lacking PNPLA3 do not exhibit any overt phenotype pertaining to insulin resistance, NAFLD or obesity (122,123). In accordance to these studies, overexpressing the I148M mutated PNPLA3 enhances TAG

content, but overexpressing wild type PNPLA3 in the liver does not affect hepatic TAG levels (120), suggesting a more complex mechanism underlying the role of PNPLA3 in hepatic TAG metabolism.

Another known TAG hydrolase is arylacetamide deacetylase (AADA), which is located in the lumen of the ER (124) and shares protein sequence homology with the active site of HSL (125). AADA is expressed in the liver and intestine (125). Expression of AADA is upregulated during fasting and is attenuated in in PPARα deficient mice (126). When AADA is overexpressed in McA-RH7777 cells, TAG content is attenuated and FA oxidation is increased while apoB-containing lipoprotein is reduced (124). Based on these studies, there are other hepatic TAG hydrolases that may play a role in the pathogenesis of metabolic abnormalities including insulin resistance and NAFLD.

Current Objectives

Dysfunctional lipid metabolism plays a key role in the development of numerous metabolic diseases. Hepatic TAG accumulation leads to the development of NAFLD, which increases hepatic insulin resistance, glucose synthesis, and VLDL production. These changes increase the risk of cardiovascular diseases and Type 2 Diabetes and play a key role in the development of obesity-related comorbidities given that nearly all obese patients have NAFLD. Therefore, it is of interest to advance our understanding of the mechanisms that are involved in hepatic TAG accumulation in order to prevent or alleviate NAFLD and its associated metabolic complications.

Given that the research examining the pathogenesis of NAFLD has focused on the role of TAG synthesis, the importance of TAG catabolism is relatively unknown. Since the discovery of ATGL in 2004, most of the studies on this lipase have been performed in adipose tissue. Although ATGL is expressed at a lower level in liver compared to adipose tissue, ATGL serves an important role in non-adipose tissue as evidenced by ectopic lipid accumulation in other tissues such as liver and cardiac muscle in ATGL knockout mice even when serum free fatty acid (FA) levels are decreased (71). Furthermore, these knockout mice have lower hepatic TAG hydrolase activity supporting a role for ATGL in hepatic TAG hydrolysis.

In order to investigate the role of hepatic ATGL in energy metabolism and whether its dysfunction may lead to NAFLD and insulin resistance, we utilized adenovirus-mediated approaches to manipulate hepatic ATGL expression in both

mouse and tissue culture models. The studies presented herein aim to answer the following questions: What is the major role of hepatic ATGL in lipid and systemic energy metabolism? Does ATGL contribute to the development of NAFLD and insulin resistance? What are the mechanisms through which ATGL regulates hepatic energy metabolism?

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CHAPTER 2

Adipose Triglyceride Lipase is a Major Hepatic Lipase that Regulates Triacylglycerol Turnover and Fatty Acid Signaling and Partitioning

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Despite advances in our understanding of the ways in which nutrient oversupply and triacylglycerol (TAG) anabolism contribute to hepatic steatosis, little is known about the lipases responsible for regulating hepatic TAG turnover. Recent studies have identified adipose triglyceride lipase (ATGL) as a major lipase in adipose tissue, although its role in the liver is largely unknown. Thus, we tested the contribution of ATGL to hepatic lipid metabolism and signaling. Adenovirus-mediated knockdown of hepatic ATGL resulted in steatosis in mice and decreased hydrolysis of TAG in primary hepatocyte cultures and in vitro assays. In addition to altering TAG hydrolysis, ATGL was shown to play a significant role in partitioning hydrolyzed fatty acids between metabolic pathways. Although ATGL gain and loss of function did not alter hepatic TAG secretion, fatty acid oxidation was increased by ATGL overexpression and decreased by ATGL knockdown. The effects on fatty acid oxidation coincided with decreased expression of peroxisome proliferator-activated receptor alpha (PPAR-α) and its target genes in mice with suppressed hepatic ATGL expression. However, Ppar-α agonism was unable to normalize the effects of ATGL knockdown on Ppar-α target gene expression, and this suggests that ATGL influences PPAR-α activity independently of ligand-induced activation. Conclusion: Taken together, these data show that ATGL is a major hepatic TAG lipase that plays an integral role in fatty acid partitioning and signaling to control energy metabolism.

Introduction

Hepatic steatosis represents the most common form of liver disease in both adults and children in the US (1–3). In addition to being a precursor to fibrosis, cirrhosis and cancer, hepatic steatosis is also tightly linked to Type 2 Diabetes, obesity and cardiovascular disease (4). Because TAG content defines steatosis, the regulation of hepatic lipid metabolism is an integral part of disease etiology. To date, most studies on hepatic steatosis have focused upon enzymes involved in TAG synthesis or how energy oversupply leads to TAG accumulation. However, little is known about lipases responsible for controlling hepatic TAG hydrolysis and how this process contributes to the development of steatosis.

Hormone-sensitive lipase has received the most research attention for its role in regulating lipolysis especially in adipose tissue. However, in 2004, several groups indentified a novel TAG lipase that is highly expressed in adipose tissue (5–7). This lipase, which is commonly known as ATGL (aliases include desnutrin, phospholipase A2-ζ and pigment epithelium-derived factor receptor), is a member of the patatin domain-containing family. Characterization of this lipase revealed that it has high substrate specificity for TAG [10 fold over diacylglycerol (DAG)] especially compared to hormone-sensitive lipase, which preferentially hydrolyzes DAG (6). ATGL null mice have impaired rates of lipolysis and, consequently, have increased adipose tissue mass (6,8). Because of its high expression and prominent role in white adipose tissue, most research has focused upon ATGL in the context of this tissue. However, ATGL is expressed at lower levels in non-adipose tissues such as heart, muscle and liver (6,9). Its

importance outside of adipose tissue is evidenced by the ectopic lipid accumulation in most tissues of ATGL null mice including increased TAG in cardiac muscle (21-fold), skeletal muscle (3-fold) and liver (2.3-fold) (6,8). Despite these changes it is difficult to determine if the effects of global ATGL ablation on hepatic metabolism are direct or due to the broad effects of ATGL on other tissues. In this report, we show that ATGL is an important hepatic lipase that governs TAG turnover and lipid partitioning and signaling to influence the development of steatosis.

Materials and Methods

Animals, diets and adenoviral administration. All animal protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. Eight week old C57/Bl6 male mice were purchased from Jackson Laboratory and housed under controlled temperature and lighting (20-22°C; 12:12-h light-dark cycle). Mice were allowed to acclimate for 1 wk prior to adenoviral injections. Adenoviruses that encode mouse ATGL shRNA and control shRNA that targets non-specific mRNA sequence were generated as described previously (10). Mice were injected with 1 x 10^9 plaque forming units of adenovirus containing ATGL shRNA or non-targeting shRNA control via the tail vein. Mice had free access to water and were fed with either chow (TD.94045) or a 45% fat diet (TD.09404) from Harlan Teklad Premier Laboratory Diets following adenovirus administration. The chow diet contained 19% protein, 64% carbohydrate and 17% fat as a % of total calories and the fat source was soybean oil (70g/kg). The high fat diet contained 19% protein, 35% carbohydrates and 45% fat with lard (195 g/kg) and soybean oil (30 g/kg) comprising the fat sources. Exactly one week following adenovirus injection, mice were sacrificed for tissue and serum collection after an overnight fast. For studies involving administration of fenofibrate, starting one day after adenovirus injection, mice were gavaged daily with fenofibrate (125 mg/kg body weight) suspended in 0.5% carboxymethylcellulose. This dosage of fenofibrate is within the range of doses used to affect PPAR-α and hepatic energy metabolism in mice (11–13).

***Primary hepatocyte isolation and culture*.** Mouse primary hepatocytes were isolated from 10-12 wk old C57/Bl6 male mice with free access to water and chow diet by the collagenase perfusion method. Hepatocytes were isolated and cultured exactly as we have described previously (14).

***Cell adenoviral transduction, radiolabeling, and lipid analysis*.** Adenovirus expressing ATGL or green fluorescent protein (GFP) that serves as a control virus was generated as described previously (10). After 4 h of plating, cells were exposed to either adenovirus expressing GFP or ATGL at 10 MOI for 24 h. For knockdown studies, cells were treated with adenovirus containing ATGL shRNA and control shRNA and were cultured in maintenance media for 66 h unless otherwise noted. After 24 h for overexpression studies and 66 h for knockdown studies, cells were pulsed with 500 μM [1-^{14}C]oleate bound to fatty acid-free BSA in a 3:1 molar ratio for 1.5 h. Some cells were harvested to measure radiolabel incorporation into cellular lipid fractions. Parallel incubations were washed with PBS and the wells were replaced with new media lacking labeled fatty acid for an additional 6 h of chase period followed by collection of cells for lipid extraction. Lipid extracts were further fractionated and radioisotopes quantified (14). Fatty acid oxidation to acid-soluble metabolites (ASM) and CO_2 was measured as described previously (15).

RNA isolation, qRT-PCR and immunoblotting. RNA was processed and gene expression was measured with qRT-PCR (14). For all analysis, gene expression was normalized to ribosomal protein L32 and melting curve analysis was performed on all samples to verify primer specificity. Primers are listed in Supporting Table 1. Electrophoreses and Western blotting of liver samples were performed as described previously (14) using antibodies against ATGL (Cell Signaling Technology) and β-actin (Sigma).

TAG hydrolase activity assay. Liver tissues were homogenized in Buffer 1 (100 mM potassium phosphate, pH 7.0) and were subsequenlty centrifuged at 100,000 x g at 4°C for 1 h. The infranatant layer was isolated and its protein concentration was determined. For preparation of substrate, 445 µg of triolein [oleoyl-9, 10-^{3}H(N)] (60 Ci/mmol) and 33 µg of phosphatidylcholine were reconstituted in Buffer 2 (0.25 mM sucrose, 1 mM EDTA, 1 mM DTT, pH 7.0) and 25% BSA. Subsequently, the substrate was emulsified by sonication. To initiate hydrolase activity assay, 50 µg of total protein lysate in 100 µL of Buffer 1 and 100 µL of substrate were incubated at 37°C for 1 h with shaking. The reaction was terminated by adding 3.25 ml of methanol:chloroform:heptane (10:9:7) and 1 ml of 0.1 M potassium carbonate, 0.1 M boric acid (pH 10.5). Following centrifugation at 800 x *g* for 15 min, 750 µL of the top heptane phase containing radiolabeled fatty acids were counted for radioactivity.

Liver TAG analysis. TAG was extracted from liver tissues or hepatocytes according to a method from Folch (16). Snap-frozen liver tissues were homogenized in sterile water and extracted twice with chloroform:methanol. The chloroform layer was isolated and dried under nitrogen gas and lipids were dissolved in Triton X-100. Liver TAG was quantified with a TAG colorimetric enzymatic kit (Stanbio). For analysis of TAG composition, lipid extracts were separated with TLC as described (17) and bands corresponding to TAG were scraped and methylated with HCl-Methanol. Fatty acid methyl esters were extracted with multiple hexane washes followed by GC analyses with a fused silica capillary column (Supelco Omegawax, model 122-7032), 30 m x 0.25 mm inner diameter (ID) x 0.25 μm film thickness, and Hewlett-Packard Agilent 5890 GC system with FID. The temperature program was as follows: 50°C with a 2 min hold; ramp: 10°C/min to 250°C with a 15 min hold. Constant pressure of 20 psi was applied throughout the run of 37 min per sample. Analyses were initiated by injection of 1 μL of sample at a split ratio of 20:1 and injector temperature of 250°C. The FID temperature was set at 300°C with air and hydrogen flow rates of 433 and 37 ml/min. Specific fatty acid methyl esters were identified based on retention time using a reference standard purchased from Nu-Chek Prep, Inc.

***Serum measurements*.** Colorimetric enzymatic assay kits were used to measure serum fatty acid levels (Wako Chemicals) and serum TAG and β-hydroxybutyrate (Stanbio). Serum glucose and insulin concentrations were

quantified using a glucose autokit (Wako Chemicals) and Ultra Sensitive mouse insulin ELISA kit (Crystal Chem), respectively.

Determination of hepatic TAG secretion. One week following adenovirus treatment, overnight fasted mice were injected via the tail vein with 500 mg/kg of Tyloxapol (Sigma-Aldrich), an inhibitor of triglyceride clearance. Blood samples were collected 0, 1, 2 and 3 h after Tyloxapol administration and serum TAG was determined as described above.

***Histological analysis*.** Liver tissues were fixed in 10% formalin, embedded in paraffin, sectioned and stained for hematoxylin and eosin. For Oil Red O staining in hepatocytes, plates containing cells were rinsed with PBS followed by fixation of the cells with 3.7% formaldehyde for 2 min. Cells were then washed again with PBS and 0.3% Oil Red O solution (in isopropanol) was added to the plate for 30 min at room temperature to stain the cells. Cells were repeatedly washed with PBS and were subsequently imaged with Carl Zeiss Axiovert 200 inverted fluorescent microscope.

Statistical analysis. Results are expressed as mean ± standard error of the mean. Statistical analysis was performed using unpaired Student t-test. Values of $P<0.05$ were considered statistically significant.

Results

Adenovirus delivery of ATGL shRNA efficiently suppresses ATGL expression and TAG hydrolase activity. To determine the effects of hepatic ATGL, we injected mice via the tail vein with adenoviruses expressing scrambled shRNA (cont shRNA) or shRNA targeted against ATGL (ATGL shRNA). After 7 d, hepatic ATGL mRNA in ATGL shRNA treated mice was reduced ~70% (Fig. 1A) and ATGL protein was suppressed ~80% (Fig. 1B) compared to mice treated with cont shRNA. We next measured hepatic TAG hydrolase activity from the different treatment groups using [^{3}H]trioleate as the substrate. Consistent with a robust knockdown of ATGL, the ATGL shRNA decreased TAG hydrolase activity ~40% relative to controls (Fig. 1C). ATGL knockdown did not influence body weight in mice fed chow or high fat diets for 7 d following transduction (Fig. 1D); a negative control group injected with saline showed similar ATGL expression and body weight as those receiving cont shRNA (data not shown). High fat feeding for 7 d following administration of adenoviruses resulted in a significant increase in gonadal fat pad weight in control mice, however, this increase was completely abrogated in mice treated with ATGL shRNA (Fig. 1E) suggesting that manipulating hepatic ATGL impacted adipose metabolism.

Hepatic ATGL knockdown causes steatosis. Despite its relatively low expression in the liver, ATGL null mice have hepatic steatosis although it is unclear if this is a direct effect of loss of hepatic ATGL or due to changes in

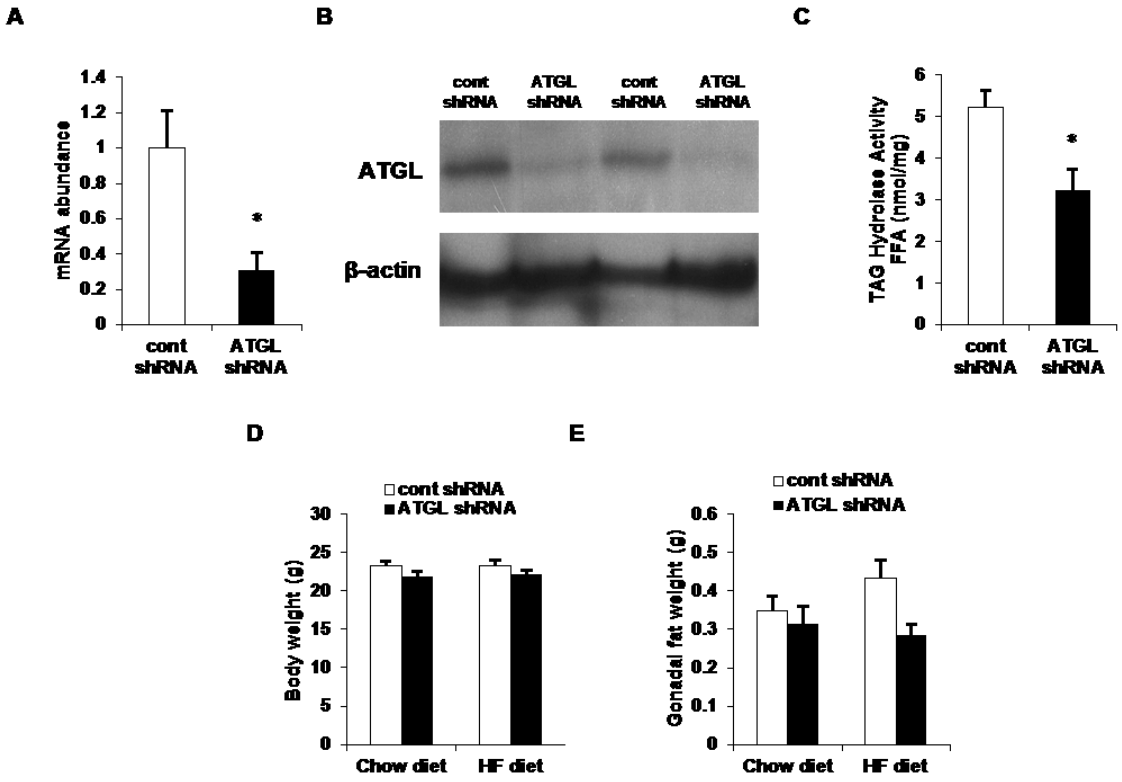


Figure 1. Adenovirus-mediated shRNA treatment suppresses hepatic ATGL expression and TAG hydrolase activity. C57/Bl6 mice at 8-10 weeks of age were infected with control or ATGL shRNA adenovirus (n = 8-10 per group) and were fed with either chow or high fat (HF) diets. After an overnight fast, animals were sacrificed 7 d post-infection followed by determination of mRNA (A) and protein (B) expression of hepatic ATGL liver cytosolic extracts from chow fed mice. (C) Cytosolic extracts were also used to measure hepatic TAG hydrolase activity from chow-fed mice (n = 3-4). Body (D) and gonadal fat (E) weights of mice fed chow or HF diets. Data are presented as means ± SEM. *P<0.05 versus control shRNA group.

extrahepatic metabolism (6, 9). Thus, we evaluated the liver-specific effects of ATGL knockdown on the development of steatosis. After 7 d following adenoviral treatments, ATGL knockdown resulted in a ~30% increase in liver weight (Fig. 2A) regardless of diet. Histological analysis of liver specimens revealed abundant lipid droplet accumulation following ATGL knockdown in chow and high fat diets (Fig. 2B). Further analysis revealed that hepatic TAG content was more than doubled in mice treated with ATGL shRNA confirming that suppression of ATGL leads to steatosis (Fig. 2C). Additionally, the role of ATGL on influencing TAG composition is not known. Therefore, we quantified the composition of fatty acids in TAG from mice treated with cont or ATGL shRNA. Knockdown of ATGL caused a significant reduction in C16:0, C18:0 and C18:3, but increased C18:1 content in TAG by ~40% (Fig. 2D). Thus, ATGL is an important hepatic lipase that regulates both TAG content and composition.

Hepatic ATGL alters TAG turnover. Given the increase in hepatic TAG content and reduced TAG hydrolase activity in mice with suppressed hepatic ATGL expression, we next sought to characterize the effects of ATGL on TAG turnover. To do so, we performed pulse-chase experiments with [1-^{14}C]oleate in primary mouse hepatocytes treated with ATGL knockdown or overexpression adenoviruses. ATGL knockdown did not influence the amount of oleate incorporated into TAG during the 1.5 h pulse period, but blunted the loss of [^{14}C]TAG during the chase period by ~80% compared to cells transduced with control shRNA (Fig. 3A).

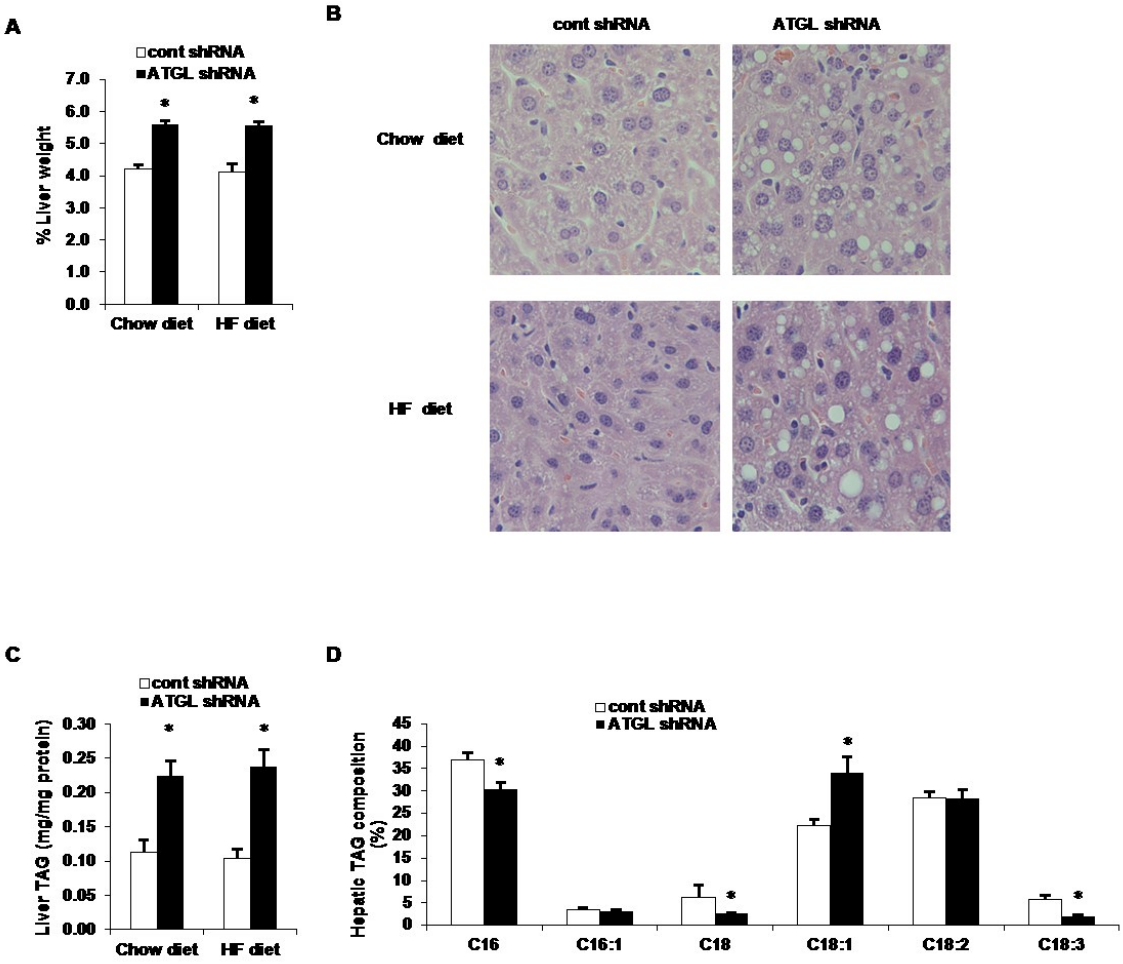
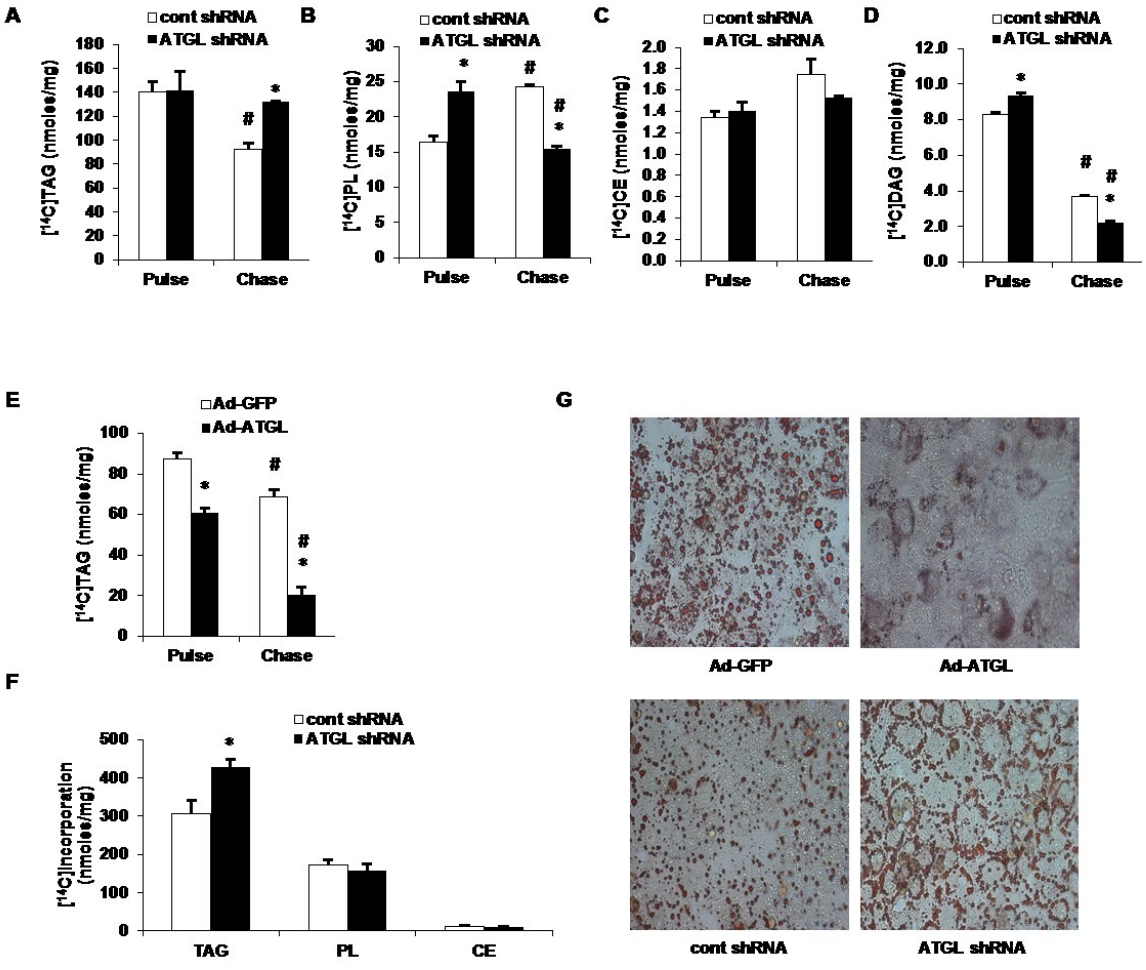


Figure 2. Hepatic ATGL knockdown induces steatosis. (A) Liver weights in mice treated with control or ATGL shRNA in both chow and HF diet groups (n = 8-10). (B) Liver sections from these mice were stained with hematoxylin and eosin (H&E) and imaged at 20X magnification. (C) Liver TAG was quantified using a colorimetric enzymatic assay kit in mice infected with control or ATGL shRNA (n = 8-10). (D) In order to determine composition of TAG, lipids extracted from livers of chow-fed mice were separated by TLC and TAG was methylated with 5% HCl in methanol to produce fatty acid methyl esters (FAMEs), which were analyzed by GC (n = 6). Data are presented as means ± SEM. *P<0.05 versus control shRNA group.

Similar effects of ATGL on hepatic TAG turnover were also observed with a second siRNA (data not shown). ATGL knockdown did not influence cholesterol ester metabolism, but did increase oleate incorporation into PL and DAG during the pulse and enhanced radiolabled PL and DAG loss during the chase period (Fig. 3B-D). Consistent with the above data, pulse-chase experiments in hepatocytes overexpressing ATGL showed that ATGL decreased incorporation of [^{14}C]oleate during the pulse period and increased the rate of [^{14}C]TAG loss during the chase period (Fig. 3E). Longer term (8 h) labeling, which more closely reflects the effects of ATGL on lipid turnover revealed that ATGL knockdown increased [^{14}C]TAG by approximately 30% without affecting PL and CE (Fig. 3F). To further characterize the role of ATGL in mediating fatty acid-induced lipid accumulation, hepatocytes were treated with overexpression or knockdown adenoviruses and then exposed to 500 μM oleate for 30 h. As shown in Fig. 3G, overexpression of ATGL almost completely prevented lipid droplet accumulation as evidenced by Oil Red O staining, whereas, ATGL knockdown resulted in increased lipid droplet formation as expected. Smaller lipid droplets were also observed in cells with suppressed ATGL expression has been previously reported (18).

Figure 3. Hepatic ATGL regulates TAG turnover. Primary mouse hepatocytes were isolated from 8-10 wk old C57/Bl6 chow-fed mice and cells were transduced with control or ATGL shRNA adenovirus for 66 h, at which time pulse (1.5 h) and chase (6 h) experiments were performed with 500 μM [1-^{14}C]oleate. (A) TAG, (B) PL, (C) CE and (D) DAG were isolated from cells by lipid extraction and separated by TLC to measure incorporation of radiolabeled oleate into different lipid species (n = 3-5). (E) Cells were transduced with Ad-GFP and Ad-ATGL virus for 24 h, at which time pulse (1.5 h) and chase (6 h) experiments were performed with 500 μM [1-^{14}C]oleate (n = 3). (F) Cells were transduced with control and ATGL shRNA for 66 h, at which time cells were pulsed with 500 μM [1-^{14}C]oleate for 8 h. TAG, PL & CE were isolated from harvested cells by TLC to measure incorporation of radiolabeled oleate into different lipid species (n = 3). (G) Primary hepatocytes were transduced with ATGL knockdown or overexpression adenovirus as described above and then exposed to 500 μM of oleate for 30 h, at which time cells were washed, fixed and stained with Oil Red O followed by imaging with light microscopy at 20X magnification (representative of 3 experiments). Data are presented as means ± SEM. CE, cholesteryl ester; DAG, diacylglycerol; PL, phospholipid. *P< 0.05 versus control shRNA group. $^{\#}$P<0.05 versus pulse period.


A
□cont shRNA
■ATGL shRNA
[14C]TAG (nmoles/mg)
Pulse
Chase
B
[14C]PL (nmoles/mg)
C
[14C]CE (nmoles/mg)
D
[14C]DAG (nmoles/mg)
E
□Ad-GFP
■Ad-ATGL
F
[14C]incorporation (nmoles/mg)
TAG
PL
CE
G
Ad-GFP
Ad-ATGL
cont shRNA
ATGL shRNA

***Hepatic ATGL does not influence TAG secretion*.** To gain insight into the effects of hepatic ATGL knockdown on whole-body fatty acid metabolism, we quantified serum FFA and TAG. Regardless of diet, the concentrations of these serum metabolites were unaltered following hepatic ATGL knockdown (Fig. 4A,B). Previous studies have shown that cytosolic TAG undergoes hydrolysis to DAG or monoacylglycerol prior to resterification and incorporation into VLDL (19). Thus, it was surprising that mice treated with ATGL shRNA had unaltered fasted serum TAG levels despite having attenuated hepatic TAG hydrolysis. Based upon these findings, we next questioned if ATGL regulates the rate of hepatic TAG secretion. To quantify rates of hepatic TAG secretion, fasted mice were injected with Tyloxapol, an inhibitor of lipoprotein lipase. In agreement with the similar fasting TAG values between treatment groups, rates of hepatic TAG secretion were unchanged in response to ATGL knockdown (Fig. 4C).

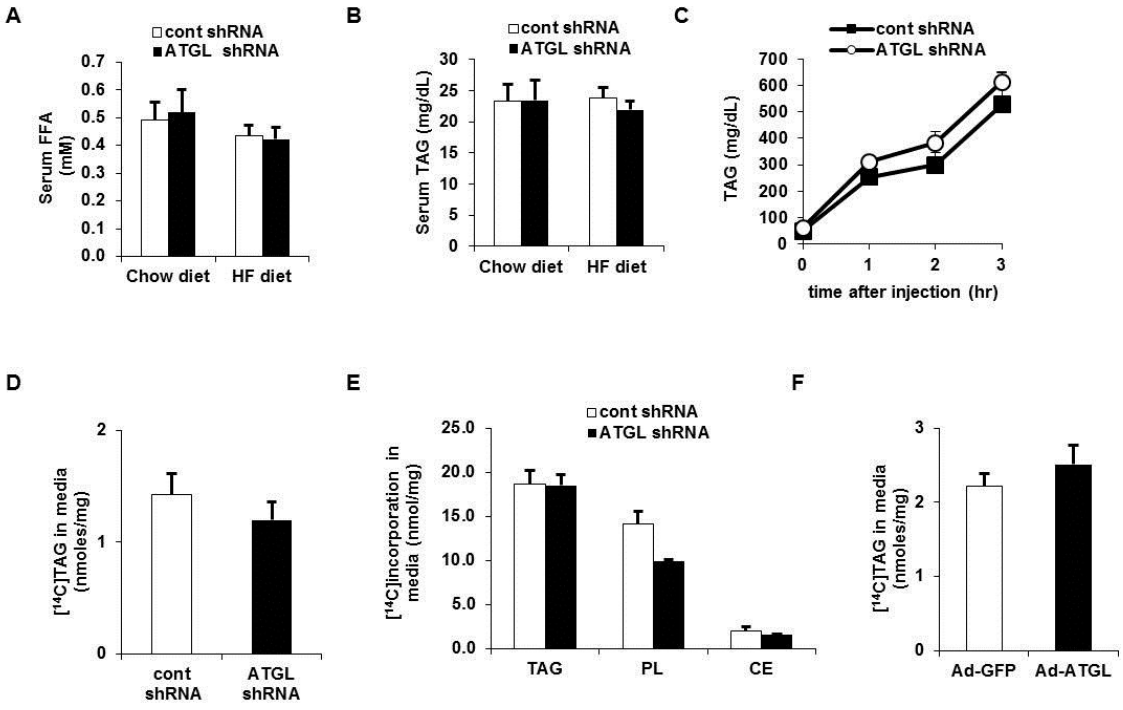
We next extended these studies to primary hepatocyte cultures. Cells were pulsed with 500 μM [1-^{14}C]oleate for 1.5 h, at which time media was changed and appearance of [^{14}C]TAG in the media was measured after 6 h to quantify the contribution of ATGL and TAG hydrolysis to hepatic TAG secretion. In support of the *in vivo* data, ATGL knockdown did not alter TAG secretion in primary hepatocytes (Fig. 4D). Similar results were obtained when cells were labeled with [1-^{14}C]oleate for 8 h (Fig. 4E). We also overexpressed ATGL and found that despite enhanced TAG hydrolysis (Fig. 3E) ATGL overexpression did not alter TAG secretion (Fig. 4F). Thus, despite potent effects on intracellular

TAG hydrolysis, ATGL does not appear to be involved in channeling hydrolyzed fatty acids to VLDL synthesis.

Hepatic ATGL promotes fatty acid oxidation. Intracellular fatty acids have two predominant routes of disposal in the liver, export via VLDL and β-oxidation. Given the pronounced effects of ATGL on hepatic TAG turnover, we next explored if ATGL influenced fatty acid oxidation. Serum β-hydroxybuytrate concentrations were similar in mice treated with control or ATGL shRNA suggesting that perhaps hepatic β-oxidation was not influenced by hepatic ATGL (Fig 5A). However, ATGL knockdown in primary hepatocytes resulted in a 30% decrease in ASM production when cells were pulsed with 500 μM [1-^{14}C]oleate (Fig. 5B). However, these data reflect primarily oxidation of fatty acid derived from exogenous uptake.

Thus, we also quantified fatty acid oxidation during the chase period, which reflects oxidation of fatty acids derived primarily from intracellular TAG hydrolysis. Under these conditions, ATGL shRNA resulted in a ~70% decrease in fatty acid oxidation to both ASM and CO_2 compared with cells treated with control shRNA (Fig. 5B,C). Similarly, we also measured fatty acid oxidation during pulse and chase periods following ATGL overexpression. In support of the above experiments, ATGL overexpression increased fatty acid oxidation to ASM 40% during the pulse period and caused an even more robust increase in fatty acid oxidation to both ASM and CO_2 during the chase period (Fig. 5D,E). Previously, we have shown that overexpression of ATGL in rat hepatocytes increases PPAR-

Figure 4. Hepatic ATGL does not regulate TAG secretion. Mice fed the chow diet were treated with control or ATGL shRNA adenovirus and were fasted overnight followed by harvesting of serum for analysis of (A) free fatty acid (FFA) and (B) TAG (n = 8-10). (C) For measurement of hepatic TAG production, overnight fasted mice fed the chow diet were treated with Tyloxapol and blood collections were performed at 0, 1, 2 and 3 h intervals (n = 4-6) and serum TAG was subsequently analyzed. (D) Primary hepatocytes were transduced with control or ATGL shRNA for 66 h, at which time pulse (1.5 h) and chase (6 h) experiments were performed with 500 μM [1-^{14}C]oleate. [^{14}C]TAG was quantified during the chase period. (E) Primary hepatocytes were transduced with control or ATGL shRNA for 66 h followed by 8 h of pulse with 500 μM [1-^{14}C]oleate. Cells were harvested and radiolabeled oleate incorporation into different lipid fractions was determined (n = 3). (F) Primary hepatocytes were also transduced with Ad-GFP or Ad-ATGL adenoviruses and after 24 h pulse (1.5 h) and chase (6 h) experiments were performed with 500 μM [1-^{14}C]oleate and [^{14}C]TAG secretion during the chase period was measured. Data are presented as means ± SEM.


A
cont shRNA
ATGL shRNA
Serum FFA (mM)
0.7
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0.3
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Chow diet
HF diet
B
cont shRNA
ATGL shRNA
Serum TAG (mg/dL)
30
25
20
15
10
5
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Chow diet
HF diet
C
cont shRNA
ATGL shRNA
TAG (mg/dL)
700
600
500
400
300
200
100
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1
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time after injection (hr)
D
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2
1
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cont shRNA
ATGL shRNA
E
cont shRNA
ATGL shRNA
[14C]incorporation in media (nmol/mg)
25.0
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TAG
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[14C]TAG in media (nmoles/mg)
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α activity (17). Moreover, gene array studies show that ATGL null mice have decreased expression of genes involved in fatty acid β-oxidation in numerous tissues (20). Since ATGL knockdown decreased fatty acid oxidation, a principal target pathway of PPAR-α, we examined the effects of ATGL knockdown *in vivo* on the expression of PPAR-α and its target genes. After 7 d following transduction, the mRNA abundance of PPAR-α and its target genes involved in fatty acid oxidation and utilization (CPT1, ACOT1, ACSL1, LCAD) and gluconeogenesis (PEPCK, PC) were decreased ~40-70% (Fig. 6A). Taken together, these data show that hepatic ATGL promotes fatty acid oxidation through altered channeling of hydrolyzed fatty acids and through changes in oxidative gene expression.

ATGL regulates fatty acid oxidation independent of PPAR-α agonism. PPAR-α is activated by numerous endogenous ligands including free fatty acids (21). Given that ATGL promotes production of fatty acids from TAG hydrolysis, it is logical to speculate that ATGL mediates PPAR-α by supplying fatty acid ligands. Thus, to test this hypothesis, mice treated with the control or ATGL shRNA adenoviruses were given daily oral gavages of carboxymethycellulose (vehicle) or 125 mg/kg fenofibrate, a PPAR-α agonist, following adenovirus administration. Fenofibrate administration had no effect on food intake or body weight (data not shown), but increased liver weight 30% (Figure 7A) as previously reported by others (22). Fenofibrate was unable to normalize liver weight or liver TAG content in ATGL shRNA treated mice compared to those

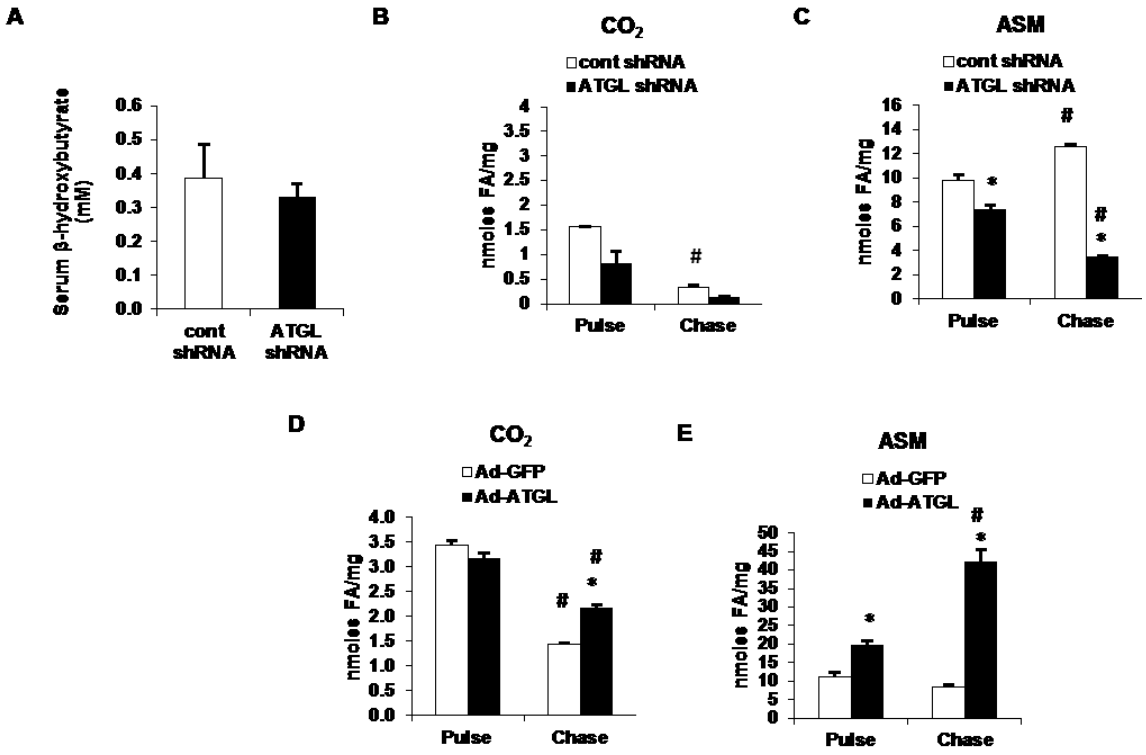

Figure 5. Hepatic ATGL promotes fatty acid oxidation. (A) Serum β-hydroxybutyrate was measured with a colorimetric enzymatic kit in serum samples collected from chow-fed mice 7 d after infection with control or ATGL shRNA adenovirus and following an overnight fast. Primary hepatocytes isolated from chow-fed mice were transduced with control or ATGL shRNA for 66 h and Ad-GFP or Ad-ATGL for 24 h, at which time pulse (1.5 h) and chase (6 h) experiments were performed with 500 μM [1-^{14}C]oleate. (B-E) Media from hepatocytes were harvested after pulse and chase, and CO_2 and ASM were quantified as outlined in the experimental procedures to measure fatty acid oxidation. Data are presented as means ± SEM. *P<0.05 versus control shRNA group. $^{\#}$P<0.05 versus pulse period.

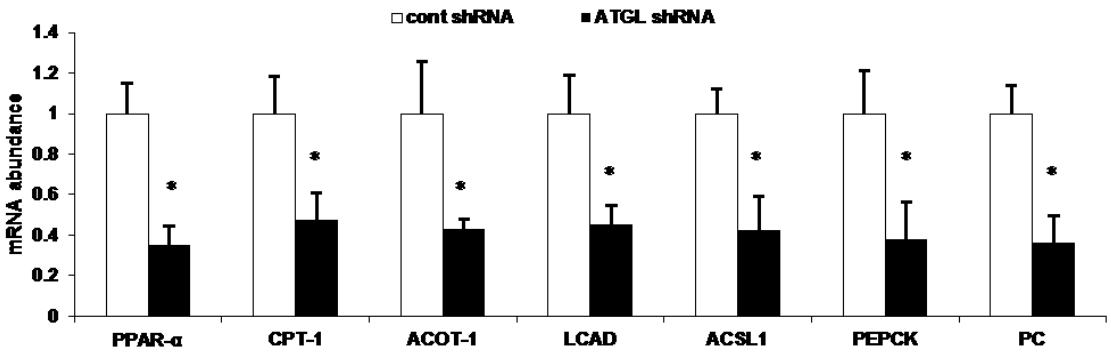

Figure 6. Hepatic ATGL regulates oxidative gene expression. Abundance of mRNA of PPAR-α and its target genes was quantified with qRT-PCR using the ΔΔCT method in livers of mice fed the chow diet for 7 d after adenoviral transduction. Data are presented as means ± SEM. *P<0.05 versus control shRNA group. PPAR-α, peroxisome proliferator-activated receptor alpha; CPT1, carnitine palmitoyltransferase I; ACOT-1, acyl-CoA thioesterase I; LCAD, long-chain acyl-CoA dehydrogenase; ACSL1, acyl-CoA synthetase 1; PEPCK, phosphoenolpyruvate carboxykinase; PC, pyruvate carboxylase.

treated with control shRNA (Fig. 7A,B). Fenofibrate caused a similar fold increase in PPAR-α target gene expression in both treatment groups, but was unable to overcome the decreased expression of PPAR-α target genes in mice treated with ATGL shRNA (Fig. 7C) suggesting that ATGL regulates PPAR-α through a ligand-independent mechanism.

Table 1. Effects of hepatic lipases and lipid droplet proteins on fatty acid metabolism.

Protein (ref)	**Effects on hepatic TAG hydrolysis**	**Effects on TAG export**	**Effects on β-oxidation**	**Effects on PPAR-α target genes**
TGH (23–25)	Increases	Increases	Decreases	Decreases
AADA[1] (26)	Increases	Decreases/ No change	Increases/ No change	ND[2]
FSP27 (27)	Decreases	No change	Decreases	ND
ADRP (28,29)	Decreases	Decreases	Decreases	ND
ATGL (30)	Increases	No change	Increases	Increases

[1]Studies performed in McArdle-RH7777 hepatoma cells. [2]ND, not determined. TGH, triacylglycerol hydrolase; AADA, arylacetamide deacetylase; FSP27, fat-specific protein-27; ADRP, adipocyte differentiation-related protein.

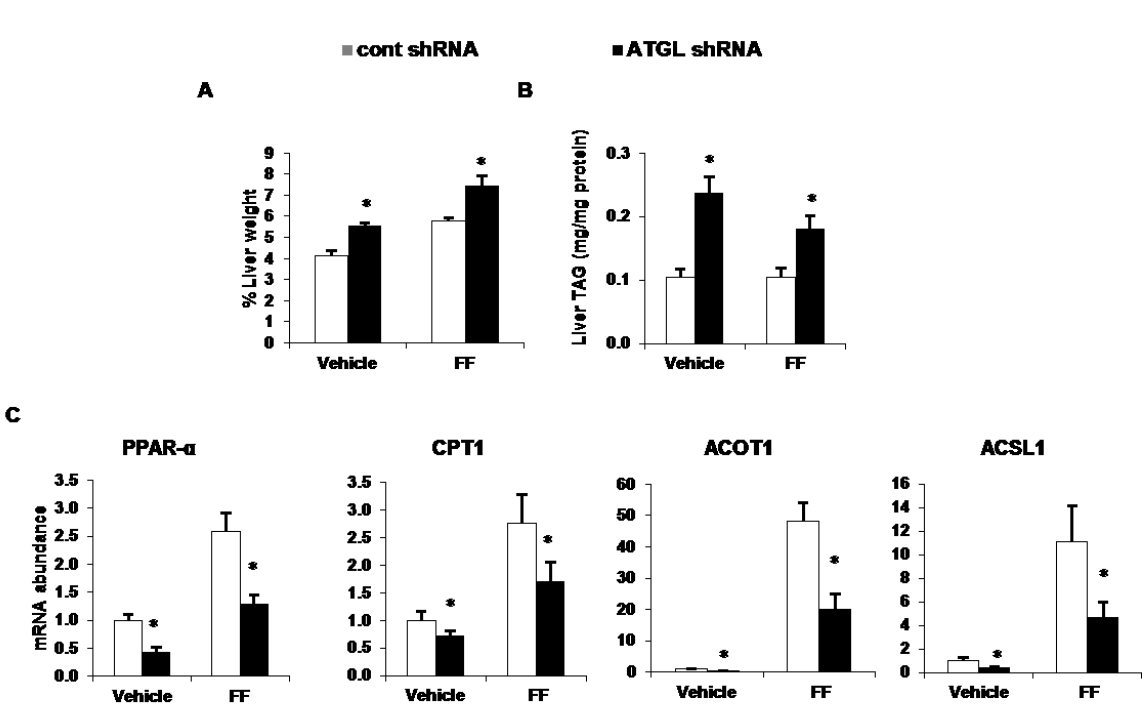

Figure 7. PPAR-α agonism does not rescue the effects of ATGL knockdown. One day after adenoviral injections, mice were treated with 125 mg/kg of fenofibrate suspended in 0.5% carboxylmethylcellulose for 6 d via oral gavage. Mice were fed with the HF diet and sacrificed 7 d after infection following an overnight fast. Liver weight (A) and triglyceride (B) were measured in mice treated with fenofibrate (FF) and compared to vehicle treated mice (n = 7-8). (C) mRNA abundance of oxidative genes was determined in liver tissues of mice treated with FF (n=6). Data are presented as means ± SEM. *P<0.05 versus control shRNA group. #P<0.05 versus pulse period.

Discussion

Despite the importance of hepatic TAG in local and systemic energy metabolism and disease etiology, the major lipases responsible for TAG mobilization in the liver are largely unknown. Herein, we show ATGL to be a principal TAG lipase in the liver consistent with its crucial role in TAG hydrolysis in other tissues such as adipose, heart and muscle. Interestingly, pulse-chase experiments in hepatocytes revealed that ATGL knockdown almost completely blocked TAG hydrolysis. However, livers from mice treated with ATGL shRNA still possessed ~60% of TAG hydrolase activity suggesting that other lipases also contribute to hepatic TAG hydrolysis. Although these studies clearly show that ATGL impacts TAG hydrolysis, the discrepancies between TAG hydrolase activity assays and TAG turnover in cells also suggest that there are many additional factors that contribute to TAG hydrolysis that are not reflected by *in vitro* assays. For example, several proteins such as comparative gene identification-58, pigment epithelium-derived factor, G0/G1 switch gene 2 and numerous lipid droplet proteins have potent effects on ATGL-mediated TAG hydrolysis and are not present on synthetic lipid droplets used for *in vitro* assays (31–33). Thus, it is likely that the contribution of ATGL to hepatic TAG turnover likely varies depending upon the presence of the various inhibitors and activators and may in fact represent more than *in vitro* assays indicate.

The current studies provide evidence that ATGL acts as a branch point in partitioning hydrolyzed fatty acids between oxidative and VLDL synthetic

pathways. As the complexity of the lipid droplet and control of TAG turnover is unraveled, it is becoming apparent that specific lipid droplet proteins and lipases differentially partition fatty acids to distinct metabolic fates. Recent studies have shown that ablation of triacylglycerol hydrolase, which is located exclusively on ER, suppresses hepatic TAG turnover and TAG export, but increases oxidation (23). Coinciding with the increased fatty acid oxidation in mice lacking triacylglycerol hydrolase is an increase in oxidative gene expression. Overexpression of arylacetamide deacetylase, which possesses TAG hydrolase activity, increases hepatic TAG turnover and fatty acid oxidation, but decreases TAG secretion (26). Taken together with the current findings, these data suggest that distinct lipases differentially channel fatty acids between β-oxidation and VLDL synthesis. Additionally, it is unclear how reducing TAG hydrolysis by both ATGL and triacylglycerol hydrolase result in opposing effects on oxidative gene expression. In addition to lipases, several lipid droplet proteins may also contribute to differential partitioning of hydrolyzed fatty acids. Overexpression of fat specific protein-27, a recently identified lipid droplet protein, decreases TAG turnover and fatty acid oxidation without altering TAG export from hepatocytes (27). Despite promoting TAG accumulation, perilipin 5 (also known as OXPAT) also promotes fatty acid oxidation consistent with its high expression levels in oxidative tissues (34). Thus, there appears to be a coordinated regulation of TAG metabolism by lipid droplet proteins and lipases that determines the metabolic fate of hydrolyzed fatty acids (Table 1). Defining the mechanism through which specific pools of TAG are hydrolyzed and channeled between

oxidative and anabolic pathways will provide valuable insight into the regulation of hepatic energy metabolism.

The effects of ATGL on fatty acid channeling highlight the importance of TAG hydrolysis in supplying substrates for β-oxidation. Although intracellular fatty acids can be derived from numerous sources, TAG hydrolysis and exogenous uptake included, the partitioning of these fatty acids between metabolic pathways appears to be dissimilar. Recently, using stable isotope infusions in humans, Kanaley et al. showed that resting muscle preferentially oxidizes fatty acids derived from TAG hydrolysis compared to those derived from exogenous uptake (35). In support, previous work in rat hepatocytes has suggested that fatty acids derived from TAG hydrolysis are more readily oxidized compared to those supplied as fatty acids in the media (36). Given the importance of intracellular TAG in supplying fatty acids for oxidation, our data would suggest that ATGL plays a critical role in controlling substrate oxidation. Additionally, although this study only focused upon male mice, effects of estrogen on ATGL have been reported (37). Thus, it remains to be determined if the effects of ATGL observed in the current study would be similar in female mice.

In addition to its direct effects on TAG hydrolysis, ATGL also appears to influence hepatic energy metabolism through changes in gene expression. The current study shows that ATGL knockdown reduces the expression of genes involved in fatty acid oxidation and gluconeogenesis. Microarray analysis of tissues from global ATGL knockout mice revealed decreased expression of oxidative genes in numerous tissues (20). Additionally, we have previously

shown that ATGL overexpression increases PPAR-α activity in rat hepatocytes (17). Thus, these data suggest that ATGL may modulate fatty acid channeling at least in part through changes in oxidative gene expression. In an attempt to recover oxidative gene expression, mice were gavaged with fenofibrate following administration of adenoviruses. Fenofibrate treatment resulted in a robust induction of oxidative gene expression between both treatment groups, but was unable to normalize gene expression in ATGL shRNA treated groups to those of control animals. Thus, these data suggest that the effects of ATGL on gene expression are independent of PPAR-α ligand binding. Although the mechanism explaining the effects of ATGL on gene expression remain to be elucidated, it is likely that the alterations in gene expression contribute to the metabolic effects of ATGL knockdown. Additionally, it could be postulated that changes in PPAR-α activity could influence fatty acid partitioning through alterations in hepatic TAG export. However, the role of PPAR-α in hepatic TAG secretion is unclear. Studies in mice and hepatocytes show that PPAR-α decreases hepatic TAG secretion (38–40) although not all data are consistent (41). Fibrate administration in humans lowers serum TAG through increased VLDL clearance, but rates of hepatic TAG secretion are unaltered (42,43). Although the direct effects of PPAR-α on hepatic TAG secretion are not resolved, perhaps changes in metabolic enzymes, such as ATGL, may mediate the effects of PPAR-α. Previous studies have shown that hepatic ATGL is activated in response to fasting and that ATGL contains a PPAR-γ responsive peroxisome proliferator

response element (44,45). Ongoing studies will further characterize the transcriptional and post-transcriptional regulation of hepatic ATGL.

The present studies shows that ATGL knockdown results in increased C18:1 and lower C16:0, C18:0 and C18:3 in hepatic TAG. Although we cannot rule out a role for ATGL in selective hepatic fatty acid uptake and esterification or in altering *de novo* fatty acid synthesis, the effects of ATGL knockdown on hepatic TAG fatty acid composition suggest that ATGL may show substrate specificity towards different fatty acids. Given the potent effects of ATGL on fatty acid partitioning and signaling, it is plausible that some of these effects could be due to changes in hydrolysis and metabolism of specific fatty acids rather than ATGL itself.

In summary, this study identifies ATGL as a major hepatic TAG lipase that has important roles in fatty acid trafficking and signaling. Given these important characteristics, alterations in hepatic ATGL expression or activity is likely to contribute to a host of metabolic diseases including non-alcoholic fatty liver disease.

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CHAPTER 3

Hepatic ATGL Knockdown Uncouples Glucose Intolerance from Liver TAG Accumulation

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Adipose triglyceride lipase (ATGL) is the predominant triacylglycerol (TAG) hydrolase in mammals, however, the tissue specific effects of ATGL outside of adipose tissue have not been well characterized. Hence, we tested the contribution of hepatic ATGL on mediating glucose tolerance and insulin action. Glucose or insulin tolerance tests and insulin signaling were performed in C57BL/6 mice administered with control (non-gene specific shRNA) or *Atgl* shRNA adenoviruses. Glucose and lipid metabolism assays were conducted in primary hepatocytes isolated from mice transduced with control or *Atgl* shRNA adenoviruses. Knocking down hepatic ATGL completely abrogated the increase in serum insulin following either 1 or 12 weeks of feeding a high fat (HF) diet despite higher hepatic TAG content. Glucose tolerance tests demonstrated that ATGL knockdown normalized glucose tolerance in HF diet-fed mice. The observed improvements in glucose tolerance were present despite unaltered hepatic insulin signaling and increased liver TAG. Mice with suppressed hepatic ATGL had reduced hepatic glucose production *in vivo* and hepatocytes isolated from *Atgl* shRNA-treated mice displayed a 26% decrease in glucose production and a 38% increase in glucose oxidation compared to control cells. Taken together, these data suggest that hepatic ATGL knockdown enhances glucose tolerance by increasing hepatic glucose utilization and uncouples impairments in insulin action from hepatic TAG accumulation.

Introduction

Insulin resistance is a metabolic state that is predictive of a host of metabolic diseases including Type 2 Diabetes and cardiovascular diseases. Hepatic steatosis has been shown to positively correlate with insulin resistance and may be an important component in the development of metabolic diseases resulting from insulin resistance (1). In fact, excessive liver fat is a stronger predictor of insulin resistance than total or visceral fat in humans (2,3). Until recently, most studies have examined the importance of TAG synthetic enzymes in linking hepatic steatosis to insulin resistance. However, the contribution of dysregulated lipolysis in non-adipose tissue to the pathophysiology of insulin resistance is unclear and has only recently gained attention among researchers.

In 2004, three separate groups discovered a novel lipase, adipose triglyceride lipase (ATGL), with higher substrate specificity for TAG than hormone sensitive lipase (HSL), which was previously thought to be the predominant TAG hydrolase (4–6). Although the expression of ATGL is lower in the liver compared to adipose tissues, we and others have shown that ATGL is a major hepatic TAG hydrolase (7,8). Since ATGL is a key TAG lipase, it would be interesting to determine whether it is involved in the pathogenesis of insulin resistance especially in the event of hepatic steatosis. The role of ATGL in insulin action was first characterized in ATGL knockout (*Atgl* KO) mice, which exhibit increased glucose tolerance and insulin sensitivity (9). This finding was unexpected given that *Atgl* KO mice have increased adipose tissue mass and ectopic TAG

deposition in insulin-sensitive tissues including skeletal muscles, heart and liver (9). Further investigation in *Atgl* KO mice indicated that insulin signaling is enhanced in skeletal muscle and white adipose tissue, but attenuated in liver and brown adipose tissue (10). Increased glucose utilization and uptake in the liver, cardiac muscle and skeletal muscle as a result of an inability to release non-esterified fatty acids (NEFA) in *Atgl* KO mice were proposed to mediate the observed improvement in glucose tolerance and insulin sensitivity.

In addition, Turpin et al. has reported that isolated hepatocytes from *Atgl* KO mice display no change in insulin action (11). However, when hepatic ATGL is overexpressed *in vivo*, whole-body glucose tolerance and Akt phosphorylation are mildly enhanced (11). This observed phenotype may be partially attributed to attenuated gluconeogenesis as depicted by decreased glucose levels during pyruvate tolerance tests and decreased gluconeogenic gene expression. Interestingly, Wu et al. has recently shown that glucose and insulin sensitivity remain unaltered in liver-specific ATGL knockout mice that were fed control or high-fat (HF) diet (8). They also noted that glucose production does not change with liver-specific ATGL deletion (8).

Given the paradoxical phenotypes in the two models and the improvement in glucose tolerance of *Atgl* KO mice, it is important to further examine liver-specific effects of ATGL on whole-body glucose tolerance and insulin action. Herein, we test the contribution of hepatic ATGL to liver energy metabolism and insulin resistance via adenovirus-mediated approach and reveal that ATGL uncouples glucose intolerance and impaired insulin signaling from steatosis.

Materials and Methods

***Animals, diets and adenovirus administration*.** All animal protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. Six to eight week old C57/Bl6 male mice were purchased from Harlan Laboratories and housed under controlled temperature and lighting (20-22°C; 12:12-h light-dark cycle). All mice were acclimatized for 1 week prior to adenovirus injections. Adenoviruses that encoded mouse *Atgl* short hairpin RNA (shRNA) and control shRNA targeting a nonspecific mRNA sequence were generated as described previously (12). The mice were injected with 1 x 10^9 pfu of an adenovirus containing *Atgl* shRNA or a non-targeting shRNA control (cont shRNA) via the tail vein. The mice had free access to water and were fed either a purified control diet (TD.94045) or a 45% fat diet (TD.09404) from Harlan Teklad Premier Laboratory (Madison, WI, USA) after adenovirus administration. The control diet contained protein (19% of total calories), carbohydrates (64%), and fat (17%) in the form of soybean oil (70 g/kg). The high-fat (HF) diet contained protein (19%), carbohydrates (35%) and fat (45%) with lard (195 g/kg) and soybean oil (30 g/kg) as the fat sources. Exactly 1 week following adenovirus injection, the mice were sacrificed for tissue and serum collection after an overnight fast. Another group of 6-8 week old C57/Bl6 male mice were fed either a control or HF diet (diet-induced obese or DIO mice) for 12 weeks. Immediately following the 12 week feeding period, mice were transduced with the control or *Atgl* shRNA adenovirus. Mice were sacrificed 1 week after adenovirus injection

to harvest tissue and serum samples. Mice fed either control or HF diets for 12 weeks were fasted for 4 h prior to experiments or euthanasia and harvesting of tissues and serum.

***Measurement of mitochondrial FA oxidation*.** Liver sections were quickly harvested from anesthetized mice and mitochondria were isolated as described previously (13). Isolated mitochondria were added to reaction medium containing [1-^{14}C]palmitate (2.13 GBq/mmol; Perkin Elmer, Waltham, MA, USA) in a 25 mL flask. The flask was quickly sealed with a double seal stopper and perchloric acid was added. The flask was shaken for 1 h at room temperature. Bovine serum albumin (BSA) and water were added to the acid-treated medium followed by centrifugation twice to separate particulate from supernatant. Supernatant containing [^{14}C]-labeled acid-soluble metabolites (ASM) was quantified with scintillation counting.

***RNA isolation, RT-PCR and real-time quantitative PCR analysis*.** RNA was extracted with Trizol from liver tissues followed by reverse-transcription with SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) to generate cDNA. Gene expression was quantified as described previously (7).

Liver TAG and glycogen analysis and serum measurements. TAG was extracted from liver tissues according to the method described by Folch (14). Liver and serum TAG levels were quantified with a TAG colorimetric enzymatic kit (Stanbio, Boerne, TX, USA). Liver glycogen content was determined as described previously (15). Serum FA and glucose levels were determined with colorimetric enzymatic assay kits from Wako Chemicals (Richmond, VA, USA) while serum insulin concentrations were assessed with Ultra Sensitive mouse insulin ELISA kit from Crystal Chem (Downers Grove, IL, USA).

Oral glucose tolerance and insulin tolerance tests. For glucose tolerance tests, a 20% dextrose solution (2 g of glucose/kg body weight) was administered via oral gavage to the mice. To perform insulin tolerance tests, mice were injected intraperitoneally with 1 U insulin/kg body weight (Humulin N from Eli Lilly, Indianapolis, IN, USA). For pyruvate tolerance tests, mice received 2 g/kg body weight of sterile-filtered sodium pyruvate. Blood glucose concentrations prior to administration and at 15, 30, 60 and 90 min post-injection were measured with AlphaTRAK glucose meter and test strips (Abbott, Chicago, IL, USA).

***Insulin signaling*.** After a 4 h fast, mice were injected intraperitoneally with 1U insulin/kg body weight or saline (vehicle). After 10 min, liver, adipose and muscle tissues were snap frozen from euthanized mice. Liver preparation, electrophoresis and immunoblotting were done as described (7). The following

antibodies were used: anti-β-actin monoclonal (Sigma-Aldrich, Saint Louis, MO, USA); anti-pIRS1 (Tyr989) polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Akt polyclonal, anti-pAkt (Ser473) polyclonal, anti-IRS1 polyclonal, anti-pIRS1 (Ser636/639) polyclonal, anti-GSK-3α and anti-pGSK-3α (Ser21) (Cell Signaling Technology, Danvers, MA, USA). Bands were visualized by ECL chemiluminescense (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Glucose production and oxidation assays. Glucose production was measured as performed previously (16). Briefly, primary hepatocytes were isolated and cultured in M199 plating media (Invitrogen) free of insulin as described previously (7). The medium was then replaced with 1 ml of glucose production buffer consisting of glucose-free DMEM (pH 7.4), without phenol red, supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate (with or without 100 nm insulin). After 3 h, 0.5 ml of medium was collected and the glucose concentration measured with a colorimetric glucose assay (Wako Chemicals) and normalized to the total protein content determined by BCA protein assay (Thermo Scientific, Waltham, MA, USA). To determine glucose oxidation in primary hepatocytes, approximately 5 x 10^5 isolated primary hepatocytes were suspended in Krebs-Ringer-phosphate buffer (KRP) containing 5.5 mM glucose, 1 μCi of [$^{14}C_6$]glucose, 135 mM NaCl, 5.4 mM KCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, and 10 mM sodium pyrophosphate (pH 7.4) in a 25 mL flask. The flask was promptly sealed with a cap containing a center well and filter paper and was shaken in a 37°C water bath. After 1 h, 200 μL of 1M sodium hydroxide was added to the

filter paper-containing well and 200 µL of 20% sulfuric acid transferred to the cell suspension to release any unbound CO_2. The flasks were shaken for an additional 1 h after which the center wells were transferred to vials for scintillation counting.

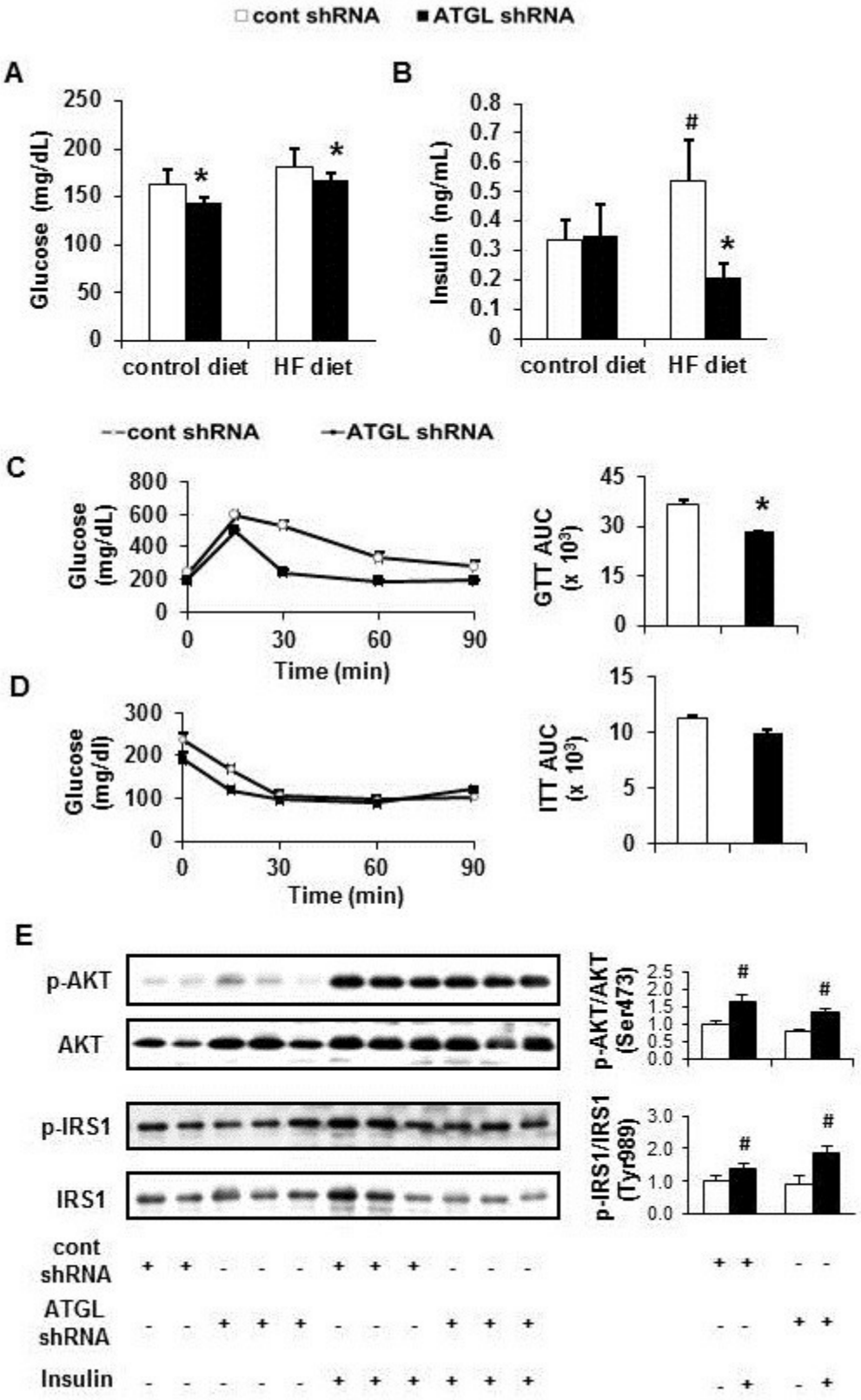
Statistical analysis. Data are expressed as mean ± SE. Statistical analyses were performed using Student's t test or ANOVA where appropriate.

Results

***Hepatic ATGL regulates serum glucose and insulin*.** To examine the effects of hepatic ATGL deficiency on serum markers of glucose tolerance and insulin sensitivity, we measured serum glucose and insulin concentrations in mice administered with adenovirus encoding scrambled shRNA (control shRNA) or shRNA targeting against *Atgl* (*Atgl* shRNA). Previously, we characterized the efficacy of the *Atgl* shRNA adenovirus and reported that hepatic ATGL knockdown in mice results in a ~45% decrease in hepatic TAG hydrolase activity and more than doubles hepatic TAG content using the same treatment regiment as described herein (7). ATGL knockdown decreased serum glucose levels in mice fed either a control or HF diet 7 days after adenovirus administration (Fig. 1A). Additionally, ATGL knockdown completely blocked the increase in serum insulin levels in mice fed the HF diet (Fig. 1B).

***Hepatic ATGL knockdown enhances whole-body glucose tolerance but not hepatic insulin signaling*.** To further characterize the role of hepatic ATGL in modulating glucose tolerance and insulin sensitivity, we performed both oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT). Mice receiving the *Atgl* shRNA adenovirus had lower glucose area under the curve (AUC) compared to control mice suggesting that hepatic ATGL deficiency confers enhanced glucose tolerance (Fig. 1C). Interestingly, there was no difference

Figure 1. Adenoviral delivery of *Atgl* shRNA increases whole-body glucose tolerance without altering hepatic insulin signaling. Serum glucose (A) and insulin (B) levels were measured 7 days after control or *Atgl* shRNA administration in mice fed a control or HF diet (n = 6-10). Following adenovirus administration, mice were fed a HF diet for 6 days followed by an overnight fast prior to oral glucose tolerance test (C) and insulin tolerance test (D) and respective area under the curve (AUC) graphs (n = 5). E) Hepatic insulin signaling was assessed by immunoblotting to determine insulin-stimulated phosphorylation of Akt and IRS1in mice fed a HF diet for 7 days (n = 6-10). $^{*}P < 0.05$ versus control shRNA group and $^{\#}P < 0.05$ versus control diet group or saline-injected group.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p-IRS1
IRS1
p-AKT/AKT (Ser473)
2.5
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1.5
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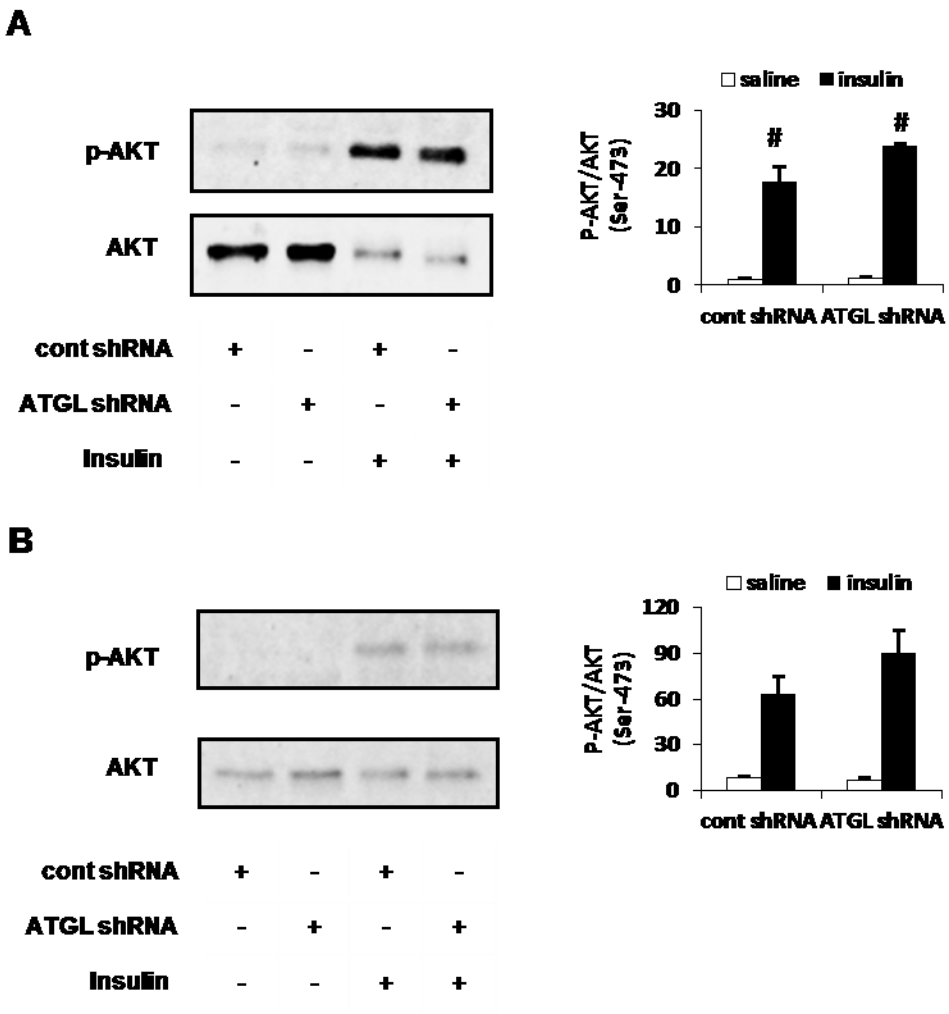
Insulin



Supplemental Figure S1. Adenoviral delivery of *Atgl* shRNA does not alter insulin signaling in adipose or muscle tissues. Following adenovirus administration, mice were fed a HF diet for 7 days and then injected intraperitoneally with 1U insulin/kg body weight or saline (vehicle) after an overnight fast. Hepatic insulin signaling was assessed by immunoblotting to determine insulin-stimulated phosphorylation of Akt in adipose (A) and muscle (B) tissues (n = 6-10). $^{\#}P < 0.05$ versus saline-injected group.

between the two adenovirus-treated groups during the ITT (Fig. 1D). Defective hepatic insulin signaling is frequently observed with increased lipid accumulation in the liver (17,18). ATGL knockdown did not affect insulin-stimulated phosphorylation of protein kinase B (Akt^{Ser473}) or insulin receptor substrate 1 ($IRS\text{-}1^{Tyr989}$) (Fig. 1E). This finding was unexpected given that *Atgl* shRNA-treated mice fed the HF diet exhibited more than a 100% increase in TAG levels when compared to control mice as previously shown (0.24 versus 0.10 mg/mg protein) (7). Insulin-stimulated phosphorylation of protein kinase B (Akt^{Ser473}) in adipose or muscle tissue also remained unchanged when hepatic ATGL was suppressed (Fig. S1). Hence, decreasing hepatic ATGL dissociates hepatic steatosis from insulin resistance.

Hepatic ATGL deficiency exacerbates hepatic steatosis in DIO mice. Given that ATGL knockdown confers glucose tolerance without changing insulin sensitivity following short-term feeding of control or HF diets, we further investigated the effects of adenoviral administration of *Atgl* shRNA on mice fed a HF diet for 12 weeks. To perform this study, we initiated an ad libitum feeding regimen of either a control or HF diet for 12 weeks. Subsequently, mice were given control or *Atgl* shRNA adenovirus injections and sacrificed 1 week later. Administration of the *Atgl* shRNA adenovirus resulted in a robust decrease in *Atgl* mRNA and protein levels regardless of diet (Fig. 2A and 2B). While body weights were not different in mice fed the control diet, hepatic ATGL knockdown decreased body weight by approximately 10% in the DIO mice when compared

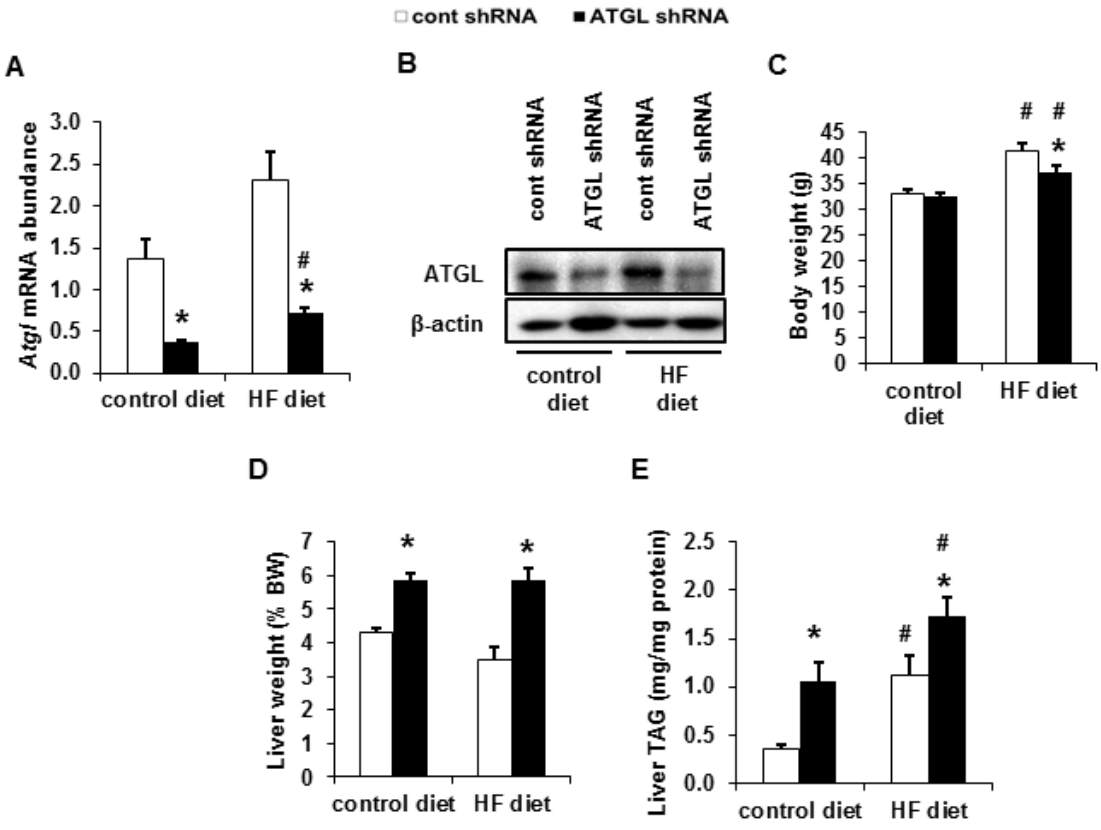


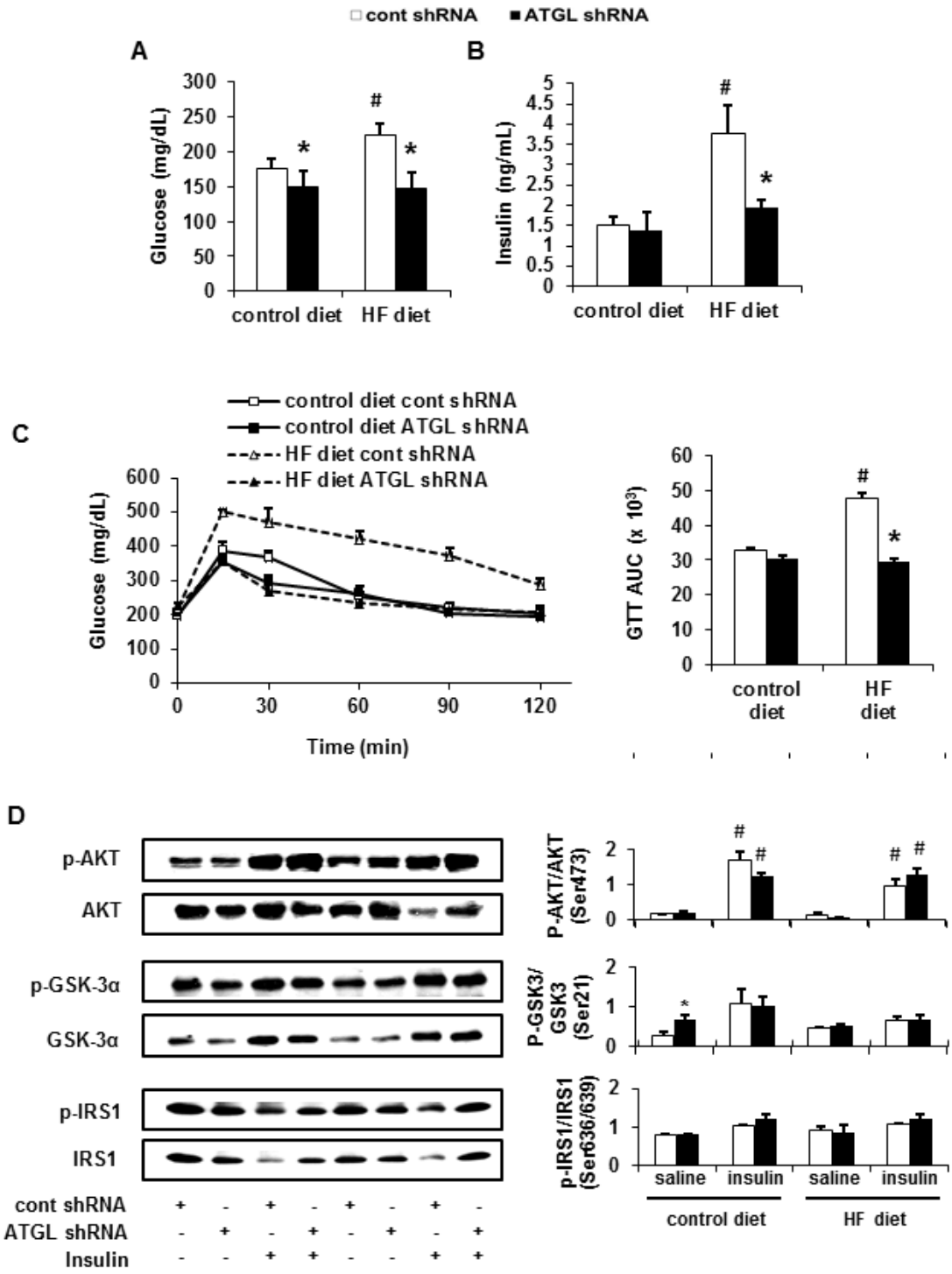
Figure 2. **Adenovirus-mediated *Atgl* shRNA alters tissue weights and liver TAG in DIO mice**. mRNA (A) and protein levels (B) of liver ATGL in mice administered with control or *Atgl* shRNA adenovirus (n= 2-5). Body (C) and liver (D) weights of mice fed control or HF diets for 12 weeks and subsequently transduced with adenoviruses (n = 8-10). E) Liver TAG concentrations were determined with a colorimetric kit (n = 8-10). *P < 0.05 versus control shRNA group and $^{\#}P$ < 0.05 versus control diet group.

to control mice 7 days later (Fig. 2C). Mice treated with *Atgl* shRNA exhibited approximately a 30% increase in liver weight regardless of diet type (Fig. 2D). The changes in liver weight can be explained by increased hepatic TAG content in the control and HF diet-fed mice (Fig. 2E). These findings show that ATGL exacerbates hepatic steatosis under a 12-week HF diet regimen.

Hepatic ATGL mediates serum glucose and insulin in DIO mice. We evaluated serum glucose and insulin levels to examine whether *Atgl* shRNA treatment could normalize serum markers of glucose action and insulin sensitivity in DIO mice. Hepatic ATGL knockdown resulted in decreased serum glucose levels within the DIO group comparable to those of mice fed the control diet (Fig. 3A). Twelve weeks of HF feeding more than doubled serum insulin levels, which were recovered to basal levels by hepatic ATGL knockdown (Fig. 3B). This observation was in agreement with our previous result in mice fed a HF diet for 1 week (Fig. 1B). Overall, the effects of ATGL knockdown were more pronounced in DIO mice.

Hepatic ATGL knockdown normalizes glucose intolerance without altering hepatic insulin signaling in DIO mice. Given that ATGL knockdown decreased serum glucose and insulin levels in DIO mice, we hypothesized that hepatic ATGL knockdown might reverse glucose intolerance commonly observed in DIO mice (19). Thus, we performed OGTT 7 days after DIO or control diet-fed mice

Figure 3. Hepatic ATGL deficiency normalizes glucose intolerance without affecting hepatic insulin signaling in DIO mice. Serum glucose (A) and insulin (B) levels were measured 7 day after control or *Atgl* shRNA administration in mice fed control or HF diets for 12 weeks (n = 5). C) Oral glucose tolerance test and corresponding AUC graph (n = 5). D) Seven days after adenovirus delivery of control or *Atgl* shRNA, mice underwent a 4 h fast and were injected intraperitoneally with saline or insulin at 1 unit /kg of body weight and sacrificed 10 min later. Hepatic insulin signaling was assessed by immunoblotting to determine insulin-stimulated phosphorylation of Akt, GSK-3α and IRS1 (n = 3-5). $^{*}P < 0.05$ versus control shRNA group and $^{\#}P < 0.05$ versus control diet group or saline-injected group.


cont shRNA
ATGL shRNA
A
Glucose (mg/dL)
control diet
HF diet
B
Insulin (ng/mL)
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HF diet cont shRNA
HF diet ATGL shRNA
Glucose (mg/dL)
Time (min)
GTT AUC (x 10³)
control diet
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D
p-AKT
AKT
p-GSK-3α
GSK-3α
p-IRS1
IRS1
cont shRNA
ATGL shRNA
Insulin
P-AKT/AKT (Ser473)
P-GSK3/GSK3 (Ser21)
p-IRS1/IRS1 (Ser636/639)
saline
insulin
saline
insulin
control diet
HF diet

were administered adenoviruses. As expected, serum glucose levels were significantly higher in DIO mice compared to control diet-fed mice receiving control shRNA treatment after an oral glucose load (Fig. 3C). However, hepatic ATGL knockdown completely normalized the glucose response in DIO mice to basal levels. Consistent with previous results in mice fed a short-term HF diet, hepatic steatosis induced by *Atgl* shRNA did not manifest in attenuated activation of insulin cascade mediators including AKT^{Ser473} or $IRS1^{Ser636/639}$ (Fig. 3D). Although activation of glycogen synthase kinase 3 alpha (GSK-$3\alpha^{Ser21}$) was enhanced with ATGL knockdown, this effect was abolished under insulin-stimulated condition. Collectively, these findings show that hepatic ATGL knockdown promotes steatosis, but improves glucose tolerance without influencing hepatic insulin signaling.

***Hepatic ATGL knockdown alters DAG content and composition depending upon dietary treatments*.** DAG is widely accepted as a key lipid metabolite involved in insulin resistance. Given that ATGL activity is a source of cellular DAG, we measured DAG content and composition. ATGL knockdown did not influence hepatic DAG content in DIO mice but resulted in a robust increase in DAG content in mice fed the control diet for 12 weeks (Fig. 4A). Interestingly, gene expression of *Dgat1* remained unchanged while *Dgat2* expression was only decreased in mice fed the HF diet but not the control diet (Fig. 4B and C). In the control diet-fed group, the percentage of palmitate, stearate and arachidonate decreased while oleate increased in response to hepatic ATGL knockdown (Fig.

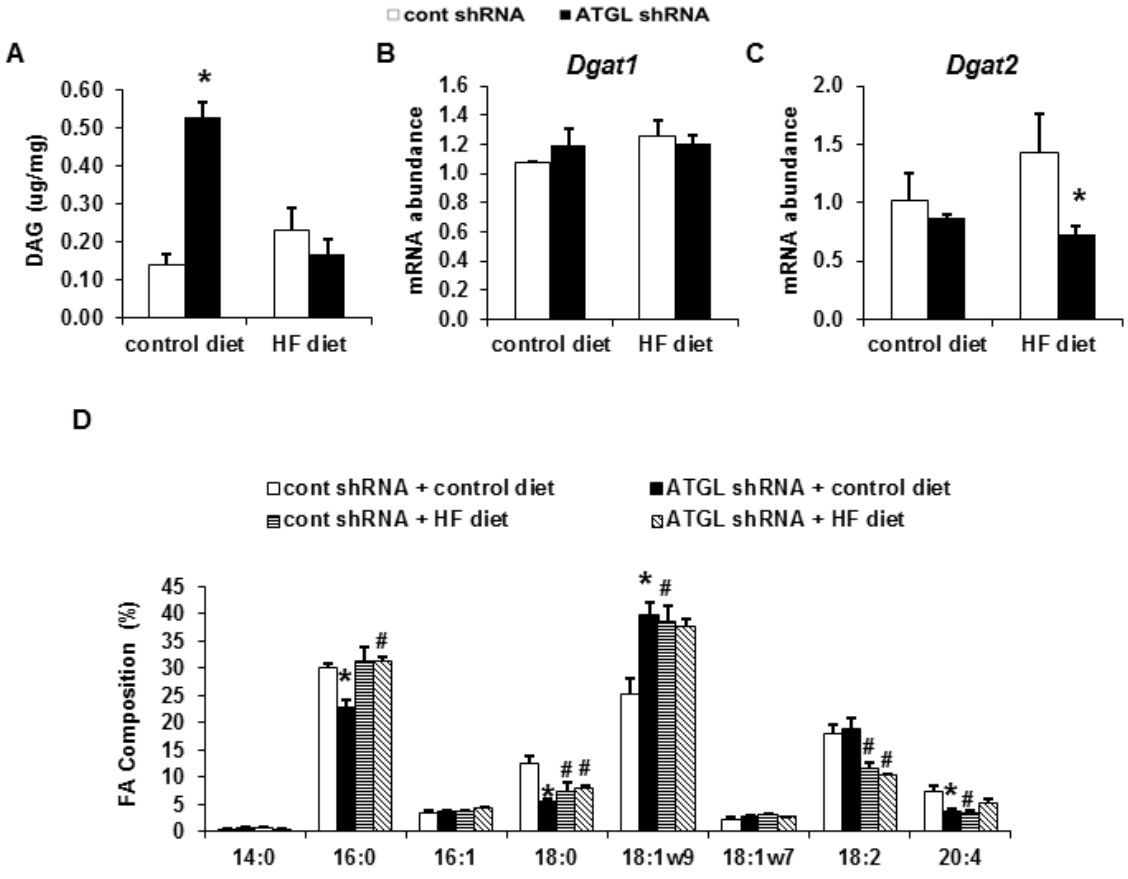

Figure 4. Hepatic ATGL knockdown diet-dependently alters DAG. A) Hepatic DAG content expressed per unit of tissue weight in mice fed control or high fat diets for 12 weeks (n = 7-9). mRNA abundance of liver *Dgat1* (B) and *Dgat2* (C) was quantified in adenovirus-infected mice. D) Fatty acid composition of DAG in mice fed the control diet or high fat diet for 12 weeks (n = 7). $^{*}P < 0.05$ versus control shRNA group and $^{\#}P < 0.05$ versus control diet group.

4D). These changes in DAG composition mirror those of TAG composition in control fed mice treated with the *Atgl* shRNA adenovirus (7). Feeding a HF diet for 3 months negated any differences in DAG composition between treatment groups (Fig. 4D).

***Hepatic ATGL alters substrate utilization in DIO mice*.** We have previously shown that hepatic ATGL knockdown results in attenuated hepatic FA oxidation in hepatocyte cultures (7). In support of the aforementioned result, we also observed that *Atgl* shRNA treatment resulted in lower mitochondrial oxidation in DIO mice (Fig. 5A). Several studies have shown that inhibition of FA oxidation leads to shift in substrate oxidation resulting in enhanced glucose catabolism (20,21). Given that DIO mice displayed enhanced glucose tolerance when deficient in hepatic ATGL, we hypothesized that hepatic glucose metabolism may be altered. Hence, we determined liver glycogen levels and observed that *Atgl* shRNA treatment decreased liver glycogen content in DIO mice without impacting control diet-fed mice (Fig. 5B). In addition, glucose oxidation was enhanced in hepatocyte suspensions isolated from mice receiving *Atgl* shRNA treatment, supporting our previous observation and hypothesis (Fig. 5C). Interestingly, however, while gene expression of hepatic glucokinase (*Gk*) was upregulated in control diet-fed mice, ATGL knockdown had no effect in DIO mice (Fig. 5D). Gene expression of pyruvate dehydrogenase kinase 2 (*Pdk2*) also showed no difference between DIO mice receiving *Atgl* and control shRNA treatment (Fig. 5D).

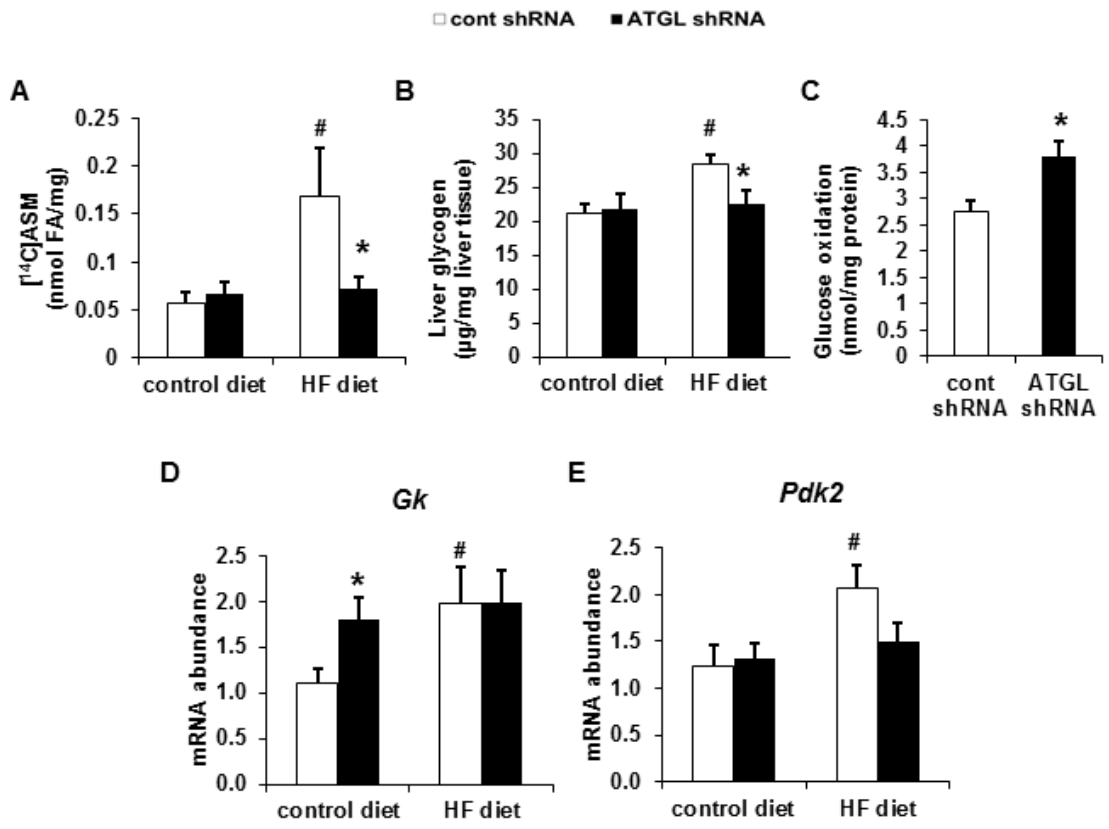

Figure 5. Hepatic ATGL knockdown alters substrate utilization in DIO mice. A) Acid soluble metabolite (ASM) production from mitochondria incubated with [1-^{14}C]palmitate (n = 4). B) Liver glycogen levels in mice fed a control or HF diet for 12-weeks (n = 6-8). C) Cell suspensions isolated from liver tissues of control or *Atgl* shRNA-treated mice fed a control diet were mixed with radiolabeled glucose to measure glucose oxidation (n = 4). mRNA abundance of glucokinase (D) and pyruvate dehydrogenase kinase 2 (E) (n = 6-8). *P < 0.05 versus control shRNA group and $^{\#}P$ < 0.05 versus control diet group. Abbreviations: *Gk*, glucokinase; *Pdk2*, pyruvate dehydrogenase kinase 2.

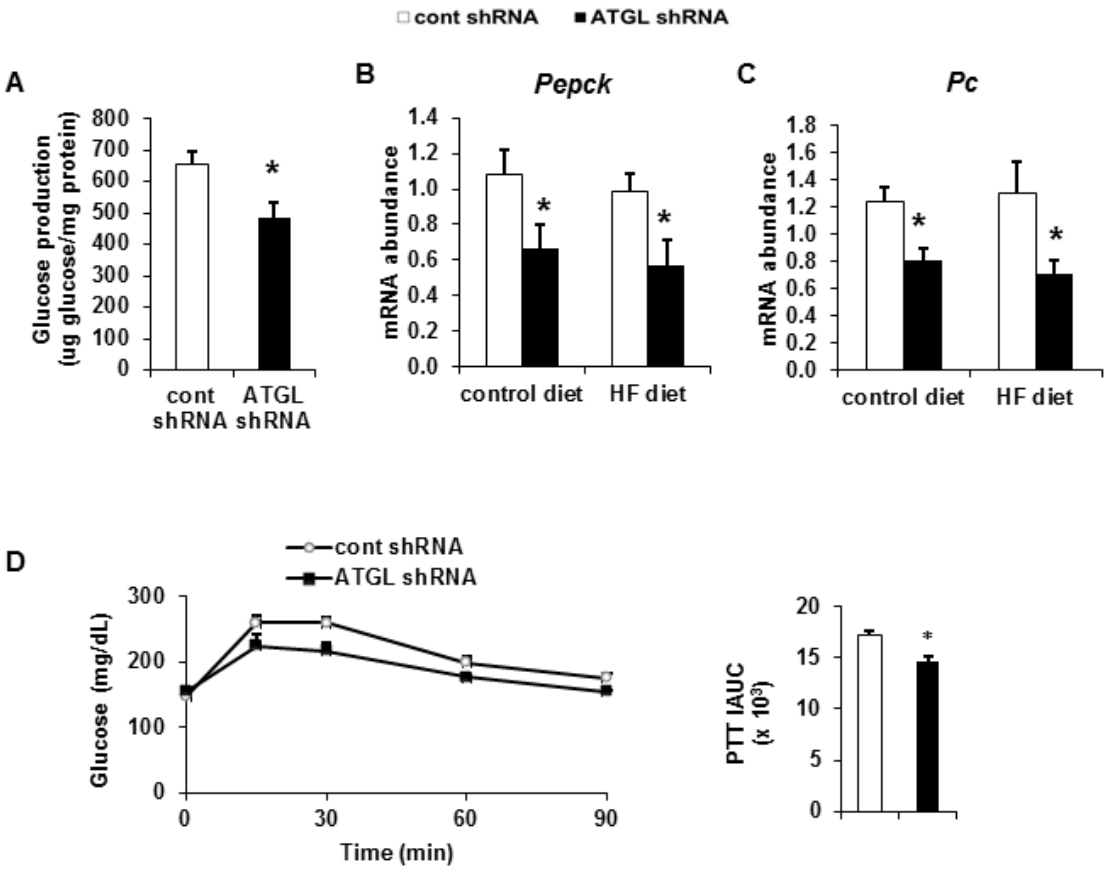

Figure 6. Adenoviral delivery of *Atgl* shRNA reduces glucose production in DIO mice. A) Gluconeogenesis assay in hepatocytes isolated from mice fed the HF diet for 7 days after adenoviral transduction (n = 4). mRNA abundance of phosphoenolpyruvate carboxykinase (B) and pyruvate carboxylase (C) was quantified in the livers of mice fed a control or HF diet for 12-weeks after adenovirus treatment (n = 6-8). D) Following adenovirus administration, mice were fed a HF diet for 6 days followed by an overnight fast prior to pyruvate tolerance test. Incremental area under the curve (IAUC) is depicted (n = 4). *P <

0.05 versus control shRNA group. Abbreviations: *Pepck*, phosphoenolpyruvate carboxykinase; *Pc*, pyruvate carboxylase.

***Hepatic ATGL regulates gluconeogenesis*.** Increased hepatic glucose production is a hallmark of insulin resistance and glucose intolerance (22,23). Thus, we tested if hepatic ATGL influenced glucose production. ATGL knockdown led to an approximately 25% reduction in production of glucose in primary mouse hepatocytes isolated 7 days after adenoviral transduction (Fig. 6A). Quantitative real time PCR analysis revealed that mice receiving the *Atgl* shRNA adenovirus had approximately 40% less expression of phosphoenolpyruvate carboxykinase (*Pepck)* or pyruvate carboxylase *(Pc)* whether fed control or HF diet for 12 weeks (Fig. 6B and C). To further corroborate these findings, we performed pyruvate tolerance test (PTT) in mice fed the HF diet for 1 week and found that mice infected by *Atgl* shRNA adenovirus had reduced glucose production compared to control mice (Fig. 6D). Taken together, these data suggest the improvements in glucose tolerance following hepatic ATGL knockdown are due to increased glucose utilization and reduced hepatic glucose output.

Discussion

While the role of the hepatic TAG synthetic pathway in elucidating the pathological association between hepatic steatosis and insulin resistance has been the focus of much research, the contribution of the TAG catabolic pathway has been less characterized (24–26). To delineate the function of hepatic ATGL in modulating whole-body glucose tolerance and hepatic insulin sensitivity, we utilized adenoviral delivered shRNA to decrease hepatic ATGL expression. In this report, we reveal that 7 days after receiving *Atgl* shRNA treatment, mice had lower serum glucose levels regardless of diet despite enhanced hepatic TAG content. In addition, the increase in serum insulin levels resulting from consuming a HF diet was abolished by hepatic ATGL knockdown. Intriguingly, ATGL knockdown also conferred enhanced glucose tolerance but had no impact on insulin action. A separate study which overexpressed hepatic *Atgl* mRNA approximately 4-fold observed minimal changes to glucose tolerance but found that hepatic insulin signaling was enhanced (11). However, in our study, we showed that hepatic insulin signaling was not altered in hepatic ATGL deficient mice, suggestive that presence of ATGL is not necessary for normal functioning of hepatic insulin signaling.

Because ATGL deficiency in the liver led to improved glucose tolerance in mice fed HF diet for only 7 days, we next investigated whether *Atgl* shRNA could normalize glucose intolerance and insulin sensitivity in DIO mice fed a HF diet for 12 weeks. Both serum glucose and insulin levels were recovered to basal levels

7 days after administration of *Atgl* shRNA. Furthermore, glucose intolerance observed in DIO mice was normalized with hepatic ATGL knockdown. Hepatic insulin signaling remained unaltered in hepatic ATGL-deficient DIO mice compared to control DIO mice. A recent study performed in *Atgl* KO mice fed a HF diet for 4 weeks also revealed that glucose tolerance and insulin sensitivity were enhanced, suggesting that hepatic ATGL may contribute to the observed phenotype based on the findings of the current study (9). In contrast, insulin and glucose tolerance were unaltered in mice with a liver specific deletion of ATGL (8). These disparities may be accounted for by the two different methods that were used to decrease hepatic ATGL expression. It is very possible that the liver-specific *Atgl* KO mice adapted to earlier metabolic changes since birth while the effects we observed in adenovirus-mediated ATGL knockdown mice were more representative of short term ATGL deficiency. Additionally, we observed the most pronounced effects of ATGL knockdown under high fat feeding conditions, which were not tested in the previous study (8).

One of the key findings in the current study is the uncoupling of glucose intolerance and insulin resistance from hepatic steatosis. Moreover, defective insulin signaling was not observed in ATGL-deficient mice despite TAG accumulation in the liver. Several other models have also shown increased steatosis without alterations in insulin signaling or impaired glucose tolerance (25–29). Although steatosis is linked to insulin resistance, lipid intermediates such as diacylglycerol (DAG) are thought to impair insulin signaling rather than TAG itself (30). Surprisingly, hepatic ATGL knockdown in mice fed the control

diet resulted in a 4-fold increase in DAG without alterations in insulin signaling. This observation is in agreement with a separate study which showed that ATGL overexpression in the liver decreased hepatic DAG levels without affecting insulin signal transduction (11). We showed that mRNA expression of *Dgat2* was markedly downregulated in the liver of mice lacking ATGL under a HF diet consistent with a separate study performed in liver-specific *Atgl* KO mice (8). Nevertheless, the effects of altering hepatic DAG content via *Dgat2* expression manipulation on insulin action appeared to be paradoxical as demonstrated in two separate animal models (24,25). Thus, the detailed mechanisms regulating hepatic DAG levels remain poorly understood. In the current study it should be noted that although mice fed the control diet with suppressed hepatic ATGL expression had elevated DAG, the percentage of saturated acyl chains in the DAG were reduced. This is important given recent data showing that saturated DAG impairs insulin signaling in hepatocytes (31) and may explain why no alterations in insulin signaling were observed despite higher total DAG content. It should be noted that the changes in FA profile of DAG only occurred in control diet-fed mice and are consistent with the FA profile of TAG in our previous study (7).

We have previously shown that hepatic ATGL deficiency leads to lowering of β-oxidation without affecting VLDL secretion (7). Consistent with these data, hepatic mitochondrial oxidation was downregulated in DIO mice administered with *Atgl* shRNA in comparison with control DIO mice. Given that FA oxidation is inhibited, we speculate that substrate utilization will be switched to glucose

oxidation as elaborated in the Randall cycle (32,33). In fact, *Atgl* KO mice had higher respiratory quotient than control mice during fasting, indicating that the main source of energy was glucose oxidation (9). In agreement with this hypothesis, this study showed that glucose oxidation was higher in hepatocytes isolated from mice treated with *Atgl* shRNA compared to those of control mice. In support of the current study, inhibiting CPT1 has been shown to increase glucose oxidation in humans and rodents (34–36). In addition, transgenic mice overexpressing glucokinase (*Gk*) in the liver exhibited decreased blood glucose levels and increased glucose tolerance suggesting that increased glucose oxidation may contribute to enhanced glucose tolerance (37). Very similar phenotypes have also been observed in mice overexpressing extra copies of the *Gk* gene locus and transgenic mice expressing the human *Gk* gene in the liver (38,39). Chronic expression of *Gk* in HF diet-fed mice not only improved glucose tolerance but also slightly increased hepatic TAG accumulation (40). These results further corroborate the notion that the switch of substrate utilization from fat to glucose is a compensatory feedback to cope with the inability to hydrolyze TAG due to deficiency in hepatic ATGL.

In accordance with decreased hepatic mitochondrial oxidation in DIO mice lacking hepatic ATGL, we have previously demonstrated that mice deficient in liver ATGL also exhibited approximately 40-70% less in gene expression of peroxisome proliferator-activated receptor alpha (PPAR-α) and its target genes involved in fatty acid utilization and glucose production (7). Several studies have been suggestive of a functional association between PPAR-α and gluconeogenic

genes (41–43). A group has recently shown that PPAR-α can bind to putative PPAR-response elements in glucose-6-phosphatase and PEPCK promoters in human and mouse hepatocytes (44). Hence, these findings indicate that the observed downregulation of hepatic gluconeogenic genes in mice lacking liver ATGL may occur by modulating activity of PPAR-α. The detailed mechanism through which ATGL controls PPAR-α activity has not been elucidated, but studies are currently underway to further define this regulation.

In summary, this study demonstrates that hepatic ATGL deficiency manifests in hepatic steatosis without impairing hepatic insulin sensitivity. Instead, glucose tolerance is improved especially in DIO mice. Decreased hepatic glucose production and enhanced glucose oxidation are two mechanisms contributing to the improvement in glucose tolerance. These important findings provide new insights into the role of hepatic ATGL and TAG hydrolysis in hepatic energy metabolism and in the etiology of steatosis and glucose intolerance.

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CHAPTER 4

Hepatic ATGL Mediates Fatty Acid Oxidation and PPAR-α Signaling Through an LFABP-Independent Mechanism

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Adipose triglyceride lipase (ATGL) catalyzes the rate-limiting step in triacylglycerol (TAG) hydrolysis in most tissues. We have shown that hepatic ATGL preferentially channels hydrolyzed fatty acids (FAs) to β-oxidation and induces peroxisome proliferator-activated receptor-α (PPAR-α) target gene expression. Nevertheless, the mechanisms that mediate this pathway are unknown. We hypothesized that the FA carrier liver fatty acid binding protein (LFABP) may be involved in transporting FAs from lipid droplets to the nucleus for ligand delivery or mitochondria for β-oxidation. Thus, we suppressed or overexpressed hepatic ATGL expression in wild type (WT) or LFABP knockout (LFABP KO) mice and primary hepatocytes to determine the contribution of LFABP in mediating the channeling of hydrolyzed FA. ATGL knockdown increased liver weight and TAG content and ATGL overexpression decreased liver weight to a similar extent in both strains. Surprisingly, manipulating ATGL expression in LFABP KO mice resulted in similar changes in PPAR-α and its target genes compared to WT mice suggesting that LFABP is not required to carry FA from ATGL to PPAR-α. Furthermore, LFABP did not impair the effects of ATGL on channeling of hydrolyzed FAs to oxidative pathways in primary hepatocyte cultures. Taken together, we conclude that LFABP is not required to channel ATGL-hydrolyzed FAs to mitochondria for β-oxidation or mediate its effects on PPAR-α.

Introduction

Adipose triglyceride lipase (ATGL) is a major TAG hydrolase in the liver (130,131). Intracellular free FAs can be exported from the liver via VLDL secretion or metabolized by β-oxidation. ATGL knockout mice exhibit reduced levels of ketone bodies, suggesting that ATGL may promote β-oxidation. In support of this notion, we have previously shown that hepatic suppression of ATGL leads to the inhibition of FA β-oxidation without affecting VLDL secretion (130). Consistent with these results, several other studies have also observed that ATGL overexpression or deletion affects FA oxidation but not VLDL secretion *in vitro* and *in vivo* (131,132). In addition, FA oxidative genes including peroxisome proliferator-activated receptor alpha (PPAR-α) and its downstream target are downregulated in the mice receiving ATGL shRNA compared to control mice (130). Others have also reported that ATGL regulates PPAR-α in heart, small intestine and brown adipose tissues (79,133,134). Nevertheless, the mechanisms through which ATGL channels hydrolyzed FAs to the mitochondria for oxidation or the nucleus for transcriptional activity are currently unknown.

Liver fatty acid binding protein (LFABP) is a FA carrier involved in the long chain fatty acid (LCFA) transport throughout the cytoplasm and channeling of LCFA and LCFA-CoA to the mitochondria for oxidation (135). LFABP has been shown to interact with CPT1, the rate-limiting step in mitochondrial LCFA oxidation (135–137). In addition, small amount of LFABP has been found to associate with mitochondria (138). These observations imply that LFABP may mediate the transport of FA to the mitochondria. LFABP gene ablation in cultured

primary hepatocytes and isolated hepatocytes from LFABP null mice has been shown to suppress mitochondrial fatty acid oxidation (139). Additionally, two separate studies have determined that LFABP binds PPAR-α with a high affinity and manipulating LFABP expression alters PPAR-α transcription of genes coding for LCFA oxidative enzymes, suggesting that LFABP also regulates FA oxidation via transcriptional mechanisms (140,141).

Given the functional and physiological significance of LFABP in FA oxidation, we hypothesize that LFABP is necessary for the transport of ATGL-hydrolyzed FAs to the mitochondria for oxidation or to the nucleus to regulate gene expression. To investigate the role of LFABP in ATGL-mediated β-oxidation, we overexpressed (Ad-ATGL) or knocked down ATGL (ATGL shRNA) in wild type (WT) and whole body LFABP knockout (LFABP KO) mice. Despite the importance of LFABP as a FA carrier, our findings strongly suggest that LFABP is not required for the channeling of FAs produced by ATGL activity to the mitochondria for oxidation or to the nucleus for transcriptional regulation of PPAR-α.

Materials and Methods

***Animals, diets and adenoviral administration*.** All animal protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. Male LFABP KO mice on a C57BL/6J background were generated as previously described (142). All mice were housed under controlled temperature and lighting (20-22°C; 12:12-h light-dark cycle). Eight to ten week old wild-type or LFABP KO mice were injected via the tail vein with Ad-GFP or Ad-ATGL for overexpression studies or adenoviruses encoding ATGL short hairpin RNA (ATGL shRNA) or control shRNA targeting a nonspecific messenger RNA (mRNA). Adenoviruses were generated as described previously (23, 24). All mice had free access to water and were fed a purified diet (TD.94045) from Harlan Teklad Premier Laboratory Diets (Madison, WI) for a week following adenoviral administration. The control diet contained protein (19% of total calories), carbohydrates (64%), and fat (17%), and the fat source was soybean oil (70 g/kg). Exactly one week after adenovirus injection, mice were sacrificed for liver tissues and serum collection after an overnight or 4 h fast.

***Primary hepatocyte isolation*.** Mouse primary hepatocytes were isolated from 8-10 weeks old WT or LFABP KO male mice with free access to water and control diet by the collagenase perfusion method described previously (143). Hepatocytes were plated on collagen-coated multiwell tissue culture plates (Nunc)

for 4 h with M199 plating media (Invitrogen) that contained 23 mM HEPES, 26 mM sodium bicarbonate, 10% FBS, 1% penicillin/streptomycin, 100 nM dexamethasone, 100 nM insulin, and 11 mM glucose. M199 maintenance media contained 23 mM HEPES, 26 mM sodium bicarbonate, 1% penicillin/streptomycin, 5.5 mM glucose, 100 μM carnitine, 10 nM dexamethasone, and 10 nM insulin. Cells were maintained in a humidified incubator at 37°C, 5% CO_2.

***Cell adenoviral infection and radiolabeling*.** Adenovirus expressing ATGL or green fluorescent protein (GFP) that serves as a control virus was generated as described previously (3). After 4 hours of plating, primary hepatocytes were exposed to either adenovirus expressing GFP or ATGL at 100 multiplicities of infection for 24 hr. Cells were pulsed with 500 μM [1-^{14}C]oleate bound to fatty acid-free BSA in a 3:1 molar ratio for 1.5 hr. Some cells were harvested for measure radiolabel incorporation into cellular lipid fractions. Subsequently, cells were washed with phosphate-buffered saline solution and the wells were replaced with new media lacking labeled fatty acid for an additional 6 hr of chase period followed by collection of cells and lipid extraction. Lipid samples were separated into different fractions by thin layer chromatography (TLC) on 0.25-mm Silica Gel G plates in a hexane:ethyl ether:acetic acid (80:20:2, v/v)solvent system). TAG fractions were identified and measured by AR-2000 radio-TLC Imaging Scanner. Total radiolabeled lipids were quantified by scintillation counter (LS6000IC, Beckman) after addition of Bio-Safe II cocktail.

***Measurement of FA oxidation*.** Following chase periods as described above, radiolabeled medium were transferred to Eppendorf tubes containing 20% bovine serum albumin (BSA). Subsequently, perchloric acid was added to the tubes and vortexed. Following an overnight incubation at room temperature, the tubes were centrifuged and the supernatant fraction was transferred to another set of tubes containing 20% BSA. The supernatant that contained [^{14}C]-labeled acid-soluble metabolites (ASM) were quantified with scintillation counter.

RNA isolation, RT-PCR and real –time quantitative PCR analysis. RNA was extracted with Trizol from liver tissues followed by reverse-transcription with SuperSript III First-Strand Synthesis SuperMix (Invitrogen). Gene expression was quantified using a SYBR GreenER Two-Step qRT-PCR Kit (Invitrogen) and an Applied Biosystems StepOne Plus Real-Time PCR system. Data were analyzed using the delta-delta CT method. For all analysis, gene expression was normalized to ribosomal protein L32. Melting curve analysis was performed on all samples to verify primer specificity.

***Liver TAG and FA analysis*.** TAG was extracted from liver tissues or hepatocytes according to a method from Folch (4). Snap-frozen liver tissues were homogenized in sterile water and extracted twice with chloroform:methanol. Chloroform layer was isolated and dried under nitrogen gas, and redissolved in Triton X-100. Liver TAG was quantified with a TAG colorimetric enzymatic kit

(Stanbio). Liver free FAs were measured by a free fatty acid quantification kit (Sigma) as outlined by the manufacturer's guideline.

Serum measurements. Serum FA and β-hydroxybutyrate levels were determined with colorimetric enzymatic assay kits from Wako Chemicals while serum TAG was measured with a colorimetric assay kit from Stanbio.

Results

ATGL suppression affects liver phenotype of LFABP knockout mice. Given that the deletion of LFABP has been shown to decrease hepatic TAG accumulation (142) and ATGL is a major hepatic TAG hydrolase (130), we set out to investigate the effects of ATGL knockdown on the liver phenotype of LFABP KO mice. ATGL knockdown had no effect on body weight in WT or LFABP KO mice when compared to control shRNA treatment following 7 d of treatment(Fig. 1A). ATGL knockdown led to increased liver weight in WT mice and this effect was replicated in LFABP KO mice (Fig. 1B). These observations could be explained by enhanced hepatic TAG accumulation in both WT and LFABP KO mice treated with ATGL shRNA adenovirus (Fig. 1D). Although ATGL knockdown increased liver weights and TAG content in both WT and LFABP KO mice, the absence of LFABP attenuated this increase. Interestingly, LFABP KO mice exhibited an increase in epididymal fat weight compared to WT mice and this change was abrogated by ATGL shRNA administration (Fig. 1C). Surprisingly, however, hepatic ATGL knockdown did not alter the content of liver free FAs (Fig. 1E).

ATGL overexpression reduces liver weight of LFABP knockout mice. To further examine the importance of LFABP in mediating the effects of ATGL on hepatic lipid metabolism, we also overexpressed ATGL in both WT and LFABP KO mice. Consistent with the above data, overexpressing ATGL did not alter body weight of WT or LFABP KO mice (Fig. 2A). Increasing the expression of

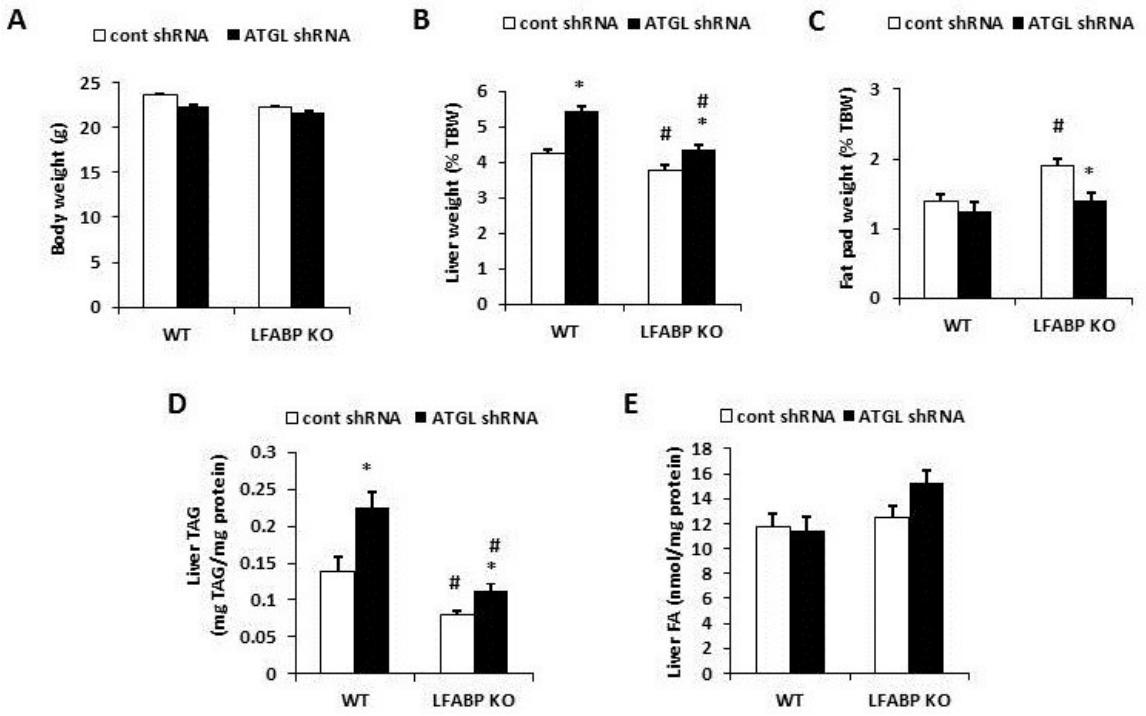

Figure 1. ATGL knockdown increases liver weight of LFABP knockout mice. WT or LFABP KO mice at 8 – 10 weeks of age were infected with control or ATGL shRNA adenovirus (n = 6 – 10 per group). Mice were fed with purified control diet and sacrificed 7 d post-infection. Body (A), liver (B) and adipose (C) weight of WT or LFABP KO mice were measured following an overnight fasting. Liver TAG (D) and FFA (E) were quantified using a colorimetric enzymatic assay kit in mice infected with control or ATGL shRNA. Data are presented as means ± SE. Comparisons between groups were made using Student's t test. *, $p < 0.05$ versus control shRNA group and $^{\#}$, $p < 0.05$ versus WT mice.

hepatic ATGL via administration of Ad-ATGL adenovirus decreased liver weight of both WT and LFABP KO mice similarly (~12% versus ~8%) (Fig. 2B). Both WT and LFABP KO mice receiving Ad-ATGL adenovirus injection showed no change in fat pad weight compared to control mice injected with GFP adenovirus (Fig. 2C). Interestingly, despite reduced liver weight, liver TAG was unaltered in ATGL overexpressing mice compared to GFP-treated mice (Fig. 2D). This observation may stem from the shorter duration of fasting and lower amount of basal liver TAG levels.

Manipulating ATGL expression does not influence serum lipids. We have previously shown that serum TAG, beta-hydroxybutyrate (BHBA) and free FAs (FFAs) do not change with ATGL knockdown (130). Nevertheless, it would be of interest to check whether absence of LFABP may mitigate lipid metabolism to influence the regulation of serum lipids by hepatic ATGL. ATGL knockdown had no effect on serum TAG, BHBA or FFAs regardless of the mouse strain (Fig. 3A-C). However, serum TAG levels in LFABP KO mice were lower than those of WT mice treated with control shRNA adenovirus after an overnight fast (Fig. 3A). Similarly, overexpressing ATGL did not alter serum TAG, BHBA or FFAs in WT or LFABP KO mice (Fig. 3D-F). These results suggest that the effects of ATGL manipulation on serum lipids are LFABP-independent.

LFABP ablation does not attenuate ATGL-mediated β-oxidation in primary hepatocytes. Although serum BHBA levels were not affected by ATGL

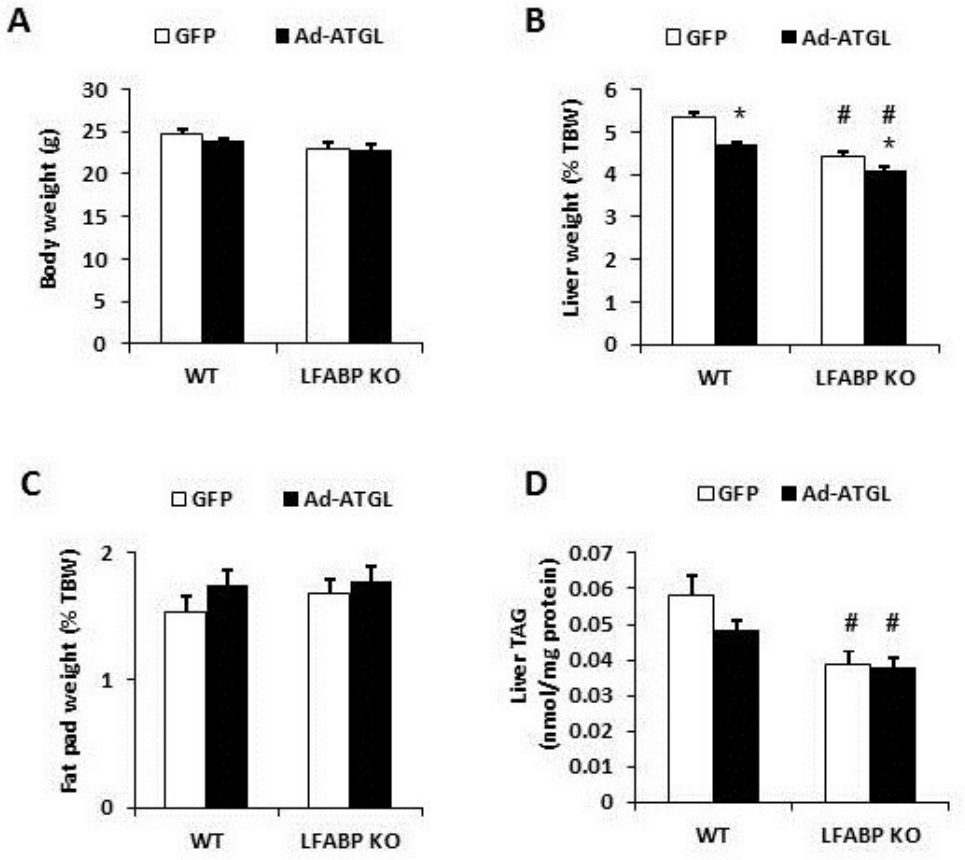

Figure 2. ATGL overexpression reduces liver weight of LFABP knockout mice. WT or LFABP KO mice at 8 – 10 weeks of age were injected with Ad-GFP or Ad-ATGL adenovirus (n = 6 – 8 per group). Mice were fed with purified control diet and sacrificed 7 d post-infection. Body (A), liver (B) and adipose (C) weight of WT or LFABP KO mice were measured following a 4 h fasting. Liver FFA (D) was quantified using a colorimetric enzymatic assay kit in mice infected with Ad-GFP or Ad-ATGL shRNA Data are presented as means ± SE. Comparisons between groups were made using Student's t test. *, $p < 0.05$ versus control shRNA group and $^{\#}$, $p < 0.05$ versus WT mice.

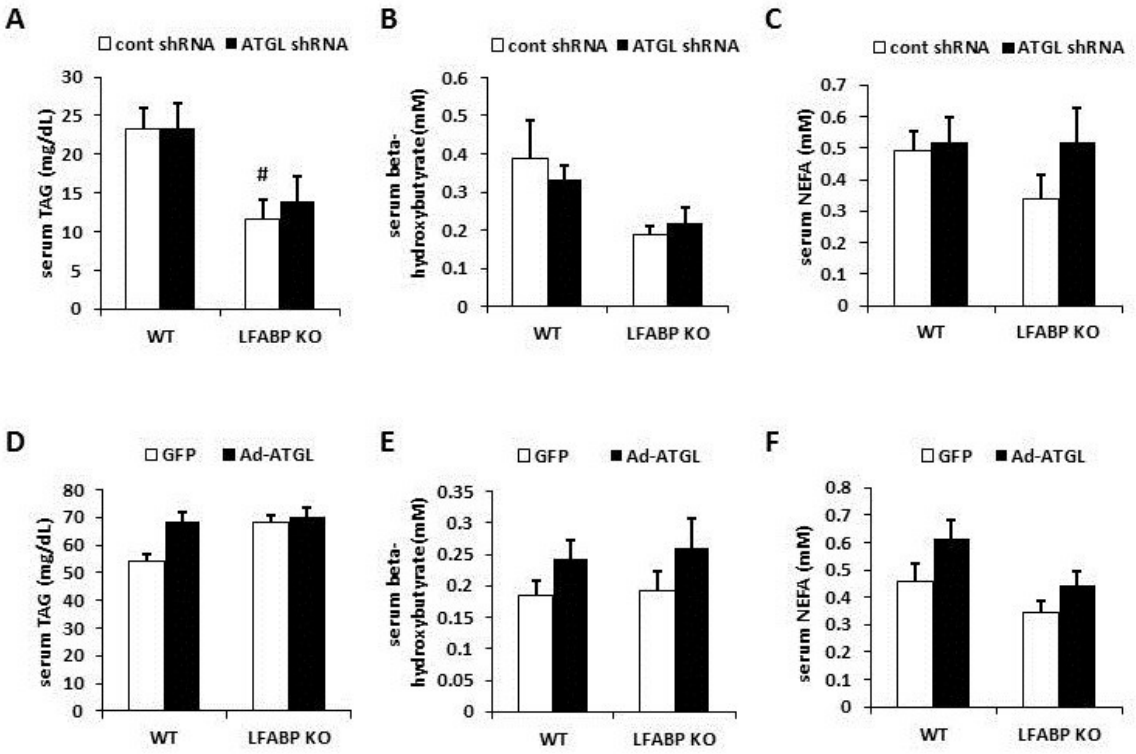

Figure 3. ATGL manipulation does not modulate serum lipid in LFABP KO mice. WT or LFABP KO mice at 8 – 10 weeks of age were transduced with i) control or ATGL shRNA adenovirus (n = 6 – 10 per group) and ii) Ad-GFP or Ad-ATGL adenovirus (n = 6 – 8 per group). Mice were fed with purified control diet and sacrificed 7 d post-infection. Serum TAG (A & C), β-hydroxybutyrate (B & D) and FFA (C & E) were measured with a colorimetric enzymatic kit from WT or LFABP KO mice after an overnight (i) or 4 hr fasting (ii). Data are presented as means ± SE. Comparisons between groups were made using Student's t test. $^{\#}$, $p < 0.05$ versus WT mice.

knockdown or overexpression *in vivo*, our previous study has shown that hepatic ATGL regulates β-oxidation in primary hepatocyte cultures (130). Thus, we extended our *in vivo* studies to primary hepatocyte cultures to gain insights into the role of LFABP in ATGL-mediated β-oxidation. We performed pulse-chase experiments with [1-^{14}C]oleate in primary mouse hepatocytes isolated from WT or LFABP KO mice. During the pulse period, overexpressing ATGL decreased incorporation of [^{14}C]oleate into the TAG fraction of primary hepatocytes derived from both WT and LFABP KO mice to a similar extent (Fig. 4A & D). Intriguingly, LFABP deletion abolished the ATGL overexpression-induced increase in pulse-labeled ASM, indicating that LFABP regulates oxidation of exogenous FAs (Fig. 4B). To determine oxidation of FAs derived primarily from intracellular TAG hydrolysis (primarily ATGL-hydrolyzed FAs), we quantified ASM as a measure of FA oxidation during the chase period. ATGL overexpression increased ASM by almost two fold in the WT mice-derived hepatocytes and this increase was duplicated in primary hepatocytes isolated from LFABP KO mice, suggesting that LFABP is not needed for ATGL-mediated increase in β–oxidation (Fig. 4C). To ensure that this observation was not due to a compensatory effect by other FABP isoforms, we also utilized a pan-specific FABP inhibitor*,* HTS01037 (100 μM), during the chase period in primary hepatocytes isolated from WT mice. Administration of Ad-ATGL doubled ASM while addition of HTS01037 during the chase period failed to abolish the Ad- ATGL induced increase in ASM (Fig. 4E). Taken together, these data indicate that LFABP is not a major carrier of ATGL-hydrolyzed FAs and may be more important for channeling exogenous FAs for

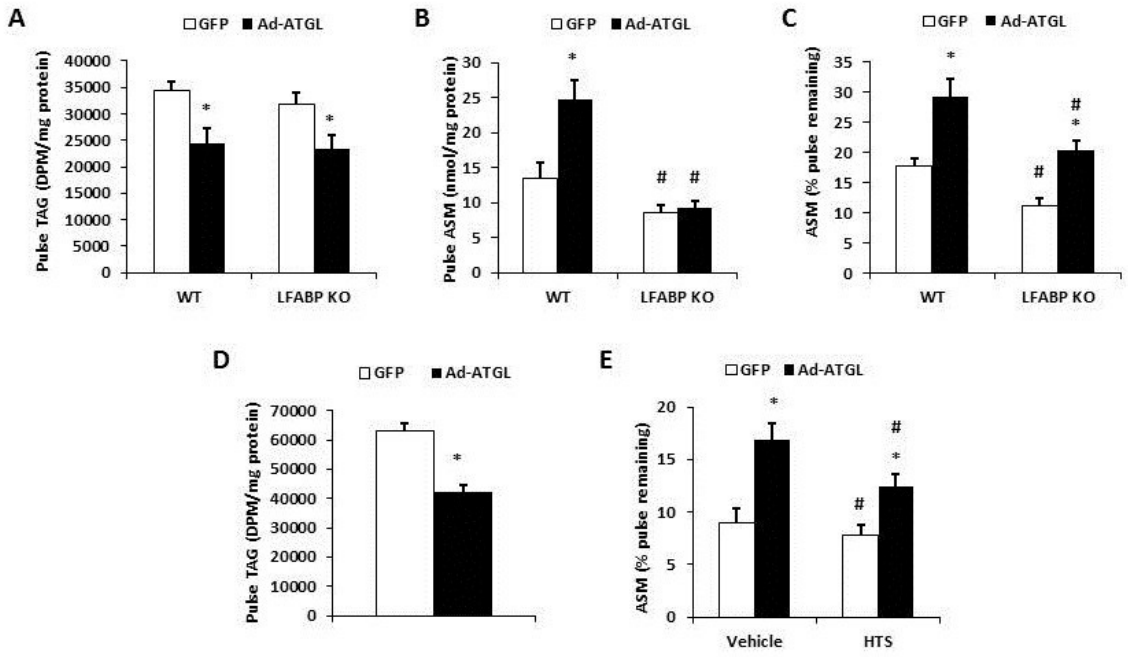

Figure 4. LFABP deletion does not block ATGL-mediated β-oxidation. Primary mouse hepatocyes were isolated from 8 – 10 weeks old WT or LFABP KO mice using collagenase perfusion method as outlined in the experimental procedures section. Cells were transduced with Ad-GFP or Ad-ATGL for 24 hr, at which time pulse (1.5 hr) and chase (6 hr) experiments were performed with 500 μM [1-^{14}C] oleate. A: TAG fractions were isolated from harvested cells by thin layer chromatography to measure incorporation of radiolabeled oleate into TAG. Media from hepatocytes were harvested at the end of pulse (B) and chase (C) periods followed by quantification of acid soluble metabolites (ASM) as outlined in the experimental procedures to measure fatty acid oxidation. During chase, a pan-specific FABP inhibitor, HTS01037 was added followed by measurement of TAG incorporation (D) and ASM E) as outlined above. Data are presented as means ± SE. Comparisons between groups were made using Student's t test. *, $p < 0.05$ versus control shRNA group and $^{\#}$, $p < 0.05$ versus WT mice.

the purpose of mitochondrial oxidation.

LFABP is not necessary to transcriptionally regulate oxidative genes downstream of ATGL. To further examine whether LFABP serves as a carrier of ATGL-hydrolyzed FAs to the nucleus to influence oxidative genes, we also quantified PPAR-α target gene expression in WT or LFABP KO mice administered with Ad-ATGL adenovirus. In WT mice, hepatic ATGL overexpression led to approximately 0.4 to 2-fold increase in PPAR-α and its target genes (Fig. 5A-F). Despite the absence of LFABP, overexpressing ATGL in the liver still induced gene expression of PPAR-α, ACOT1, LCAD and ACSL1 (0.3 to 2.5-fold) in LFABP KO mice (Fig. 5A, C, D & E). These results show that LFABP does not mediate the effects of ATGL on PPAR-α activity and suggest that an alternate mechanism links changes in ATGL activity to transcriptional regulation.

LFABP deletion does not enhance the effects of ATGL knockdown on PPAR-α and its target genes. We have previously shown that hepatic ATGL promotes fatty acid oxidation via changes in the expression of PPAR-α and its target genes (130). Interestingly, LFABP has been shown to bind directly to PPAR-α and may play a role in PPAR-α activation (140). Thus, we investigated the effect of ATGL knockdown on PPAR-α-regulated genes in LFABP KO mice. Interestingly, only some of the genes such as PPAR-α, ACOT1 and ACSL1 were

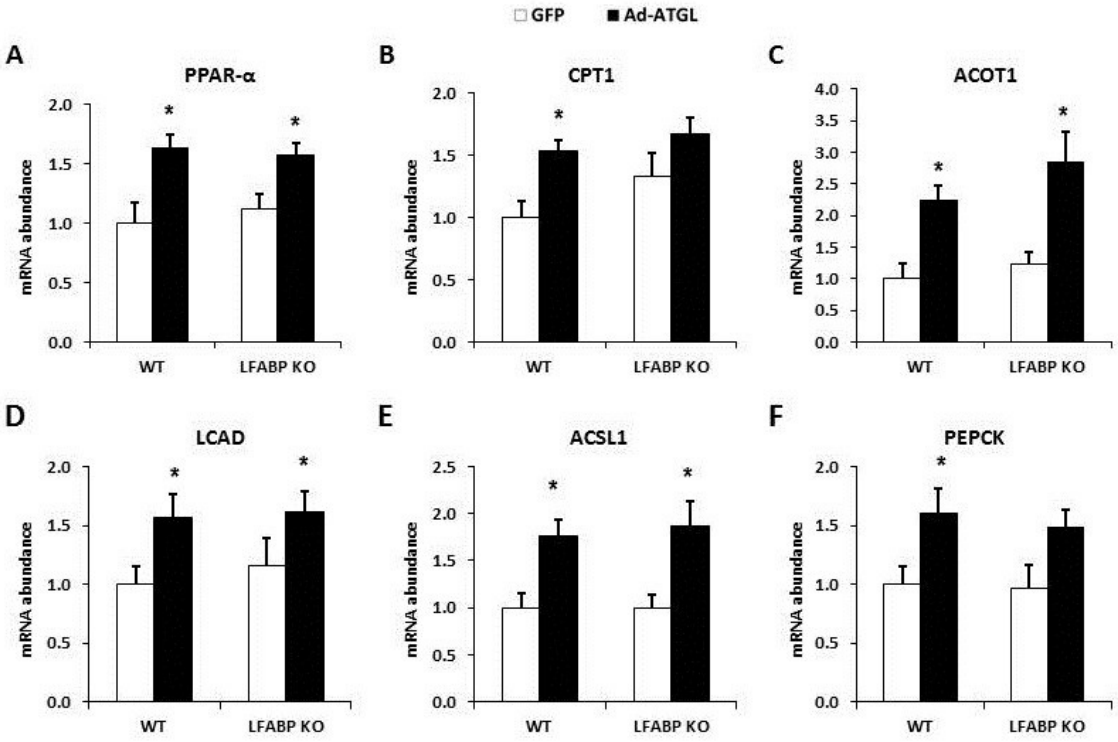

Figure 5. LFABP is not required for transcriptional regulation of oxidative genes downstream of ATGL. WT or LFABP KO mice at 8 – 10 weeks of age were transduced with Ad-GFP or Ad-ATGL adenovirus (n = 6 – 8 per group). Liver tissues were collected 7 d post-transduction after a 4 h fasting. Liver tissues were used to synthesize cDNA via reverse transcription PCR from isolated mRNA. mRNA expression of oxidative genes including PPAR-α (A), CPT1 (B), ACOT1 (C), LCAD (D), ACSL1 (E) and PEPCK (F) were quantified with real-time quantitative PCR using the ΔΔCT method. Data are presented as means ± S.D. Comparisons between groups were made using Student's t test. *, $p < 0.05$ versus control shRNA group and $^{\#}$, $p < 0.05$ versus WT mice. PPAR-α, peroxisome proliferator-activated receptor alpha; CPT1, carnitine palmitoyltransferase I; ACOT1, acyl-CoA thioesterase I; LCAD, long-chain acyl-CoA dehydrogenase; ACSL1, acyl-CoA synthetase 1; PEPCK, phosphoenolpyruvate carboxykinase.

downregulated in LFABP KO compared to WT mice injected with control shRNA adenovirus (Fig. 6A, C & E). These discrepancies have been observed in several studies due to differences in experimental model (*in vitro* versus *in vivo*), fasting duration, diet type, age and sex (144–146). The fold change between the two genotypes within the same virus treatment group is very similar across all the genes, which further supports the data in the ATGL overexpression model showing that ATGL mediates PPAR-α through a LFABP-independent mechanism (Fig. 6A-F).

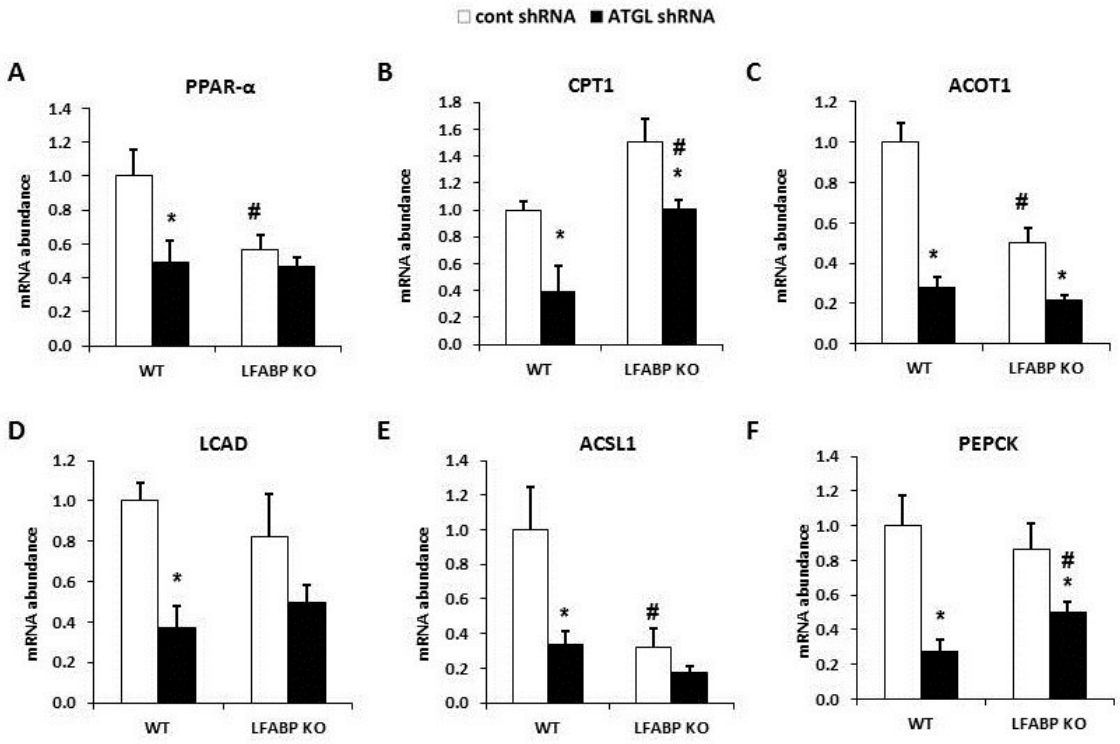

Figure 6. LFABP deletion does not enhance suppression of PPAR-α target genes induced by ATGL knockdown. WT or LFABP KO mice at 8 – 10 weeks of age were infected with control or ATGL shRNA adenovirus (n = 6 – 10 per group) followed by harvesting of liver tissues 7 d later after an overnight fasting. Liver tissues were used to produce cDNA via reverse transcription PCR from isolated mRNA. mRNA expression of oxidative genes including PPAR-α (A), CPT1 (B), ACOT1 (C), LCAD (D), ACSL1 (E) and PEPCK (F) were quantified with real-time quantitative PCR using the ΔΔCT method. Data are presented as means ± S.D. Comparisons between groups were made using Student's t test. *, $p < 0.05$ versus control shRNA group and #, $p < 0.05$ versus WT mice. PPAR-α, peroxisome proliferator-activated receptor alpha; CPT1, carnitine palmitoyltransferase I; ACOT1, acyl-CoA thioesterase I; LCAD, long-chain acyl-CoA dehydrogenase; ACSL1, acyl-CoA synthetase 1; PEPCK, phosphoenolpyruvate carboxykinase.

Discussion

The first evidence suggesting that ATGL regulates FA oxidation was shown by ATGL knockout mice exhibiting lower plasma ketone bodies compared to control mice (71). Interestingly, hepatic overexpression of ATGL in McA-RH7777 cells also promotes FA oxidation (132). Our group was the first to reveal that ATGL preferentially exports TAG-hydrolyzed FAs to β-oxidation instead of VLDL production and secretion in the liver (130). Moreover, the effect of ATGL on β-oxidation appears to be mediated by transcriptional changes in PPAR-α and its target genes (130). Wu et al. also showed that hepatic palmitate oxidation and PPAR-α gene expression are downregulated in liver-specific ATGL knockout mice (131). However, the ATGL-mediated channeling of FA to the β-oxidation pathway is currently not well understood. Given that ATGL activity yields FA, it is reasonable to speculate that the FA may be transported to the mitochondria for oxidation or to the nucleus for modulating transcriptional activity of PPAR-α target genes.

Hence, we hypothesized that a hepatic FA carrier such as LFABP may mediate this function. While LFABP has been shown to play an essential function in regulating both FA oxidation and transcriptional activity of PPAR-α, its role in channeling specifically ATGL-hydrolyzed FAs to the mitochondria or nucleus is unclear. To examine this role, we manipulated ATGL expression in WT or LFABP KO mice. From this study, we found that either knocking down or overexpressing ATGL in the liver of LFABP KO mice is still sufficient to influence liver weight or

TAG, suggesting that the TAG hydrolase activity of ATGL is not dependent on LFABP. Consistent with our previous study in WT mice, ATGL knockdown or overexpression does not influence serum lipid markers in LFABP KO mice. Despite not observing a change in ketone bodies (serum beta-hydroxybutyrate) when hepatic ATGL is suppressed *in vivo*, ASM (FA oxidation) is reduced in primary hepatocyte cultures transduced with ATGL shRNA adenovirus (130).

Therefore, we also explored whether LFABP is needed to regulate FA oxidation mediated by ATGL. The fold induction of FA oxidation as measured by ASM in primary hepatocytes isolated from LFABP KO mice is similar to those derived from WT mice. Furthermore, using a pan-inhibitor to suppress activity of all FABP isoforms has no effect on attenuating the induction of ASM by overexpressing ATGL. This observation is consistent with a previous finding that shows other isoforms of FABP are not upregulated in LFABP KO mice (142). While LFABP has been shown to be critical for acute FA uptake (147), hepatic free FA levels do not differ between WT and LFABP KO mice under fasting condition (142,147). In addition, acute hepatic deposition of radiolabeled oleate is substantially lower in LFABP KO mice compared to WT mice under fed state in relation to fasted state (147). In agreement with these *in vivo* data, we show that LFABP deletion abrogates any increase in ASM induced by ATGL overexpression. These findings strongly suggest that LFAPB may be more important for channeling of exogenous FAs but not hydrolyzed FAs. Taken together, these findings strongly suggest that ATGL-mediated β-oxidation does not require LFABP or any other FABP family members as a FA transporter.

The next logical step is to speculate that other FA transporters which can potentially mediate the shuttling of FAs to the mitochondria would be upregulated as a compensatory mechanism in LFABP KO mice. Interestingly, Newberry et al. have shown that other putative FA transporters including sterol carrier protein (SCP-2 & SCP-x) and members of the fatty acid transporter family are not enhanced in response to the ablation of LFABP in the liver during fasting despite reduction in hepatic FA oxidation (142). However, another study has indicated that LFABP deletion results in upregulation of SCP-2 and acyl-CoA binding protein (ACBP), by 45 and 80%, respectively (148). ACBP has previously been observed to partition fatty acids between esterification reactions and beta-oxidation (149). Despite this compensation effect, LFABP KO mice still exhibit a reduction in ketone bodies or FA oxidation when fasted for 12 hr. Hence, the functions of other putative FA transporters especially SCP-2 and ACBP are unlikely to involve the shuttling of FAs to the mitochondria for β-oxidation.

Now that we have largely discounted the role of FA transporters, another potential mechanism may involve a direct association or interaction between lipid droplets (LDs) and mitochondria where ATGL-hydrolyzed FAs are handed off directly from the LD to the mitochondrion for oxidation. In fact, mitochondria are often observed to be closely located to LDs in adipocytes, heart and liver (150–155). Given that oxidative and metabolically active tissues often have higher rates of lipolysis and FA oxidation, conditions that increase energy demand may enhance these interactions. In fact, in exercise-trained dogs, goats and humans, every LD in muscle tissue is observed to be in contact with at least one

mitochondrion (156,157). In addition, mitochondria and LDs also form chains of alternating organelles in type I fibers of human skeletal muscle (158). When lipolysis is activated with resveratrol in hepatocytes, more mitochondria are observed to directly attach to LDs than those treated with vehicle (159). Notably, perilipin 5, a LD protein commonly found in oxidative tissues, plays an important role in linking LDs to mitochondria in cardiomyocytes and muscle tissues (160,161). However, a separate study has presented that knocking down PLIN5 in hepatocytes stimulates lipolysis and slightly increases the mitochondrial content and level of fatty-acid β-oxidation in the mitochondria, suggesting that PLIN5 is antagonistic towards β-oxidation.

Although several studies have shown that LFABP may mediate the effects of FAs on activating PPAR-α gene expression and activity (140,162,163), whether LFABP is critical in regulating activation of PPAR-α by ATGL-hydrolyzed FAs is unknown. Herein, we reveal that LFABP ablation does not abolish the effect of manipulating ATGL on the expression of PPAR-α or its target genes in comparison with control mice, suggesting that LFABP is not a major transporter of ATGL-hydrolyzed FAs to the nucleus. These observations are in agreement with a study which has shown that LFABP is not required for the action of PPAR-α during fasting (139). Given that free FAs are derived primarily from adipose tissue during fasting, LFABP is possibly a major carrier of exogenous-derived FAs but not TAG-hydrolyzed FAs. In fact, a recent study has indicated that LFABP delivers polyunsaturated FAs (PUFA) including omega-3 FAs to the nuclei to augment induction of PPAR-α target genes involved in β-oxidation (163).

LFABP has been shown to bind and deliver fluorescent-labeled omega-3 PUFA into the nucleus or nucleoplasm (164). In addition, saturated FAs but not PUFA could normalize diet-induced obesity and hepatic steatosis in female LFABP KO mice, suggesting that LFABP's role in FA trafficking may be dependent on FA species (165). Given that PUFA is an essential FA that can only be obtained from the diet, it is very likely that LFABP is a key player in the delivery of exogenous FAs but not ATGL-hydrolyzed FAs.

In short, this study demonstrates that LFABP, despite being a major FA carrier and an abundant cytosolic protein, is not necessary for the process of shuttling FAs broken down by ATGL to the mitochondria. Furthermore, LFABP plays no essential role in delivering ATGL-hydrolyzed FAs to the nucleus to modulate oxidative genes. These finding allude to a more complex mechanism linking ATGL to downstream changes in hepatic energy metabolism.

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CHAPTER 5

Conclusions and Perspectives

Kuok Teong Ong wrote this chapter in its entirety.

Over the last several decades, an increase in the supply of high calorie foods has led to a substantial weight gain among Americans. In America, the average daily caloric intake has increased by 570 kcal between 1977 and 2006 (166) while daily occupation-related energy expenditure has decreased by more than 100 calories per day in the last 50 years (167). Thus, it is no surprise that the incidence of individuals who are overweight and obesity have increased by two- and three-fold, respectively, over the last five decades (168). The health risks of obesity are significant as this condition is highly correlated with diabetes, NAFLD and cardiovascular diseases, collectively referred to as metabolic syndrome. The healthcare costs of metabolic syndrome are estimated to be $600 billion annually and increase about 25% for each additional risk factor associated with metabolic syndrome (169). Even though weight loss is an effective option for metabolic syndrome treatment, difficulties in achieving sustained weight loss are common. Consequently, many have sought for pharmacological solutions. While there are drugs on the market for treating obesity or diabetes, there is no known medication that specifically targets NAFLD. Thus, it is critical to understand the pathophysiology of NAFLD and its association with the other components of the metabolic syndrome.

The discovery of ATGL has piqued the interest of many researchers to characterize this lipase and its importance in the development of NAFLD, obesity and diabetes. Our group is the first to show that ATGL is a predominant TAG hydrolase in the liver that preferentially channels hydrolyzed FAs to the mitochondria for β-oxidation (130). Nevertheless, hepatic ATGL knockdown does

not alter serum ketone bodies under fasting condition. This discrepancy indicates that a systemic regulation of β-oxidation may play a role to maintain homeostasis when hepatic ATGL is suppressed. Thus, it will be interesting to investigate whether extra-hepatic tissues secrete protein hormones or factors as signaling molecules to regulate hepatic ATGL and lipid metabolism under different physiological conditions.

Given that NAFLD may result from impaired β-oxidation, it is imperative to understand the role of ATGL in the pathogenesis of NAFLD. We have shown that a specific FA carrier, LFABP, is not necessary for ATGL-mediated β-oxidation or PPAR-α expression. Other potential FA carriers including SCP-2 and ACBP are unlikely to play this role given that their upregulation could not normalize the reduction of ketones bodies or FA oxidation in LFABP KO mice (148). If no FA transporter is needed to carry ATGL-hydrolyzed FAs to the mitochondria, LD may interact directly with the mitochondria to facilitate the transfer of FAs. While perilipin 5, a LD protein linking LDs to mitochondria, has been shown to negatively regulate lipolysis and β-oxidation, it is possible that other LD proteins may positively regulate both lipolysis and β-oxidation via LD-mitochondria interaction (103). For example, synaptosomal-associated protein 23 is a mediator of mitochondria-LD interaction and its suppression leads to attenuated β-oxidation (170). Thus, future proteomics and functional analyses of LDs may reveal new LD proteins that are required to facilitate transfer of ATGL-hydrolyzed FAs via LD-mitochondria interaction.

Apart from FAs, ATGL activity also yields DAG. Given that DAG is a secondary messenger molecule and our study has shown that ATGL influences DAG composition, it will be of interest to investigate whether distinct DAG species (product of ATGL activity) may regulate downstream pathways including FA oxidation and gluconeogenesis, and the molecular and transcriptional mechanisms underlying these processes. Moreover, ATGL exhibits DAG transacylase activity (116) but it has yet to be determined if different DAG species influences their fate (being acylated or hydrolyzed by HSL) and if a switch or regulatory mechanism exists to control the two opposing activities.

Aside from ATGL, there are other known TAG hydrolases including TGH and AADA. Both TGH and AADA are localized to the ER and have different effects on TAG export or β-oxidation (116,130). Thus, separate pools of FAs may exist and their fate may depend on the specific lipase and intracellular localization. It is currently not known whether these ER lipases are upregulated with ATGL deficiency or knockdown. A recent study has revealed that ATGL knockout, but not HSL knockout, induces the expression of other TAG lipases including esterase 1, esterase 10 and esterase 21 in white and brown adipose tissues (171). It will be very interesting to investigate whether these lipases interact with ATGL or other LD proteins to form a complex that regulates the metabolism of LD. Recent studies have also revealed autophagy as a regulator of TAG hydrolysis and LD metabolism (172,173). Whether ATGL is involved in autophagy-mediated lipid metabolism is not known and warrants further investigation into its role and potential mechanisms.

Although NAFLD is frequently correlated with insulin resistance and type II diabetes (40,174), several animal models have shown a dissociation of these processes (175). In agreement with these models, mice lacking hepatic ATGL are not insulin resistant in comparison with control mice despite enhanced TAG accumulation (176). The improvement in the glucose tolerance of ATGL knockdown mice is due to attenuated hepatic glucose production and increased glucose utilization, potentially a mechanism to compensate for impaired FA oxidation. It has been reported that free FAs can induce hepatic glucose production (177,178). To date, it remains unknown whether hepatic ATGL-hydrolyzed FAs can serve as a ligand or signal to induce hepatic gluconeogenesis. Hence, it will be of interest to further investigate the mechanisms underlying the crosstalk between gluconeogenesis and ATGL action. Our preliminary data (not shown) show that proteins involved in glucose metabolism may be present on LDs based on proteomics analysis. This observation suggests that ATGL may interact with other glycolytic or gluconeogenic proteins to regulate both lipid and glucose metabolism.

In short, ATGL is a major player in lipid catabolism in the liver and may contribute to the etiology of NAFLD. Future research will be needed to understand the crosstalk between ATGL and other proteins involved in glucose and LD metabolism and how it relates to the pathogenesis of NAFLD and its associated comorbidities. Further understanding may pave way to the first therapeutics specifically targeting NAFLD.

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