GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE ISOFORMS: HEPATIC TRIACYLGYCEROL SYNTHESIS AND THE DEVELOPMENT OF INSULIN RESISTANCE

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ABSTRACT

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Glycerol-3-Phosphate Acyltransferase Isoforms: Hepatic Triacylglycerol Synthesis and the Development of Insulin Resistance
(Under the direction of Rosalind A. Coleman)

Elucidation of the metabolic pathways of triacylglycerol synthesis is critical to the understanding of chronic metabolic disorders such as obesity, cardiovascular disease, and diabetes. Glycerol-*sn*-3-phosphate acyltransferase (GPAT) and *sn*-1-acylglycerol-3-phosphate acyltransferase (AGPAT) catalyze the first and second steps in *de novo* triacylglycerol synthesis. These enzymes have multiple isoforms with different subcellular locations and tissue distributions. The specific function of each isoform and its product in the regulation of TAG synthesis and intracellular signaling pathways is not completely understood. This dissertation describes two separate projects. The first project examines the role of GPAT1 and *de novo* glycerolipid synthesis in the development of hepatic steatosis and hepatic insulin resistance. The second project describes the identification of a novel GPAT isoform, GPAT4.

In the first project, we used an adenoviral construct to overexpress glycerol-*sn*-3-phosphate acyltransferase-1 (Ad-GPAT1) to determine whether increased glycerolipid flux can, by itself, cause hepatic insulin resistance. We found that hepatic overexpression of GPAT1 caused hepatic TAG accumulation and hyperlipidemia without affecting body weight or adiposity. This GPAT1-mediated increase in hepatic *de novo* glycerolipid synthesis

caused both systemic and hepatic insulin resistance, which was associated with elevated hepatic diacylglyerol and lysophosphatidic acid content, suggesting a role for these lipid metabolites in the development of hepatic insulin resistance. We identified a potential DAG/protein kinase C-mediated mechanism for hepatic insulin resistance, and we did not find any evidence for an immune mediated mechaninsm.

In the second project, we characterized the acyltransferase activity of AGPAT6. AGPAT6 was one of eight AGPAT isoforms identified through sequence homology, but the enzyme activity for AGPAT6 had not been confirmed. The NEM-sensitive GPAT specific activity was 65% lower in liver and brown adipose tissue from *Agpat6*$^{-/-}$ mice than in tissues from wild type mice, but the AGPAT specific activity was similar in wild type and *Agpat6*$^{-/-}$ mice. Cos-7 cells transfected with AGPAT6 had higher NEM-sensitive GPAT specific activity and no change in AGPAT activity. Thus, we have identified a novel NEM sensitive GPAT isoform that is expressed in liver and brown adipose tissue. We have named this isoform GPAT4.

To my father W. William Nagle, Jr.; my mother, Ashley V. Fine Nagle; and my grandfathers, W. William Nagle, M.D. and Valentine L. Fine.

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ACC	acetyl-CoA carboxylase
ACS	acyl-CoA synthase
AICAR	5-aminoimidazole-4-carboxamide-1-beta-D ribonucleoside
AGPAT	1-acyl-*sn*-glycerol-3-phosphate acyltransferase
ALT	alanine aminotransferase
AMPK	adenosine monophosphate-activated protein kinase
AST	aspartate aminotransferase
BAT	brown adipose tissue
CHO	Chinese hamster ovary
ChRE	carbohydrate response element
ChREBP	carbohydrate response element binding protein
CK2	casein kinase 2
CPT-1	carnitine palmitoyl transferase-1
CRP	C reactive protein
DAG	diacylglycerol
DGAT	diacylglycerol acyltransferase
EGFP	enhanced green fluorescent protein
FAS	fatty acid synthase
G3P	glycerol-3-phsophate
G6Pase	glucose-6-phosphatase
GPAT	glycerol-3-phosphate acyltransferase;
IκB	inhibitory factor B

IKKβ	inhibitory factor B kinase beta
IL-1β	interleukin factor-1-beta
IL-6	interleukin-6
IR	insulin receptor
IRS-1/2	insulin receptor 1/2
L-FABP	liver fatty acid binding protein
LPA	lysophosphatidic acid
LXR	liver-X-receptor
NEM	*N*-ethylmaleimide
NFκB	nuclear factor-kappa B
NF-Y	nuclear factor-Y
PA	phsophatidic acid
PC	phosphatidyl choline
PAI-1	plasminogen activator inhibitor-1
PEPCK	phosphoenolpyruvate carboxykinase
PI	phosphatidyl insositol
PKCε	protein kinase Cε
PL	phospholipids
PLC	phospholipase C
PLD	phospholipase D
PPARγ	peroxisome proliferator-activated receptor gamma
PS	phosphatidyl serine
RE	response element
SCD-1	stearoyl-CoA desaturase-1
SOCS	suppressor of cytokine signaling
SRE	sterol response element

SREBP	sterol regulatory binding protein
TAG	triacylglycerol
TNFα	tumor necrosis factor alpha
WAT	white adipose tissue
VLDL	very low density lipoprotein

CHAPTER I INTRODUCTION

Excessive storage of triacylglycerol (TAG) in hepatocytes is associated with increased secretion of TAG in very low density lipoproteins (VLDL). Both fatty liver and increased VLDL have been implicated in the pathogenesis of insulin resistance and type 2 diabetes, and they are also well known risk factors for atherosclerosis and cardiovascular disease. Prevention and treatment of these chronic diseases of lipid metabolism requires a better understanding of the regulation of hepatic TAG synthesis.

Glycerol-3-phosphate acyltransferases (GPAT) catalyze the first committed step in *de novo* synthesis of TAG and other glycerolipids. There are 3 cloned GPAT isoforms - one located in the endoplasmic reticulum,GPAT3, and 2 located in mitochondria, GPAT1 and GPAT2 [1]. Approximately 50% of total liver GPAT activity is an N-ethylmaleimide (NEM) resistant activity located in mitochondria, and the other 50% is an ER-located NEM-sensitive activity. GPAT1, which is resistant to NEM, is highly expressed in liver and adipose tissue. Studies with GPAT1$^{-/-}$ mice indicate that GPAT1 is the primary mitochondrial GPAT isoform in liver [2]. GPAT2 is highly expressed in testes, and its activity in liver is low under normal conditions [3]. GPAT3, the only identified erGPAT, is NEM-sensitive, and its mRNA expression is high in white adipose tissue, but not liver [4, 5]. Thus, GPAT3 may not be the primary erGPAT in liver. GPAT3 was originally classified by Vergnes et al. as 1-sn

acylglycerol-3-phsophate acyltransferase-8 (AGPAT8) based on amino acid sequence homology to AGPAT1 and AGPAT2, which share similar conserved acyltransferase motifs with GPAT isoforms [4]. Another AGPAT isoform, AGPAT6, is 66% homologous to AGPAT8, and AGPAT6 is relatively highly expressed in liver tissue. Mice deficient in AGPAT6 have reduced TAG mass in adipose tissue and liver, indicating that AGPAT6 is important for hepatic TAG synthesis. **The phenotype of AGPAT6$^{-/-}$ mice is consistent with a deficiency in either AGPAT or GPAT activity, but the specific acyltransferase activity of AGPAT6 had not been confirmed.**

Work in our laboratory with GPAT1$^{-/-}$ mice and with primary hepatocytes that over-express GPAT1 suggests an important role for GPAT1 in the synthesis of TAG destined for intracellular storage. GPAT1$^{-/-}$ mice have low liver and VLDL TAG mass, and decreased VLDL secretion [2]. However, adenoviral over-expression of GPAT1 in rat primary hepatocytes increases TAG accumulation without increasing TAG secretion into culture media. Because systemic GPAT1 deficiency decreased plasma TAG levels but GPAT1 overexpression failed to increase secretion of labeled TAG from hepatocytes, **the role of GPAT1 in the synthesis of TAG for incorporation into VLDL is not clear.**

Despite the association of hepatic steatosis with insulin resistance, and the amelioration of hepatic triacylglycerol accumulation with improved insulin sensitivity, it is still unclear whether insulin resistance causes the increase in hepatic triacylglycerol or whether the increase in glycerolipid intermediates or triacylglycerol itself plays a causal role in hepatic or systemic insulin resistance [6-12]. Most animal models of hepatic steatosis and

insulin resistance have been created through high-fat or high-sucrose feeding or through genetic disruption of insulin or leptin signaling pathways. Diet-induced hepatic steatosis, however, is not a good model for isolating the role of the liver in the pathogenesis of insulin resistance because high-fat diets cause weight gain and obesity, which independently contribute to the development of systemic insulin resistance. Systemic deficiencies in leptin or insulin signaling also cause obesity by increasing centrally-mediated food intake. **An animal model that isolates the accumulation of triacylglycerol in liver from its accumulation in other tissues may provide a better understanding of the role of hepatic lipid synthesis or accumulation in the development of hepatic and peripheral insulin resistance.**

Hepatic overexpression of GPAT1 may be an ideal model to examine the role of hepatic *de novo* glycerolipid synthesis in the development of insulin resistance. GPAT1$^{-/-}$ mice have reduced hepatic lysophosphatidic acid (LPA), DAG and TAG content [13]. When fed a high-fat safflower oil diet for 3 weeks, GPAT1$^{-/-}$ mice are protected from high-fat diet-induced insulin resistance, suggesting that a glycerolipid metabolite may be involved in the mechanism of high-fat-diet-induced hepatic insulin resistance [14]. GPAT1 mRNA expression is increased in livers from *ob/ob* and high-fat-fed mice, suggesting that enhanced GPAT1 activity could play a role in the pathogenesis of insulin resistance [15]. **However, the specific role of hepatic *de novo* glycerolipid synthesis in the development of systemic insulin resistance is not completely understood.**

1.1 SPECIFIC AIMS:

Aim 1. To determine whether liver-specific overexpression of GPAT1 causes fatty liver and hyperlipidemia

Aim 2: To determine whether GPAT1 over-expression in liver leads to the development of hepatic or whole-body insulin resistance.

Aim 3: To determine the mechanism(s) of hepatic insulin resistance in Ad-GPAT-treated rats.

Aim 4. To identify and characterize the mouse liver erGPAT isoform(s).

CHAPTER II. LITERATURE REVIEW

2.1 Triacylglycerol Metabolism in Health and Disease

The regulation of triacylglycerol (TAG) synthesis and metabolism plays an important role in whole body energy homeostasis in mammals. TAG is synthesized in hepatocytes when glucose is abundant. Newly synthesized TAG is either stored within hepatocytes in intracellular droplets as an energy reserve, or packaged in VLDL particles that are released into the blood and carried to skeletal muscle, heart, and adipose tissue for oxidation or storage. In mammals, the primary site of TAG storage is in adipose tissue, which is designed to accumulate large quantities of stored TAG. When glucose supply to the body is limited, stored TAG is released from adipose stores as free fatty acids (FFA) and glycerol, which are oxidized by other tissues. However, when TAG oxidation and synthesis are not properly regulated, TAG can accumulate in non-adipose tissues as well as in adipose tissue, and the excessive accumulation of intracellular TAG has been implicated in the pathogenesis of obesity, cardiovascular disease, insulin resistance, and type 2 diabetes [16]. Cardiovascular disease (CVD) is the number one cause of death in U.S. adults, and the prevalence of obesity and type 2 diabetes, both risk factors for CVD, are increasing at alarming rates [17]. Expanding our basic understanding of TAG metabolism, its regulation, and its role in the pathogenesis of chronic diseases is important for developing effective treatments and prevention strategies for obesity-related disorders.

2.2 *De novo* Triacylglycerol Synthesis

Triacylglycerol (TAG) synthesis begins with activation of long chain fatty acids to acyl-Coenzyme A (CoA) thioesters by the enzyme acyl-Coenzyme A synthetase. Then, *sn*-glycerol-3-phosphate acyltransferase (GPAT) esterifies fatty acids to glycerol-3-phosphate at the *sn*-1 position, forming lysophosphatidic acid (LPA). This first esterification step commits fatty acids to TAG or phospholipid synthesis (Figure 2.1). LPA is acylated by 1-acyl-sn-glycerol 3-phosphate acyltransferase (AGPAT) at the *sn*-2 position to form phosphatidic acid (PA). Phosphatidate phosphohydrolase (also known as lipin) hydrolyzes the phosphate group at the *sn*-3 position of PA to form diacylglycerol (DAG). Diacylglycerol acyltransferase (DGAT) acylates DAG at the *sn-3* position to complete the final step in TAG synthesis [18]. All of the enzymes of *de novo* TAG synthesis have multiple isoforms, but the exact role of each isoform in the regulation of *de novo* glycerolipid synthesis and in the intracellular channeling of glycerolipid intermediates to specific downstream pathways has not been elucidated [19].

2.3 Glycerol-*sn*-3-Phosphate Acyltransferase Isoforms

Glycerol-*sn*-3-phosphate acyltransferase acylates glycerol-3-phosphate (G3P) at the *sn*-1 position of the glycerol backbone to form 1-acyl-*sn*-glycerol-3-phosphate, or lysophosphatidic acid (LPA) [20, 21] (Figure 2.1). This step commits acyl-CoAs to *de novo* glycerolipid synthesis, and it may be the rate-limiting step, as GPAT has the lowest specific activity of all of the enzymes in that pathway [22, 23]. When this dissertation project was initiated there were two known mammalian GPAT activities – a mitochondrial activity that was resistant to inhibition by the sulfhydryl reagent N-ethylmaleimide (NEM) (called

mtGPAT), and a microsomal activity that was sensitive to inhibition by NEM (called erGPAT) [24]. The enzyme responsible for the NEM-resistant mitochondrial activity had been purified and cloned (GPAT1) [25-27], but no NEM-sensitive erGPAT had been cloned. In the last several years, multiple mitochondrial and microsomal GPAT isoforms have been identified and cloned. There are now 4 known GPAT isoforms, 2 mitochondrial GPATs [GPAT1 (formerly mtGPAT) and GPAT2], and two microsomal GPATs (GPAT3 and GPAT4). GPAT isoforms 1-3 are described in this chapter, and the identification of GPAT4 will be described in Chapter 3.

2.3.1 GPAT1

2.3.1.1 GPAT1 Enzyme Structure:

GPAT1 has been cloned from both mouse (827 a.a.) and rat (828 a.a) liver, and has also been purified from rat liver [25-27]. Located in the outer mitochondrial membrane, GPAT1 has 2 transmembrane domains (472-493 a.a. and 576-592 a.a.), with the N and C terminal ends facing the cytosol and a loop structure (494-575 a.a.) facing the intermembrane space of the mitochondrion [28]. The active-site is located on the N terminal domain and faces the cytosol, indicating that LPA is released into the cytosol or into the cytosolic face of the mitochondrial bilayer. LPA synthesiszed by GPAT1 is either acylated by a mitochondrial AGPAT isoform (none have been cloned), or it must travel from the mitochondria to the endoplasmic reticulum, where the downstream enzymes of glycerolipid synthesis are located [22]. Although the exact mechanism for the transport of LPA from mitochondria to the ER are not known, liver-fatty acid binding protein (L-FABP) has been shown to transport LPA from mitochondria to the ER for PA synthesis *in vitro* [29].

GPAT1 shares conserved active site and substrate binding motifs with other acyltransferases, including AGPAT isoforms **(Table 2.1)**[29]. Site-directed mutagenesis of conserved amino acids in Motifs I-IV of *E. coli* GPAT indicate that Motifs I, III, and IV are involved in catalysis, whereas Motifs II and III are important for glycerol-3-phosphate binding [30]. Although the essential catalytic motifs are on the N-terminus, site-directed mutagenesis of the loop structure that faces the intermembrane space decreases enzyme activity, suggesting a critical structural or regulatory role of this domain [28]. Truncation of the C-terminus also inactivates GPAT1, suggesting that the N- and C-termini are both required for GPAT activity [31]. Crosslinking studies of GPAT1 truncation mutations indicate that the N- and C-terminal regions interact [31]**.** This interaction of termini may be involved in the post-translational regulation of GPAT1 by adenosine monophosphate activated protein kinase (AMPK), as phosphorylation of two AMPK consensus binding domains on the C-terminal end could alter the interaction between the termini, and thus decrease enzyme activity [31].

2.3.1.2 GPAT1 Tissue Expression and Activity:

Because GPAT1 activity is resistant to NEM, its activity can be distinguished from all other known GPAT isoforms in total cellular membrane fractions in liver. Purified GPAT1 has a substrate preference for 16:0-CoA compared to 18:0-CoA or 18:1-CoA, but it can use a variety of long-chain acyl-CoAs [25]. GPAT1 mRNA is expressed in most tissues, but it is most highly expressed in liver, adipose tissue, muscle, kidney, and brain [29, 32]. Protein, mRNA, and activity levels do not correlate well to each other, and the correlation varies in

Table2.I: Acyltransferase motifs in *E. coli* and mouse GPAT and AGPAT isoforms

	Motif I[a]	Motif II[a]	Motif III[a]	Motif IV[a]	Motif V[f]
Function[a]	**Catalysis**	**G-3-P binding**	**G-3-P binding**	**Catalysis**	
***E. coli* GPAT** (plsB) (BAE78043)	306 **H**RSHM**D**	349 GAFFI**R**	383 **F**V**EG**GRSR	417 ITLI**P**IYIG**Y**E	
GPAT1[b] (NP_032175)	230 **H**RSHI**D**	272 GGFFI**R**	313 **F**L**EG**TRSR	347 ILVI**P**VGIS**YD**	
GPAT2[c] (NP_001074558)	202 **H**KSLL**D**	231 T*C*SPAL**R** or 235 ALRALL**R** or 247 LGGFLPP[33]	291 GSP**G**RLSA or 285 **F**L**E**EPPGS[33]	320 ATLV**P**VAIA**YD**	
GPAT3 (AGPAT8[d]) (NP_766303)	229 **H**RTRV**D**	268 VHIFIH**R**	301 **F**P**EG**T*C*LT	324 GTIY**P**VAIK**Y**N	366 **V**WYMPPMTREEGE**D**
GPAT4 (AGPAT6[e]) (NP_061213)	248 **H**TSPI**D**	286 PHVWFE**R**	320 **F**P**EG**T*C*IN	343 ATVY**P**VAIK**YD**	385 **V**WYLPPMTREKDE**D**
AGPAT1 (NP_061350)	104 **H**QSSL**D**	140 GIIFID**R**	173 **F**P**E**G**G**TRNH	200 VPII**P**IVNSSY	231 **V**LPPVSTEGLTPD**D**
AGPAT2 (NP_080488)	98 **H**QSLI**D**	137 GVYFIN**R**	170 YP**EG**TRND	197 VPII**P**VVYSSF	227 **V**LDAVPTNGLTDA**D**
AGPAT3 (NP_443747)	96 **H**NFEI**D**	138 EIVFCK**R**	174 YC**EG**TRFT	202 YHLL**P**RTKGF	
AGPAT4 (NP_080920)	96 **H**KFEI**D**	138 EMIFCT**R**	174 HC**EG**TRFT	202 HHLL**P**RTKGF	
AGPAT5 (NP_081068)	93 **H**QSTV**D**	137 GGIYVK**R**	171 FP**EG**TRYN	202 HVLT**P**RIKAT	
AGPAT7 (NP_997089)	129 **H**STFF**D**	166 QAILVS**R**	201 FP**EG**T*C*SN	224 VPVQ**P**VLIRY	

[a]Acyltransferase motifs as we described in [30]. Bolded residues are highly conserved across these GPAT family members. Underlined amino acids are important for GPAT1 activity as determined by mutagenesis [29, 30]. *Cysteines* that may be targets for *N*-ethylmaleimide are highlighted and italicized. [b]Previously called mtGPAT or mtGAT. [c]Also identified as xGPAT1 [33]. [d]Identified in GenBank as AGPAT8, but has only GPAT activity [34]. [e]Identified in GenBank as AGPAT6, but has only GPAT activity as reported here. [f]Motif V as described in [35].

different tissues [32]. Rat liver and adipose tissue have the highest GPAT1 specific activities [32]. The discrepancies between GPAT1 mRNA and protein expressions and activities indicate that GPAT1 may be regulated post-transcriptionally and post-translationally [32]. In most tissues, GPAT1 activity accounts for 10-20% of the total GPAT activity, whereas NEM sensitive GPAT activity accounts for 80-90% of the total GPAT activity [29]. In liver, however, GPAT1 activity accounts for 40-50% of the total GPAT activity, suggesting a specific role for GPAT1 in liver glycerolipid synthesis [29].

2.3.1.3 GPAT1 As a Key Regulator of TAG Synthesis

Despite its location on the mitochondrial outer membrane and the fact that the terminal enzymes required for TAG synthesis are located in the ER (Figure 1), GPAT1 plays an important role in the synthesis of TAG [2, 36-38]. When GPAT1 is overexpressed in Chinese hamster ovary (CHO) cells in the presence of trace [^{14}C]oleate, label incorporation into DAG and TAG increase 2-fold, and TAG mass also increases 2-3-fold, whereas label incorporation into phospholipids decreased by 30% [38]. However, in primary hepatocytes treated with trace [^{14}C]oleate, a slight increase in PC synthesis (28%) and a >2-fold increase in DAG synthesis is observed in the absence of a significant change in TAG synthesis [36]. When primary hepatocytes are incubated with 100-750 μM oleate, [^{14}C]oleate incorporation into DAG and TAG increase >2-fold, while acid soluble metabolites and CO_2 production are reduced 50-80% [36, 37]. Slight increases in PL synthesis are also observed when GPAT1-treated primary hepatocytes were incubated with exogenous fatty acid, but the increases in DAG and TAG were more pronounced [36, 37]. Although an increase in hepatic TAG might cause increased TAG secretion into the cell culture media from hepatocytes, no significant

increase in media TAG was observed in two independent studies of GPAT1 overexpression in primary hepatocytes [36, 37]. These studies in cell culture systems suggested that GPAT1 primarily directs exogenous fatty acids toward TAG synthesis, rather than toward PL synthesis pathways or β-oxidation, but the role of GPAT1 in the regulation of TAG secretion from liver cells is not clear from these *in vitro* models.

Several animal models have been developed to examine the role of GPAT1 in TAG synthesis *in vivo*. Compared to wild-type mice, mice that are deficient in GPAT1 ($GPAT1^{-/-}$) have 37% lower liver TAG content, lower TAG secretion, lower body weight, lower gonadal fat pad weight, and lower plasma cholesterol levels[2]. Incorporation of palmitate (16:0) into TAG and PL was significantly lower in liver from $GPAT1^{-/-}$ mice, supporting the preference of GPAT1 for palmitate. When fed a high sucrose/high saturated fat (HH) diet for 4 months or a high-fat safflower oil diet for 3 weeks, $GPAT1^{-/-}$ mice had a 60% lower hepatic TAG content than wild-type controls, and a 2-fold increase in plasma β-hydroxybutyrate concentration, indicating that lack of GPAT1 in liver altered fatty acid metabolism to favor oxidation over TAG synthesis [13, 14]. Liver-specific knock-down of GPAT1 with antisense oligonucleotides in obese and diabetic *ob/ob* mice also reduced hepatic TAG and DAG content with little effect on hepatic phospholipid or cholesterol content, further supporting a role for GPAT1 in the regulation of TAG synthesis [39]. While these studies all suggest that GPAT1 is important in regulating hepatic TAG synthesis, the effects of GPAT1 on plasma TAG concentrations were not clear, as $GPAT1^{-/-}$ mice had lower plasma TAG concentrations compared to wild-type controls in

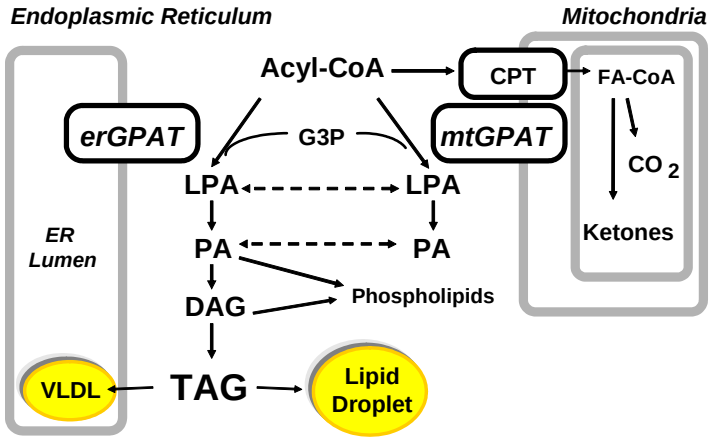

Figure 2.1: GPAT isoforms located in mitochondria (GPAT1 and GPAT2) and ER (GPAT3 and GPAT4) catalyze the initial step in the *de novo* synthesis of phospholipids and triacylglycerol (TAG). Acyl-CoA use for β-oxidation or TAG synthesis is regulated reciprocally by carnitine palmitoyl transferase 1 (CPT-1) and GPAT1 (mtGPAT). Lysophosphatidic acid (LPA), phosphatidic acid (PA), diacylglycerol (DAG), very low density lipoprotein (VLDL).

some studies [2, 14], and not in others [13, 39]. The effects of GPAT1 on plasma cholesterol content were more consistent, with reduced hepatic GPAT1 activity correlating with lower plasma cholesterol in 3 out of 4 studies [2, 13, 14, 39].

Hepatic overexpression of GPAT1 in mice [15] and rats (see Chapter 3)[40] also supports a role for GPAT1 in TAG synthesis. Mice treated with an adenovirus that expresses GPAT1 had a 3.5-fold increase in hepatic TAG, and fatty acid oxidation was reduced in liver slices from Ad-GPAT1-treated mice [15]. Taken together, studies of GPAT1 overexpression and knock-down studies in rodents support the hypothesis that GPAT1 and CPT1 compete for acyl-CoAs at the mitochondrial membrane, and that GPAT1 primarily channels these acyl-CoAs towards TAG synthesis. The role of GPAT1 in the secretion of TAG from liver is still not completely understood, but over expression of GPAT1 in rodents increases TAG and plasma cholesterol concentrations (see Chapter 3)[15, 40].

2.3.1.4 GPAT1 Transcriptional Regulation

Transcription of the GPAT1 gene is primarily regulated by insulin, which is consistent with the hypothesis that GPAT1 plays a prominent role in regulating TAG metabolism. Hepatic GPAT1 mRNA levels decrease dramatically in rats that were fasted for 48 h, and both gene expression and enzyme activity levels increase when the rats are re-fed a high sucrose, low-fat diet [32]. The response of GPAT1 gene expression to fasting and re-feeding is regulated by insulin, as hepatic GPAT1 gene expression does not respond to fasting and re-feeding in streptozotocin-induced diabetic rodents, which do not produce insulin [41]. When insulin is administered to these rodents, liver GPAT1 mRNA levels

increase, as do sterol regulatory element binding protein-1c (SREBP1-c) mRNA levels.[41, 42]. SREBP1-c is a transcription factor that regulates the expression of multiple lipogenic enzymes, including FAS, ACC, and SCD-1 [43-46]. Insulin up regulates the transcription of SREBP1-c, and active SREBP-1c binds to SREs or E-box motifs in the promoters of its target genes [29]. The murine GPAT1 promoter contains response elements for the transcription factors sterol SREBP-1c and for NF-Y (nuclear factor Y), and GPAT1 expression increases in 3T3-L1 adipocytes with ectopic expression of SREBP-1c or SREBP-1a [47]. GPAT1 gene expression is also up regulated in liver and adipose tissue by both SREBP-1a and -1c [48].

Carbohydrate may increase GPAT1 gene expression by enhancing the binding of carbohydrate response element binding protein (ChREBP) to carbohydrate response elements (ChRE) in the GPAT1 promoter [49]. ChREBP is a transcription factor that is activated by xyulose 5-P, and it regulates many of the genes involved in hepatic lipogenesis [50, 51]. In ChREBP$^{-/-}$ mice, an SREBP-1c agonist still fully activates the GPAT1 transcript, suggesting that ChREBP is not required for the SREBP-1c-mediated activation of GPAT1 expression [52]. However, it is still unknown whether ChREBP can activate GPAT1 transcription independently of SREBP-1c. The liver X receptor (LXR) is the key lipogenic regulatory transcription factor in liver because it regulates the expression of both SREBP-1c and ChREBP [52]. Thus, activation of LXR through insulin signaling or oxysterol binding to LXR can activate GPAT1 expression indirectly through its activation of SREBP-1c expression [52].

2.3.1.5 Acute Regulation of GPAT1

Although no one has shown direct phosphorylation or dephosphorylation of GPAT1, there is mounting evidence that GPAT1 activity is acutely regulated by phosphorylation and dephosphorylation events. GPAT1 activity is negatively regulated by the cellular energy sensor, AMP-activated protein kinase (AMPK). AMPK becomes active when cellular ATP levels fall and cellular AMP levels rise, and it inactivates anabolic pathways in favor of catabolic pathways [53]. AMPK phosphorylates and inactivates ACC, thus decreasing cellular concentrations of malonyl-CoA, a potent inhibitor of CPT-1, and increases acyl-CoA entry into mitochondria for β-oxidation [53]. Incubation of rat liver total membranes with purified recombinant AMPK inhibits GPAT1 activity, and treating primary rat hepatocytes with 5-amino-4-imidazolecarboxamideriboside (AICAR), an activator of AMPK, inhibits radio labeled glycerol incorporation into glycerolipids [54]. Exercise, which increases cellular energy needs, activates AMPK, and decreases GPAT1 activity in liver and adipose tissue, but not in skeletal muscle [55]. Thus, AMPK reciprocally regulates the partitioning of acyl-CoAs into oxidative and glycerolipid synthetic pathways by inhibiting GPAT1 while it activates CPT-1 by inhibiting ACC and producing malonyl-CoA.

In addition to acute regulation by AMPK, GPAT1 activity is also regulated by casein kinase II (CK2). Casein kinase II is a constitutively active cytosolic serine/threonine kinase that is involved in multiple cellular processes, including regulation of the cell cycle and DNA repair [56]. Only a few metabolic enzymes are known to be regulated by CK2, including ACC and glycogen synthase [56]. Treating rat mitochondria with CK2 increases GPAT1 activity 2-fold, and there are several putative CK2 binding sites in the GPAT1 protein

sequence [57]. Because CK2 is constitutively active, its physiological role in GPAT1 regulation is not clear.

2.3.2 GPAT2:

2.3.2.1 Structure of GPAT2

GPAT2 is a 798-801 a.a. protein that is 27%-32% identical to GPAT1 [58, 59]. The active site region of GPAT2 contains acyltransferase motifs I and IV, but motifs II and III differ from those of GPAT1 and are more closely related to those of squash chloroplast GPAT [59]. At least two transmembrane domains are predicted for the GPAT2 protein structure, one at 446-474 a.a., and another at 156-177 a.a. [59]. Two other potential transmembrane domains (204-226 or 301-329 a.a.) seem unlikely because they include the active site motifs and would place portions of the active site on opposite sides of the membrane [59]. Assuming that the two transmembrane domains do not include the active site motifs, the predicted topology for GPAT2 would place both N and C termini in the intermembrane space, with the active site in the loop region facing the cytosol [59]. The open reading frame for GPAT2 encodes an 88 kDa protein, but GPAT2 undergoes posttranslational modification in some tissues (brown adipose tissue) and cell culture lines (Cos-7 cells and HEK cells) that results in an 80 kDa protein [59]. The significance of this post-translational modification on GPAT2 activity, subcellular location, or function, is not known.

2.3.2.2 GPAT2 Activity and Tissue Expression

Although structurally similar, GPAT1 and GPAT2 have different sensitivities to sulfhydryl reagents, have different substrate preferences, and have different patterns of expression in mouse tissues. Unlike GPAT1, GPAT2 is an NEM sensitive mitochondrial GPAT [3]. GPAT2 is expressed in mouse testis, heart, liver, spleen, kidney, white and brown adipose tissue, and in 3T3-L1 pre- and post adipocytes [58, 59]. GPAT2 does not prefer palmitoyl-CoA as its substrate, and it does not have a substrate preference [3]. Unlike GPAT1, GPAT2 has low expression and activity in normal mouse liver, but it was up regulated in GPAT1$^{-/-}$ mouse liver, where its activity was originally characterized [3, 59]. This up regulation indicates that GPAT2 may have functional redundancies with GPAT1, but the redundancies are not significant enough to suppress the phenotype of GPAT1$^{-/-}$ mice. Thus, GPAT2 may also have unique functions that are not shared by GPAT1. When GPAT2 is overexpressed in Cos-7 cells, there is an 85% increase in [^{14}C]-oleate incorporation into TAG, while label incorporation into phospholipids is reduced 13 % [59]. These results suggest that GPAT2 is involved in TAG synthesis, but because GPAT2 activity and expression are not readily detectible in wild-type mouse liver, GPAT2 may not play an important role in liver TAG synthesis under normal physiologic conditions. Unlike GPAT1, rat liver GPAT2 expression and activity are not regulated by fasting and re-feeding, indicating that GPAT2 does not play a major role in TAG synthesis in response to nutritional status [59]. GPAT2 may play a unique role in testis lipid metabolism, and the promoter of GPAT2 contains hormone response elements for glucocorticoids, estrogen, and progesterone that could regulate its expression in testis [59]. Further studies are needed to identify the specific role of GPAT2 in the regulation of TAG and PL synthesis in liver and other tissues.

2.3.3 GPAT3

Recently, the gene product previously called AGPAT8 in the NCBI database was shown to have NEM-sensitive GPAT activity and to lack AGPAT activity; this gene product was re-named GPAT3 [34]. GPAT3 is a 438 a.a. protein that contains all four conserved acyltransferase motifs, and like GPAT1 and 2, it is predicted to have at least 2 transmembrane domains [5]. GPAT3 is located in the endoplasmic reticulum, making it the first erGPAT to be identified [5]. Like GPAT2, GPAT3 recognizes a broad range of acyl-CoA species, and it has the highest activity with l6-18 carbon acyl-CoA species [5]. GPAT3 was highly expressed in mouse white adipose tissue (WAT), small intestine, brown adipose tissue (BAT), kidney, heart and colon[5]. The expression and activity of GPAT3 increase during the differentiation of 3T3-L1 preadipocytes, and siRNA knockdown of *Gpat3* in 3T3-L1 adipocytes reduces total GPAT activity by ~60% [4, 34]**,** it is likely that GPAT3 is important for TAG synthesis in WAT. Expression of GPAT3 in WAT may be regulated by the transcription factor peroxisome proliferator gamma (PPARγ), because GPAT3 mRNA increased 5-fold in WAT from *ob/ob* mice treated with rosiglitizone [5]. Although GPAT3 appears to be the major erGPAT isoform in WAT, GPAT3 is unlikely to play a major role in TAG synthesis in liver where its mRNA expression is very low [4, 5]. Since 50% of the GPAT activity in liver is a microsomal NEM-sensitive activity, it is possible that another erGPAT isoform exists (see Chapter 3).

2.4 1-Acyl-sn-Glycerol-3-Phosphate Acyltransferase Isoforms

1-Acyl-*sn*-glycerol-3-phosphate acyltransferase (AGPAT) acylates LPA at the *sn*-2 position to form PA. There were 8 named AGPAT isoforms (AGPAT1-8), all of which share sequence homology with GPAT isoforms at the conserved motifs I-IV (Table 2.1) [29]. AGPAT1 and AGPAT2 have been shown to have robust AGPAT activity [60, 61], but the activities of AGPAT3-7, which were identified by sequence homology to AGPAT1 and 2, have either not been characterized, or were reported to have very low AGPAT activity compared to AGPAT1 and 2 [62, 63]. AGPAT8 has recently been re-characterized as an NEM-sensitive GPAT and has been re-named GPAT3 [5].

2.4.1 AGPAT1

Human AGPAT1 is a 283 a.a. protein that is located in the endoplasmic reticulum when overexpressed in CHO cells [60]. AGPAT1 prefers 16:0, 18:0, or 18:1-CoA over 20:0 or 20:4-CoA, suggesting that AGPAT1 is not responsible for the enrichment of glycerolipids with 20:4 at the *sn*-2 position [29, 60]. AGPAT1 is expressed in most tissues, with high levels of expression in mouse BAT, brain, and visceral WAT, and human liver, heart, immune cells, and epithelium [4, 61, 64, 65]. Although few functional studies of AGPAT1 have been done, overexpression of AGPAT1 in adipocytes suggests that AGPAT1 is involved in TAG synthesis **[66]**. AGPAT1 may play an important role in mediating PA signaling for immune responses, as the AGPAT1 gene has been mapped to the class III region of the human major histocompatibility complex [60]. Additionally, overexpression of AGPAT1 can enhance IL-1β-mediated TNFα and IL-6 release from ECV304 cells [65].

2.4.2 AGPAT2

Human AGPAT2 is a 278 a.a. protein that shares 35% amino acid identity with AGPAT1 [65]. Unlike AGPAT1, AGPAT2 has a preference for 20:4-CoA, suggesting that it is the AGPAT isoform responsible for the common placement of arachidonic acid at the *sn*-2 position of phospholipids [61]. AGPAT2 mRNA is expressed in most human tissues, but its expression is highest in heart and liver in humans and in adipose tissue in mice [4, 61]. Patients with congenital generalized lipodystrophy are deficient in adipose tissue stores of TAG, and this deficiency in adipose tissue TAG has been associated with loss-of function mutations in AGPAT2 [67, 68]. Pre-adipocytes deficient in AGPAT2 do not differentiate into mature adipocytes, and C/EBPβ and PPARγ induction during early adipocyte differentiation is suppressed [69]. Knock-down of AGPAT2 in mature adipocytes reduces TAG mass by 50%, suggesting a key role for AGPAT2 in TAG synthesis [69]. Thus, AGPAT2 may play an important role in generating PA both for TAG synthesis and for intracellular signaling events important for preadipocyte differentiation [69]. In addition to its role in adipose tissue differentiation and metabolism, AGPAT2 may play an important role in carcinogenesis, as AGPAT2 expression in up regulated in several tumor cell lines [70]. PA generated by AGPAT2 supports Raf activation in the Ras/Raf/ERK pathway that enhances cell proliferation, and pharmacological inhibition of AGPAT2 inhibits cellular growth and initiates apoptosis in tumor cells [71]. Thus, AGPAT2-derived PA seems to be important for both TAG synthesis and for intracellular signaling pathways related to cellular differentiation and survival.

2.4.3 AGPAT3-5, and 7

No functional studies of AGPAT3-5 or 7 have been reported, so their role in TAG synthesis is not known. AGPAT activities for AGPAT3-5 have been reported, but the activities were barely above background AGPAT activity in Cos-7 cells, and they were also low in comparison to those measured for AGPAT2 in the same cells [63]. Thus, it is possible that AGPAT3-5 catalyze other acyltransferase activities besides AGPAT activity. AGPAT3-5 expression increases during 3T3-L1 adipocyte differentiation, but the level of expression is much lower than that of AGPAT2, indicating that these isoforms may not be as important for adipocyte TAG synthesis as AGPAT2 [69]. In mice, AGPAT3 and 5 are expressed in liver, WAT, and BAT [4]. Unlike other AGPATs, AGPAT4 and AGPAT7 have very high mRNA expression in mouse brain [4]. Further studies are needed to characterize the roles of these AGPAT isoforms in TAG and PL synthesis and in intracellular signaling pathways, and to determine whether they have GPAT activity.

2.4.4 AGPAT6 Knock-out Mice

AGPAT6 plays an important role in TAG synthesis in subdermal adipose tissue, WAT, and BAT, as mice deficient in AGPAT6 have a 90 % reduction in subdermal TAG, and a 40% and 30% reduction in TAG in WAT and BAT, respectively [4]. Liver TAG content and plasma TAG concentration are reduced 50% in AGPAT6$^{-/-}$ mice, suggesting that AGPAT6 is important for liver TAG synthesis and for the secretion of TAG from the liver. AGPAT6 deficiency protected *ob/ob* and high-fat/high-carbohydrate fed mice from obesity [4]. AGPAT6 is also important for TAG synthesis in mammary epithelial cells, as AGPAT6^{-}

$^{/-}$ dams produce milk deficient in DAG and TAG, and the pups must be fostered to survive [35]. Together, these studies indicate an important role for AGPAT6 in TAG synthesis in adipose tissue, mammary tissue, and liver. No studies have been done to examine the transcriptional regulation of AGPAT6, and the specific acyltransferase activity of AGPAT6 was not confirmed when it was overexpressed in insect cells [35]. The phenotype of the AGPAT6$^{-/-}$ mice is consistent with a deficiency in a glycerolipid acyltransferase. Thus, AGPAT6 could have GPAT activity (see Chapter 4).

2.5 Are There More Than 3 GPAT Isoforms?

Evidence that more than 3 isoforms of GPAT exist is growing. For several decades, only two GPAT activities were recognized, a mitochondrial NEM-resistant activity, and a microsomal NEM-sensitive activity. The NEM-sensitive activity is generally 90% of the activity in most tissues, while the NEM-resistant activity accounts for 10% of the activity [24]. In liver, however, the NEM-resistant and NEM-sensitive activities each account for ~50% of the total GPAT activity[24]. The recent identification of GPAT2 and GPAT3 complicates the picture of GPAT activity in hepatocytes and other cells. Each known GPAT isoform has a different profile of tissue expression, suggesting that each isoform plays a specific role in different tissues. For instance, GPAT2 may have an important role in testis glycerolipid metabolism, but it probably has a minor role in hepatic TAG synthesis [59]. GPAT3 is the first erGPAT identified, and its tissue expression pattern and role in 3T3-adipocyte differentiation indicate that it is important for regulating TAG synthesis in WAT [4, 5, 72]. Approximately fifty percent of total hepatic GPAT activity is NEM-sensitive and microsomal, but GPAT3 may not be responsible for all this activity because GPAT3 gene

expression in liver is very low compared to its expression in WAT [4]. Thus, another NEM-sensitive erGPAT isoform may exist. GPAT3 was originally classified as an AGPAT (AGPAT8) based on amino acid sequence homology to AGPAT1 and AGPAT2, and the specific acyltransferase activity of several other named AGPAT isoforms has not been confirmed (Vergnes, 2006). AGPAT6 shares 66% amino acid sequence homology with GPAT3 (AGPAT8), and its expression in liver is relatively high [4, 5]. AGPAT3 and AGPAT5 are also expressed at relatively high levels in liver. The specific acyltransferase activity of these "AGPAT" isoforms needs to be characterized to confirm that they have AGPAT activity. It is possible that one or more of the "AGPAT" isoforms actually has GPAT activity.

2.6 Why Are There So Many GPAT and AGPAT Isoforms?

The identification of multiple GPAT and AGPAT isoforms with different subcellular locations and tissue expression patterns is somewhat puzzling. Why does a cell need so many enzymes to make precursors for phospholipid and TAG? One possibility is that different GPAT and AGPAT isoforms are assigned to channel acyl-CoAs to PL synthesis vs. TAG synthesis. In this case, GPATs designated to channel LPA to PL synthesis might be in close proximity on the mitochondrial or endoplasmic reticulum membranes to AGPATs designated to make PA for PL synthesis. The fact that functional studies of all 3 of the known GPAT isoforms suggest that all 3 are involved in TAG synthesis makes this first idea seem unlikely [19]. Another possibility is that the reason for multiple GPAT and AGPAT isoforms is to regulate the synthesis of their products, LPA and PA, as intracellular signaling molecules that can affect cellular processes in addition to PL and TAG synthesis [19]. LPA

secreted into plasma by immune cells can bind to extracellular G-protein-coupled receptors (EDGE receptors) that are involved in the regulation of cell growth [73]. Additionally, intracellular LPA may activate transcription factors such as PPARγ [74]. PA can activate kinases in the Ras/Raf/ERK signaling pathway that regulates cellular differentiation and survival, and it may also activate survival pathways through activation of mTOR [71, 75]. Additionally, the DAG product of phophatidic acid phosphohydrolases (Lipin), can activate conventional and novel forms of protein kinase C, which are involved in multiple cell signaling pathways (Parker, 2004). Traditionally, these signaling glycerolipids were thought to be generated from membrane phospholipids, not from *de novo* glycerolipid synthesis, but the increased association of PKC isoform activation and DAG accumulation in insulin resistant tissues whose TAG content is elevated suggests that intermediates of *de novo* triacylglyerol synthesis could be involved in activating intracellular signaling pathways. Further studies are necessary to identify the particular roles of GPAT and AGPAT isoforms in channeling acyl-CoAs to PL and TAG synthesis, as well as their potential roles in regulating intracellular signaling by LPA, PA, and DAG.

2.7 Fatty Liver and the Metabolic Syndrome

Once thought to be a benign hallmark of excessive alcohol consumption, hepatic triacylglycerol (TAG) accumulation has recently been acknowledged as a significant metabolic disorder. Non-alcoholic fatty liver disease (NAFLD) is associated with obesity, insulin resistance, type 2 diabetes, and cardiovascular disease [76-79]. Accumulation of TAG in hepatocytes is associated with increased very low density lipoprotein (VLDL) secretion from the liver, leading to high plasma TAG and cholesterol levels [79]. Elevated

plasma lipids can induce TAG accumulation in extra-hepatic tissues, such as skeletal and heart muscle, and TAG accumulation in these tissues may contribute to the development of whole-body insulin resistance. Although associations between insulin resistance and NAFLD are well documented, the role of hepatic steatosis in the development of whole-body and hepatic insulin resistance is not completely understood.

2.7.1 The Role of Fatty Liver in Insulin Resistance:

The pathogenesis of whole-body insulin resistance is complex, involving multiple organs and endocrine systems that are involved in the regulation of energy homeostasis. Four of the main organs affected by diabetes and insulin resistance include the liver, pancreas, adipose tissue, and skeletal muscle, and it has been hypothesized that alterations in the distribution of fat between adipose tissue, liver, and muscle are primarily responsible for causing insulin resistance [80]. Each of these tissues has a unique role in the maintaining glucose and lipid homeostasis, and insulin resistance in any one of these tissues has been shown to contribute to whole-body insulin resistance and type 2 diabetes [81, 82]. Liver-specific insulin receptor knock-out mice exhibit severe insulin resistance and impaired glucose tolerance, while muscle-specific and adipose-specific insulin receptor knock-out mice have normal glucose tolerance, suggesting that the liver could play an important role in the pathogenesis of insulin resistance [81-83]. A recent study of glucose tolerance and insulin sensitivity in obese patients with normal glucose tolerance tests indicates that hepatic steatosis can increase insulin resistance independently of obesity [84]. In another study, patients with type 2 diabetes and hepatic steatosis were substantially more insulin resistant than those with type 2 diabetes and no hepatic steatosis [85], suggesting that liver fat

accumulation can play a causal role in the development of insulin resistance and type 2 diabetes in humans.

Hepatic insulin resistance develops when the liver is less sensitive to the suppressive effects of insulin on hepatic glucose output (HGO) and VLDL TAG production. Hepatic steatosis and hepatic insulin resistance co-exist in many animal models, including, high-fat and high-sucrose diet-induced obesity in rats [86, 87], obese Zucker rats [88], leptin deficient mice [89], A-ZIP/F-1 fatless mice [90], CD36 fatty acid transporter KO mice [10], and ethanol fed rats [91]. Furthermore, hepatic steatosis in animal models is highly correlated with systemic insulin resistance [92]. Additionally, the amelioration of steatosis has been associated with increased insulin sensitivity. A-ZIP/F1 transgenic fatless mice accumulate TAG in liver and muscle and are insulin resistant. Hepatic and muscular steatosis and insulin resistance is ameliorated when adipose tissue from wild-type mice is transplanted into the A-ZIP/F1 mice [90]. Administering a PPARα agonist to *ob/ob* mice increases β-oxidation, lowers hepatic TAG accumulation, and improves hepatic insulin sensitivity [93].

Despite these correlations, there are models of steatosis where hepatic insulin resistance is not evident. Buettner et al. (2004) recently demonstrated that rats fed a high fat diet for 6 weeks do not have hepatic insulin resistance, despite hepatic steatosis and mild whole-body insulin resistance [94]. In another model, PTEN, a lipid phosphatase that negatively regulates the insulin-stimulated PI3K/Akt pathway, was selectively knocked out in mouse liver. PTEN-LKO mice have hepatic steatosis but have increased hepatic insulin sensitivity compared to wild-type mice [95, 96]. Mice fed a methionine-choline-deficient

diet develop steatohepatitis, but they have lower blood glucose levels and increased hepatic insulin sensitivity compared to control-fed mice [97]. Additionally, treatment of insulin resistant, steatotic mice with PPARγ agonists such as rosiglitazone improves hepatic insulin sensitivity despite an increase in hepatic TAG accumulation [86].

The reason for improved insulin sensitivity in some models of steatosis and for insulin resistance in others is not understood, but differences in the specific types of lipids that accumulate in tissues may explain the differences in insulin sensitivity. Increased *de novo* lipogenesis will not only increase TAG synthesis, but will also increase the synthesis of other lipid metabolites, such as acyl-CoAs, LPA, PA, DAG, or ceramide that could accumulate in the liver and affect insulin sensitivity. Another possibility is that hepatic steatosis caused by an increase in *de novo* glycerolipid synthesis is more likely to cause insulin resistance than steatosis caused by a reduction in secretion of TAG from the liver in VLDL particles. In this case, lipid metabolites generated during the synthesis of TAG could act as signaling molecules that block insulin signaling. TAG that accumulates in hepatocytes as a result of a block in TAG secretion would not increase lipid intermediates that could interfere with the insulin signaling pathway.

2.8 Insulin signaling and insulin resistance

Insulin is a peptide hormone that is secreted by pancreatic beta cells in response to elevated blood glucose levels during and following a meal. Insulin binds to extracellular tyrosine kinase receptors on cells and initiates signaling cascades that mediate a variety of cellular processes involved in cellular metabolism, cell growth, and cell survival. When

insulin binds to the insulin receptor, the receptor's tyrosine kinase activity autophosphorylates the receptor at several tyrosine residues. Activation of the insulin receptor leads to recruitment and activation of insulin receptor substrate-1 and -2 (IRS-1/2) (Figure 2.2). IRS-1/2 can activate phosphatidylinostitol 3-kinase (PI3K), which converts the phospholipid phosphoinositol-4,5, bisphosphate (PIP2) to phosphoinositol-3,4,5-trisphosphate (PIP3). PIP3 attracts other kinases to the plasma membrane through their plextrin homology domains, which bind PIP3. Insulin's regulation of glucose and lipid metabolism in hepatocytes is mediated through signaling pathways down-stream of PI3K [98]. Recent studies with IRS-2 KO mice suggest that signaling pathways down-stream of IRS-2 are primarily responsible for insulin's regulation of glycogen synthesis and gluconeogenesis [99]. Signaling downstream of IRS-2 leads to the phosphorylation of Akt/PKB, which then phosphorylates GSK3. Akt/PKB-mediated phosphorylation of GSK3β inactivates GSK3β, and allows glycogen synthase to become dephosphorylated and activated. Signaling down-stream of GSK-3 may also activate PEPCK gene expression, thus inhibiting gluconeogenesis [100]. Insulin also increases the expression of many lipogenic enzymes in liver through increased transcription of SREBP-1c, thus promoting FA and TAG synthesis. Insulin's regulation of SREBP-1c is down-stream of IRS-1 [101, 102], but the details of this regulation pathway are not known.

In response to elevated blood glucose levels, insulin acts on skeletal muscle cells and adipocytes to increase glucose uptake into cells through increased translocation of GLUT4 glucose transporters to the plasma membrane. In adipocytes, insulin signaling also results in increased lipogenesis and decreased lipolysis. Insulin acts to increase glycogen synthesis in

skeletal muscle, as well as in liver, and it also decreases the activity and expression of liver enzymes involved in gluconeogenesis. Therefore, the overall action of insulin is to increase glucose uptake into cells, to decrease hepatic glucose output, and to increase lipogenesis in adipose tissue and liver.

2.8.1 Lipid mediators of insulin resistance

The frequent association of TAG accumulation with insulin resistance in liver and other tissues implies that either TAG itself, or some other lipid product synthesized in parallel with TAG may act as a mediator that antagonizes insulin signaling. TAG can be synthesized from acyl-CoAs derived from *de novo* lipogenesis or from re-esterification of acyl-CoAs derived from plasma FFAs. *De novo* synthesis of fatty acids occurs in the liver in response to a meal high in carbohydrate and in response to increased insulin stimulation of enzymes involved in FA synthesis. An increase in *de novo* lipogenesis and glycerolipid synthesis results in increased production of several lipids that could affect hepatic insulin sensitivity, such as malonyl-CoA, ceramide, acyl-CoA, LPA, PA, DAG, or TAG. Of these, malonyl-CoA, ceramide, acyl-CoA, DAG, and TAG accumulation have been implicated in insulin resistance.

Malonyl-CoA is a potent inhibitor of CPT-1, the enzyme responsible for shuttling long-chain fatty acids into mitochondria for beta-oxidation. Thus, an increase in *de novo* lipogenesis increases malonyl-CoA levels and results in lowered rates of beta-oxidation, encouraging synthesis and accumulation of lipids. Adenoviral over-expression of malonyl-

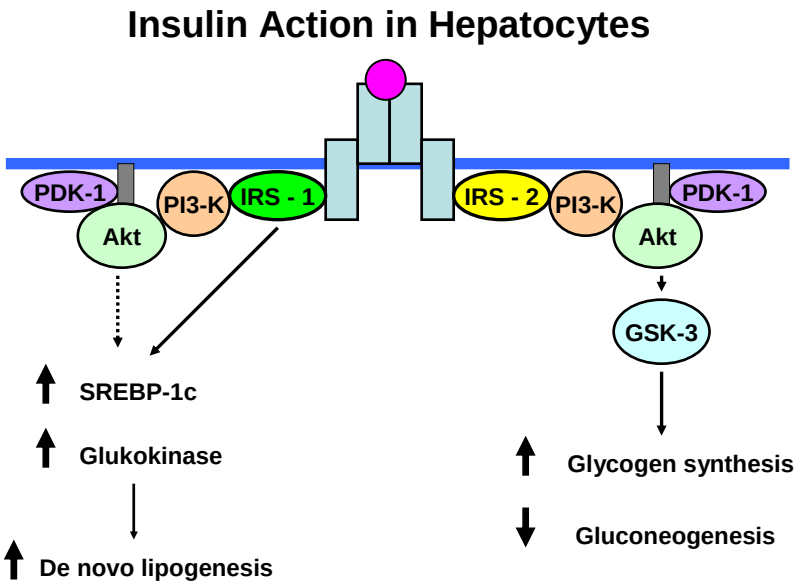

Figure 2.2. Basic insulin signaling pathways in hepatocytes affects lipid and glucose metabolism. Protein dependent kinase 1 (PDK-1), Protein kinase B (Akt/PKB), phosphoinositol 3-kinase (PI3K), insulin receptor substrate (IRS), glycogen synthase kinase 3 (GSK3).

CoA decarboxylase in the livers of rats fed a high fat diet decreased malonyl-CoA levels and protected rats against diet-induced insulin resistance and hepatic steatosis [103].

Ceramide can be synthesized from palmitoyl-CoA and serine by serine palmitoyltransferase (SPT). Since palmitoyl-CoA is the primary FA synthesized in *de novo* lipogenesis, ceramide could accumulate in hepatocytes as a result of increased *de novo* lipogenesis and decreased beta-oxidation. Ceramide accumulation has been implicated in the pathogenesis of insulin resistance in several tissues, including skeletal muscle, pancreatic beta-cells, heart, adipose tissue, and liver [104-107]. Ceramide can inhibit insulin signaling through activation of PKCζ and/or protein phosphatase A2 [105].

The flux of acyl-CoA in the liver is increased both in *de novo* lipogenesis and during increased FFA influx into the liver; this flux of acyl-CoA or FFA into the liver could affect insulin sensitivity [108]. Acyl-CoA accumulation in skeletal muscle and liver has also been associated with insulin resistance [109, 110].

Glycerolipid synthesis is initiated by GPAT, and down-stream lipid products of this pathway include LPA, PA, DAG, and TAG (Figure 2). Of these lipids, DAG accumulation has been implicated in insulin resistance. DAG accumulation has been documented in several models of IR [88, 110], and increased DAG synthesis is thought to mediate inhibition of insulin signaling through activation of various PKC isoforms that can inhibit the activity of signaling molecules that are down-stream of PI3K [111].

2.8.2 DAG synthesis, PKC, and insulin resistance:

Diacylglycerol production is the primary step leading to activation and translocation of conventional and novel PKC isoforms, and there are 3 major pathways that can produce DAG: 1) Receptor-stimulation of phopholipase C (PLC) initiates the hydrolysis of inositol phospholipids (PI) to yield DAG and IP3. 2) Phospholipase D (PLD) hydrolyses phosphatidylcholine (PC) to PA, which is converted to DAG by phosphatidic acid phosphohydrolase (Lipin) 3) DAG can be made through *de novo* synthesis from the glycolytic intermediate glycerol -3-phosphate (G-3-P) via LPA and PA intermediates [112] (**Figure 2.3).** The *de novo* synthesis of DAG provides the major source of DAG in most cells, and the DAG synthesized from this pathway acts a precursor for TAG, phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine synthesis [113].

De novo synthesis of DAG from PA has been implicated in the hyperglycemia-induced DAG formation in diabetic tissues [114-116]. DAG synthesized by hydrolysis of phospholipids on cellular membranes by PLC and PLD is associated with increased protein kinase C (PKC) activity. DAG-mediated activation of PKC has been observed in insulin resistant tissues, and PKC activation has been implicated in the inhibition of insulin signaling on several levels [111, 117, 118]. Novel PKCε and δ have recently been implicated in insulin resistance in liver induced by high-fat feeding or lipid infusion [119, 120], and both isoforms are elevated in diabetic liver [121, 122]**.**

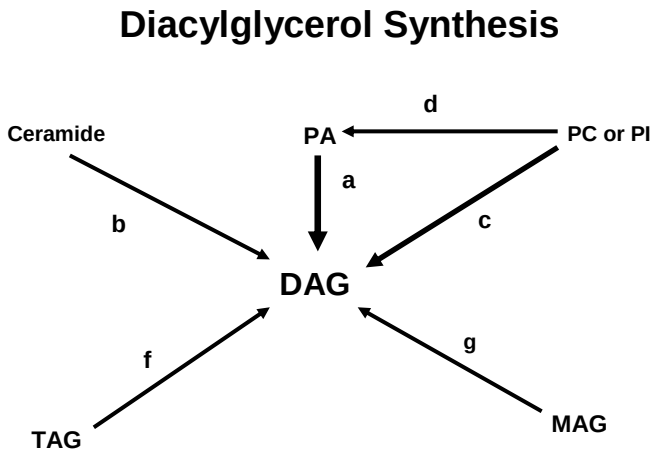

Figure 2.3. Diacylglycerol synthesis pathways. Diacylglycerol (DAG), triacylglycerol (TAG), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidic acid (PA). a, phosphatidic acid phosphohydrolase; b, sphingomyelin synthase; c, phospholipase C; d, phospholipase D; f, triacylglycerol lipase; g, monoacylglycerol acyltransferase.

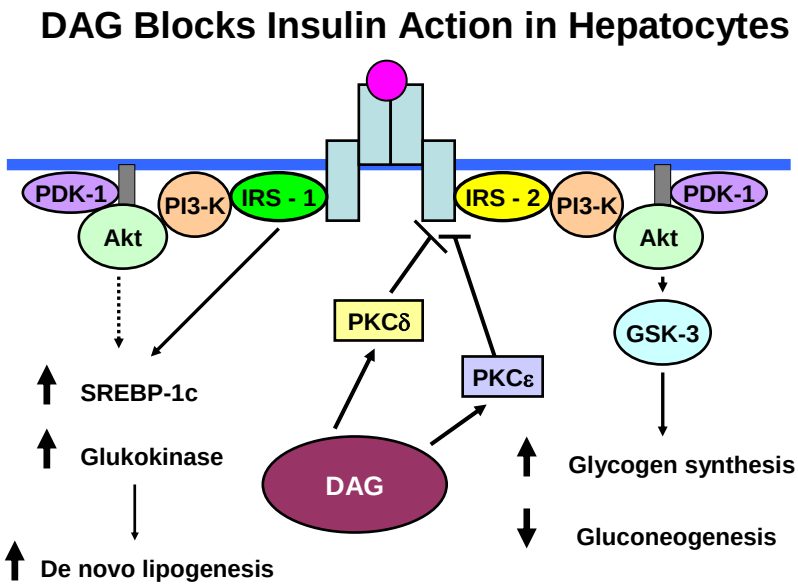

Figure 2.4. DAG activates PKCε, which associates with the insulin receptor and inhibits insulin receptor kinase activity and subsequent activation of IRS-2. DAG can also activate PKCδ, which inhibits IR activity.

2.8.2.1 Protein Kinase C (PKC)

Protein kinase C (PKC) is a serine/threonine kinase family of isoenzymes that play a central role in a wide variety of cellular signaling pathways that affect multiple biological responses, including cell proliferation and differentiation, transmembrane ion transport, smooth muscle contraction, gene expression, and glucose and lipid metabolism [112]. PKC isoforms can be classified into 3 different subfamilies. Conventional PKC isoforms (cPKC) -- α, βI, βII, and γ -- are activated by diacylglycerol DAG in the presence of phosphatidylserine and calcium ions, and they can be activated by phorbol esters. Novel PKC isoforms (nPKC) -- δ, ε, θ,η, μ -- are activated by DAG but do not require calcium for activation, while atypical isoforms -- λ,ζ-- do not require calcium or DAG for activation and cannot be activated by phorbol esters [123]. Activation of the cPKC and nPKC isoforms is thought to require recruitment to the plasma or nuclear membranes and interaction with DAG. Receptor tyrosine kinases (such as the insulin receptor) mediate the production of DAG by recruitment of PLCγ (phospholipase C) to the membrane, while G-protein coupled receptors mediate production of DAG by recruiting PLCβ [123]. Optimum activation of all three subfamily members appears to require phosphorylation by phosphoinositide-dependent kinase 1 (PDK-1) at a conserved tyrosine in the activation loop [124]. PDK-1 is recruited to the plasma membrane by PIP3, the product of PI3K. PKC isoform expression patterns vary by tissue, subcellular localization, and disease status, but very little is known about the functional differences of PKC isoforms [112]. Rat hepatocytes express PKCα, βII, δ, ε, and ζ isoforms, while whole liver also expresses the PKC λ and θ isoforms [125-127].

Alterations in PKC isoform expression and DAG content in various tissues, including liver, has been associated with insulin resistance and type 2 diabetes in humans and in animal models [112, 128]. PKC activation may down-regulate insulin-mediated glucose uptake in muscle and adipose tissue by decreasing autophosphorylation and tyrosine kinase activity of the insulin receptor, inactivation of glycogen synthase, inhibition of insulin-stimulated Akt/PKB activity, increasing insulin receptor degradation, and increasing phosphorylation of IRS [111].

2.8.2.2 Novel PKC Isoforms ε and δ in Insulin Resistance

Recent studies in skeletal muscle and liver from diabetic patients and animal models have suggested a role for novel PKC isoforms ε, θ, and δ in the inhibition of insulin signaling. Increased PKC ε and θ activity was observed in Zucker diabetic rat soleus muscle [111], and PKCε expression was elevated in diabetic Psammomy gerbil muscle and liver [122]. Streptozotocin-induced diabetes in rats led to increased PKCε, α, and βII protein expression in liver membrane fractions, and feeding Sprague-Dawley rats a high-fat safflower diet for 3 days induced hepatic steatosis, decreased insulin-stimulated gluconeogenesis, and activated PKCε [14, 120, 121]. Hepatic steatosis may cause insulin resistance through lipid-mediated activation of PKCε, which directly inhibits insulin receptor β kinase activity and tyrosine phosphorylation of IRS-2 [129] (Figure 2.3). Increased PKCε activity has also been associated with elevated c-Jun amino terminal kinase 1 (JNK1) activity [120, 129]. JNK interferes with insulin signaling [130], and can be activated by TNFα and free fatty acids [126, 131], which have been implicated in the development of insulin resistance [132]. Hepatic knock-down of PKCε prevents high-fat-diet-induced hepatic

insulin resistance in rats, suggesting that activation of PKCε is required for the mechanism of hepatic insulin resistance in this model [129].

In addition to the evidence supporting a role for PKCε activity in insulin resistance, several studies support a role for increased PKCδ activity in insulin resistant muscle and liver tissues. Lipid/heparin infusion during a hyperinsulinemic-euglycemic clamp increases human and rat skeletal muscle DAG content and membrane associated PKCδ [117, 119, 126]. This increase in PKCδ was associated with a decrease in the NFkB inhibitor IkBα, which is degraded when IKKB is activated. Activation of NFkB, a transcription factor involved in the transcription of genes involved in inflammation, has been associated with insulin resistance and type 2 diabetes [133, 134]. These studies suggest that PKCδ activity may play a role in the pathogenesis of liver insulin resistance induced by increased blood FFA levels.

2.8.3 Ceramide Synthesis:

Ceramide is a sphingolipid that serves as a second messenger in signaling pathways affecting numerous cellular functions. Ceramide is generated by two major pathways 1) *de novo* synthesis from palmitoyl –CoA and serine via serine palmitoyl transferase (SPT) and ceramide synthase 2) from hydrolysis of sphingomyelin (SM) by one of the sphingomyelinases (SMase) [135] (Figure 2.5). Synthesis of ceramide by SPT is dependent upon cellular concentrations of palmitoyl-CoA and serine. Since palmitoyl-CoA is the primary FA synthesized in *de novo* lipogenesis, ceramide could accumulate in hepatocytes as a result of increased *de novo* lipogenesis and decreased beta-oxidation. Increased SPT

activity in the liver is accompanied by increased fatty acid, TAG, and cholesterol synthesis [136]. Inhibition of CPT-I in cells treated with exogenous palmitate led to increased ceramide synthesis [137, 138]. Thus, alterations in the flux of palmitoyl-CoA towards oxidation pathways can affect ceramide production. The preferred substrate of GPAT is palmitoyl-CoA, and increased GPAT activity channels fatty acids away from CPT-1 [37]. Thus, over-expression of GPAT in rat liver could lead to increased ceramide production parallel to increased TAG synthesis if channeling of fatty acids away from CPT-1 increases palmitoyl-CoA availability for GPAT and SPT (Figure 2.6). On the other hand, increased GPAT activity could channel palmitoyl-CoA away from SPT and CPT-1, resulting in decreased ceramide synthesis (Figure 2.7).

2.8.3.1 Ceramide, PKC, PP2A, and Insulin Resistance

Several mechanisms of ceramide action have been proposed for the reduction in insulin signaling in tissues that accumulate ceramide. One mechanism involves the activation of various PKC isoforms by ceramide and subsequent phosphorylation and inactivation of members of the insulin signaling pathway. Ceramide decreases insulin-stimulated Akt/PKB translocation to the plasma membrane in 3T3-L1 adipocytes and L6 myotubes, thus preventing insulin stimulated glucose uptake [139, 140]. Also, a number of studies have demonstrated that PKCζ can interact with Akt/PKB and inhibit its activity [141, 142]. Recently, Powell et al. (2003) found that ceramide-induced activation of PKCζ in L6 myotubes prevented insulin stimulated PI3K activation of Akt/PKB, and that treatment of the cells with palmitate could induce ceramide accumulation, and PKCζ inhibition of Akt/PKB

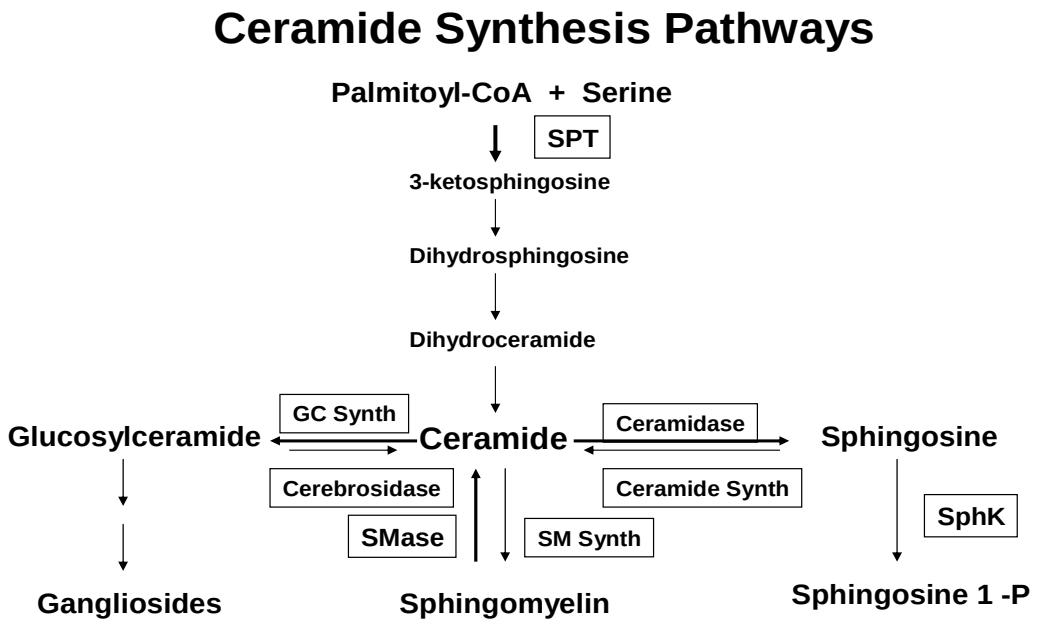

Figure 2.5. Ceramide synthesis. Serine palmitoyl-CoA transferase (SPT), glucosylceramide synthase (GC Synth), sphingomyelinase (SMase), sphingomyelin synthase (SM Synth), ceramide synthase, sphingosine kinase (SphK).

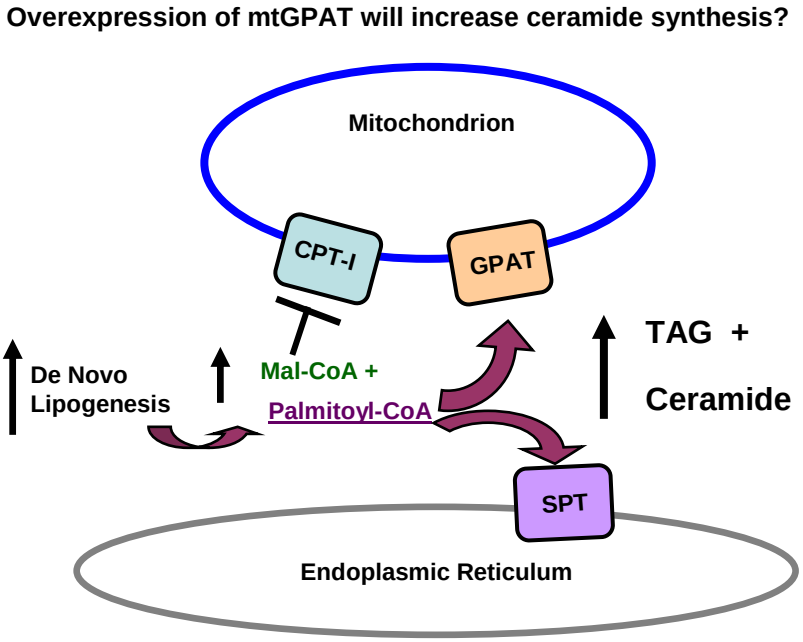

Figure 2.6. Over-expression of GPAT1 could increase the availability of palmitoyl-CoA for de novo ceramide synthesis and for glycerolipid synthesis by increasing the demand for de novo lipogenesis of fatty acids. Increased de novo lipogenesis leads to increased synthesis of mal-CoA and inhibition of CPT-1. Malonyl-CoA (Mal-CoA), carnitine palmitoyl transferase-1 (CPT-1), glycerol-3-phosphate acyltransferase (GPAT), serine palmitoyl transferase (SPT).

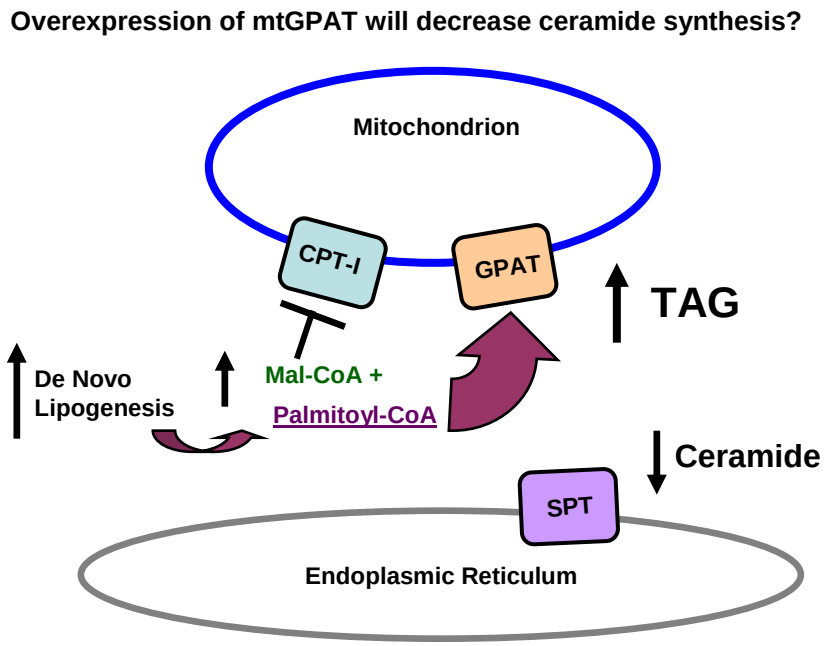

Figure 2.7. Over-expression of mtGPAT could decrease the availability of palmitoyl-CoA for *de novo* ceramide synthesis by partitioning palmitoyl-CoA towards glycerolipid synthesis and away from oxidation. Malonyl-CoA (Mal-CoA), carnitine palmitoyl transferase-1 (CPT-1), glycerol-3-phosphate acyltransferase (GPAT), serine palmitoyl transferase (SPT).

activity [143, 144]. These studies implicate ceramide and PKCζ in fatty acid-induced insulin resistance in skeletal muscle cells. The second mechanism proposed involves ceramide activation of protein phosphatase 2A (PP2A). PP2A has been shown to dephosphorylate Akt/PKB as a result of ceramide treatment of 3T3-L1 adipocytes [105]. Stratford et al. (2004) recently proposed that ceramide acts to prevent Akt/PKB activation using both mechanisms [105] (Figure 2.8).

2.8.3 Immune Mediators of Insulin Resistance

Obesity is associated with insulin resistance and fatty liver, and there is evidence for low-grade chronic systemic inflammation in patients with these disorders [145-147]. The development of obesity is associated with an increased infiltration of macrophages from the bone marrow into adipose tissue and other tissues [148]. When activated, macrophages produce a variety of cytokines, including TNFα, IL-6, and IL-1**,** and adipocytes may also contribute to cytokine production in obese patients [149, 150]. Increased circulating levels of these cytokines can cause insulin resistance, and anti-inflammatory treatments may reduce its severity [149, 151-155].

2.8.3.1 NF-kappa B

NFκB is one of the most important transcription factors that regulates inflammatory genes, and activates NFκB has been implicated in numerous immune-mediated diseases, including atherosclerosis, asthma, cancer, alcoholic liver disease, and rheumatoid arthritis [156]. The evidence that NFκB plays a critical role in the pathogenesis of insulin resistance

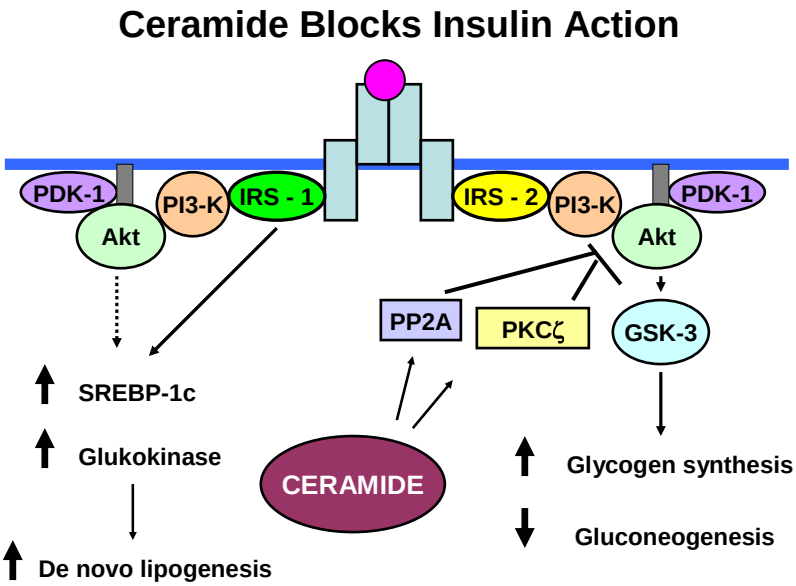

Figure 2.8. Ceramide activates PKCζ and/or PP2A, which prevents Akt/PKB translocation and phosphorylation.

is significant. Treatment of diabetics with high doses of salicylates inhibits NFκB and dampens inflammation and reduces blood glucose levels [157-159]. Chronic hyperglycemia activates NFκB in endothelial cells [160, 161], and both genetic obesity and high-fat-diet-induced obesity increase hepatic NFκB expression and expression of NFκB- regulated cytokines [133]. In liver, NFκB controls the expression of multiple inflammatory mediators, including CRP, PAI-1, IL-6, TNFα, and IL-1 [133]. TNFα and IL-6 can cause hepatic insulin resistance, and elevated plasma concentrations of CRP are common in obesity and insulin resistance [162]. IL-1β has been implicated in the suppression of insulin-mediated hepatic glycogen synthesis [163]. NFκB is activated when inhibitory-kappa B kinase (IKK-β) phosphorylates and inactivates the inhibitor of NFκB, IκB. Inhibition of IKK-β improves insulin resistance in mice, and liver specific activation of NFκB in mice causes systemic insulin resistance that can be inhibited by treatment with anti-IL-6 antibodies or salicylates [133, 152, 164]. Together, these studies suggest a prominent role for NFκB activation in the pathogenesis of both hepatic and systemic insulin resistance.

2.8.3.2 TNFα

TNFα and IL-6 are elevated in the plasma of type 2 diabetics [165-167], and can mediate hepatic insulin resistance [149, 168, 169]. TNFα production by adipocytes and macrophages is elevated in numerous animal models of diabetes, and TNFα action in adipocytes results in increased lipolysis and plasma FFA levels[170]. Plasma TAG is also increased by TNFα, which may be due to TNFα-mediated inhibition of lipoprotein lipase

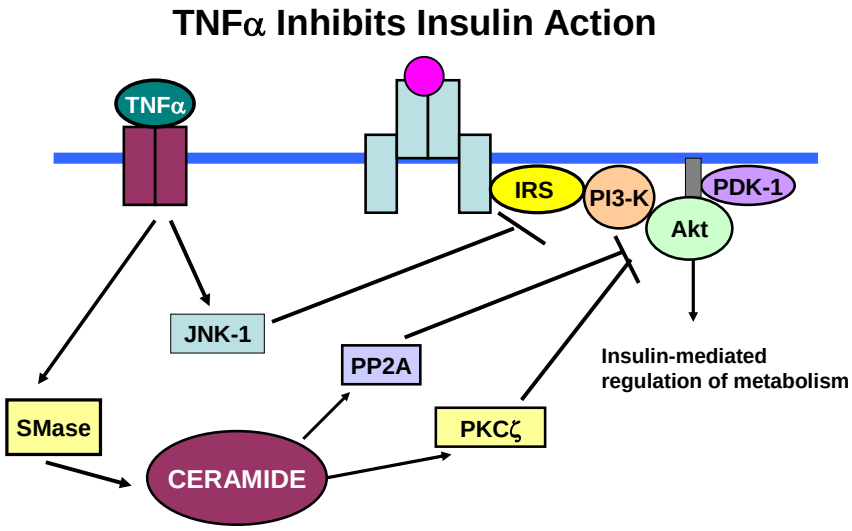

Figure 2.9. TNFα signaling activates SMase to generate ceramide. Ceramide can activate PKCζ or PPA2 to inhibit Akt/PKB activation. TNFα can also activate JNK-1, which down regulates IRS activation by the insulin receptor.

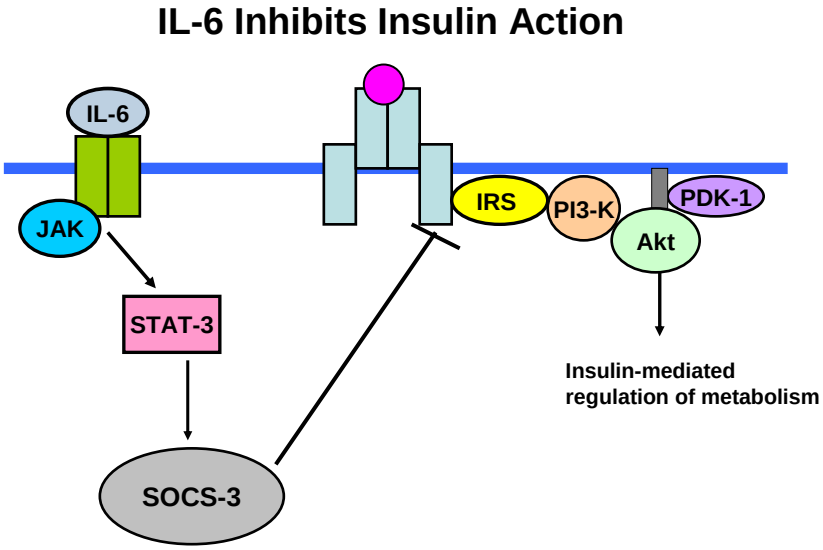

Figure 2.10. Activation of the IL-6 receptor leads to activation of STAT-3 and SOCS-3. SOCS-3 can inhibit tyrosine phosphorylation and activation of the insulin receptor.

expression in adipocytes [151]. Prolonged infusion of TNFα in rats impairs both insulin-mediated whole-body glucose disposal and insulin-stimulated hepatic glucose output [151].

In liver, resident macrophages called Kupffer cells, and infiltrating macrophages are activated when hepatocytes are injured or stressed. Kupffer cells are the main source of TNFα in the liver [171]. TNFα signaling in hepatocytes can activate inhibitory-κB kinase-beta (IKKβ), which degrades the inhibitor of NFκB, IκB, allowing NFκB translocation to the nucleus and activation of NFκB-regulated genes [133, 172]. NFκB induces the transcription of IL-6, which increases the activation of SOCS proteins, which are feedback regulators of cytokine signaling. Activation of SOCS can inhibit insulin signaling through IRS-2 because SOCS-1 directly inhibits IRS-2, and SOCS-3 competes with IRS-2 for docking sites on other proteins [169, 173] (Figure 2.10). Additionally, both SOCS-1 and -3 can ubiquinate IRS-2 [173]. TNFα signaling can also activate JNK-1, which can inactivate IRS-2 via serine phosphorylation, and TNFα may contribute to ceramide-mediated insulin resistance through activation of SMase [151, 170, 174] (Figure 2.9).

The importance of TNFα signaling in the liver was demonstrated recently in several mouse models. Mice deficient in TNFα-receptors were protected from hepatic steatosis and ob/ob mice treated with anti-TNFα antibodies had reduced hepatic steatosis that was associated with reduced IKKβ activity and increased insulin sensitivity [154, 175, 176]. Mice deficient in TNFα ($TNF\alpha^{-/-}$ mice) and wild-type mice ($TNF\alpha^{+/+}$ mice) were irradiated and had bone marrow transplants with either wild-type (WT) or TNFα deficient (KO) bone marrow. The mice were fed high-fat diets for 26 weeks. $TNF\alpha^{-/-}$/bone marrow KO mice were

protected from diet-induced hepatic steatosis and hepatic insulin resistance, while all four other treatment groups developed steatosis and insulin resistance [150]. Thus, TNFα signaling in hepatocytes may increase hepatic fat accumulation and contribute to hepatic insulin resistance.

2.8.3.3 Interleukin-6

Adipose tissue produces one third of the circulating plasma IL-6, and an increase in plasma IL-6 concentrations is associated with obesity and type 2 diabetes [174]. In hepatocytes, IL-6 inhibits insulin signaling through activation of SOCS proteins [168, 169, 173, 174]. Chronic treatment of mice with IL-6 causes insulin resistance, and when insulin resistant *ob/ob* mice are injected with anti-IL-6 antibodies, hepatic insulin sensitivity is dramatically improved, while adipocyte and muscle insulin sensitivity are not altered [155, 177]. These studies suggest that circulating IL-6 can impair hepatic insulin sensitivity, and that IL-6 released from adipocytes or macrophages during the development of obesity could contribute to hepatic insulin resistance.

2.9 GPAT1, TAG Synthesis, and Insulin Resistance:

Mice lacking a functional copy of GPAT1 ($GPAT1^{-/-}$) are resistant to high-fat-diet and high-fat/high-sucrose-diet-induced hepatic steatosis, with a reduction in hepatic TAG levels and in serum TAG concentration [13, 14]. Liver LPA and DAG content is reduced 2-fold in $GPAT1^{-/-}$ mice, while liver acyl-CoA content and serum beta-hydroxybuterate are elevated 2-fold, suggesting a shift from glycerolipid synthesis to β-oxidation [13, 14]. Mice are protected from high-fat-diet-induced insulin resistance when fatty acid oxidation is

increased and hepatic lipid accumulation is decreased, so mice deficient in GPAT1 might be expected to be more insulin sensitive than wild-type mice (Ide, 2004). However, the effect of a reduction in GPAT1 activity on the insulin sensitivity of mice fed high-fat diets is not clear. GPAT1$^{-/-}$ mice fed a high fat/high sucrose (HH) diet for 4 months were less insulin sensitive than their wild-type counterparts, despite a 50% lower hepatic TAG content and an increased rate of fatty acid oxidation [13]. However in high-fat safflower oil fed GPAT1$^{-/-}$ mice, a reduction in hepatic DAG and TAG synthesis and an increase in fatty acid oxidation has positive effects on whole body lipid homeostasis and on insulin sensitivity [14]. GPAT1$^{-/-}$ mice were protected from high-fat diet induced insulin resistance as shown by hyperinsulinemic euglycemic clamp procedures. The glucose infusion rate required to maintain constant blood glucose levels was 33% higher in the GPAT$^{-/-}$ mice compared to wild-type. Additionally, hepatic glucose production was suppressed by 80% in GPAT1$^{-/-}$ mice compared to only 20% in wild-type mice. These results suggest that GPAT1$^{-/-}$ mice have increased hepatic insulin sensitivity and that they are protected from high-fat induced insulin resistance [14]. Improved hepatic insulin sensitivity was associated with reduced hepatic DAG content and reduced PKCε activity, which may have led to reductions in IRS2 associated PI3K and Akt2 activities [14].

The reasons for the differences in the insulin sensitivity of GPAT1$^{-/-}$ mice on the HH and high-fat safflower oil diets are not clear, because hepatic TAG content was lower in GPAT1$^{-/-}$ mice on both diets. Additionally, hepatic acyl-CoA content was elevated in GPAT1$^{-/-}$ mice fed both diets, and there were no obvious differences in plasma lipid profiles. Differences in dietary lipid and sucrose content could explain the conflicting effect of these

two diets on hepatic insulin sensitivity in GPAT1$^{-/-}$ mice. The HH diet was high in medium chain fatty acids, whereas the safflower oil diet is particularly high in linoleic acid. Although hepatic acyl-CoA content was elevated in GPAT1$^{-/-}$ mice fed both diets, oleoyl-CoA was the predominant species in mice fed the HH diet, whereas linoleoyl-CoA was the predominant species in mice fed the safflower oil diet. It is not clear why the accumulation of a particular acyl-CoA species would cause insulin resistance, but it is possible that a particular species could act as a signaling molecule that affects insulin signaling pathways. Another difference between the two diets was the high sucrose in the HH diet. Because there was no high sucrose control group, it is difficult to know how this dietary difference may contribute to the insulin resistance in the HH-fed GPAT1$^{-/-}$ mice. The final difference between the two studies is the duration of high-fat feeding. In the HH study, mice were fed the HH diet for 4 months, and in the safflower oil study the mice were fed the high-fat diet for 3 weeks. It is possible that chronic exposure to a high-fat diet is more detrimental in GPAT1$^{-/-}$ mice due to increased production of hepatic lipid peroxides, which could activate immune-mediated insulin resistance pathways in the liver [178].

Although ceramide content of the livers from GPAT1$^{-/-}$ mice on either diet was not measured, it is possible that the differences in fatty acid composition of the high fat diets contributed to different levels of ceramide accumulation in the GPAT1$^{-/-}$ mice. A recent publication has shown that rats infused with soybean oil, which is high in linoleic acid, and those infused with lard oil, which is high in palmitic acid, had different mechanisms of hepatic insulin resistance [104]. The insulin resistance in rats infused with linoleate was attributed to DAG, while the insulin resistance in rats infused with palmitic acid was

associated with ceramide, despite concurrent accumulation of DAG [104]. Thus, it is possible that GPAT1$^{-/-}$ mice were protected from hepatic insulin resistance on the high-fat safflower oil diet because DAG accumulation was reduced, and ceramide did not accumulate due to the low level of palmitate in the diet. However, GPAT1$^{-/-}$ mice fed the HH diet were not protected from hepatic insulin resistance because the high saturated fat/high sucrose diet provided palmitate from dietary fatty acids and from *de novo* lipogenesis that could be used for ceramide synthesis instead of glyceorlipid synthesis (blocked due to knock-out of GPAT1) and instead of β-oxidation (overloaded due to lack of GPAT1 and influx of FFA from high fat diet).

Studies of insulin sensitivity in GPAT1$^{-/-}$ mice suggest that alterations in the systemic expression and activity of GPAT1 can alter hepatic insulin sensitivity, possibly through changes in hepatic lipid content. However, it is not clear whether GPAT1 deficiency in hepatocytes is responsible for the changes in insulin sensitivity, or whether GPAT1 deficiency in adipose tissue, pancreas, skeletal muscle, or immune cells could also be contributing to increased insulin sensitivity in these mice. There was evidence for increased insulin sensitivity in adipose tissue of GPAT1$^{-/-}$ mice fed the high-fat safflower oil diet, suggesting that the decrease in liver lipid levels was not the only factor responsible for protecting the mice from hepatic and systemic insulin resistance [14]. The specific role of liver glycerolipid accumulation in the pathogenesis of hepatic and systemic insulin resistance is not known.

CHAPTER III HEPATIC OVEREXPRESSION OF GPAT1

Although fatty liver is associated with insulin resistance, and the amelioration of fatty liver in insulin resistant animals is associated with improved insulin sensitivity in many animal models, it is still unclear whether liver triglyceride accumulation plays a causal role in whole body insulin resistance. Most existing animal models of fatty liver and insulin resistance have been created through high-fat or high-sucrose feeding or through genetic disruption of insulin or leptin signaling pathways. High-fat or high-sucrose diet-induced fatty liver and insulin resistance is not a good model for studying the role of the liver in the pathogenesis of IR, since these diets induce obesity, which, in itself, is a risk factor for IR. Whole-body deficiencies in leptin or insulin signaling also cause obesity in mice due to reduced satiety and increased feeding behavior. Since adipose tissue is thought to contribute to whole-body insulin resistance, it is not possible to isolate the role of the liver in the pathogenesis of IR in mouse models of obesity.

To examine the roles of hepatic triglyceride accumulation and increased *de novo* glycerolipid synthesis in the pathogenesis of whole-body and hepatic insulin resistance, we infected rats with an adenovirus that expresses GPAT1 (Ad-GPAT1) to overexpress GPAT1 in the liver of rats. Since GPAT1 is a key regulatory enzyme in the *de novo* triglyceride synthesis pathway, we expected to induce fatty liver without the need to feed high-fat or high-sucrose diets to the

rats. Since GPAT1 expression is regulated by SREBP-1c, and livers of insulin resistant animals have increased SREBP-1c expression, hepatic over-expression of GPAT is a realistic model for the hepatic steatosis observed in insulin-resistant animals. Additionally, by targeting GPAT1 overexpression to the liver, we have ensured that the primary site of metabolic dysfunction is in the liver.

Manuscript #1: **Hepatic overexpression of glycerol-*sn*-3-phosphate acyltransferase in rats causes insulin resistance**

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3.1 ABSTRACT:

Fatty liver is commonly associated with insulin resistance and type 2 diabetes, but it is unclear whether triacylglycerol accumulation or an excess flux of lipid intermediates in the pathway of TAG synthesis are sufficient to cause insulin resistance in the absence of genetic or diet-induced obesity. To determine whether increased glycerolipid flux can, by itself, cause hepatic insulin resistance, we used an adenoviral construct to overexpress glycerol-*sn*-3-phosphate acyltransferase-1 (Ad-GPAT1), the committed step in *de novo* triacylglycerol synthesis. After 5-7 days, food intake, body weight, and fat pad weight did not differ between Ad-GPAT1 and Ad-EGFP control rats, but the chow-fed Ad-GPAT1 rats developed a fatty liver, hyperlipidemia and insulin resistance. Liver was the predominant site of insulin resistance; Ad-GPAT1 rats had 2.5-fold higher hepatic glucose output than controls during a hyperinsulinemic-euglycemic clamp. Hepatic diacylglycerol and lysophosphatidate were elevated in Ad-GPAT1 rats, suggesting a role for these lipid metabolites in the development of hepatic insulin resistance, and hepatic PKCε was activated, providing a potential mechanism for insulin resistance. Ad-GPAT1-treated rats had 50% lower hepatic NF-κB activity and no difference in expression of TNF-α and IL-β, consistent with hepatic insulin resistance in the absence of increased hepatic inflammation. Glycogen synthesis and uptake of 2-deoxyglucose were reduced in skeletal muscle, suggesting mild peripheral insulin resistance associated with a higher content of skeletal muscle triacylglycerol.. These results indicate that increased flux through the pathway of hepatic *de novo* triacylglycerol synthesis can cause hepatic and systemic insulin resistance in the absence of obesity or a lipogenic diet.

3.2 INTRODUCTION:

Hepatic steatosis, an increasingly common health concern, is associated with obesity, insulin resistance, type 2 diabetes, and cardiovascular disease [179-182]. Despite the association of hepatic steatosis with insulin resistance, and the amelioration of hepatic triacylglycerol accumulation with improved insulin sensitivity, it is still unclear whether insulin resistance causes the increase in hepatic

triacylglycerol or whether the increase in glycerolipid intermediates or triacylglycerol itself plays a causal role in hepatic or systemic insulin resistance [6-12]. Most animal models of hepatic steatosis and insulin resistance have been created through high-fat or high-sucrose feeding or through genetic disruption of insulin or leptin signaling pathways. Diet-induced hepatic steatosis, however, is not a good model for isolating the role of the liver in the pathogenesis of insulin resistance because high-fat diets cause weight gain and obesity, which independently contribute to the development of systemic insulin resistance. Systemic deficiencies in leptin or insulin signaling also cause obesity by increasing centrally-mediated food intake. An animal model that isolates the accumulation of triacylglycerol in liver from its accumulation in other tissues may provide a better understanding of the role of hepatic lipid synthesis or accumulation in the development of hepatic and peripheral insulin resistance.

Acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT) is the committed step in the *de novo* synthesis of TAG and glycerophospholipids [29]. GPAT esterifies fatty acids to glycerol-3-phosphate at the *sn*-1 position, forming lysophosphatidic acid (LPA) (Figure 3.1). Three different isoenzymes of GPAT have been described, one located in the endoplasmic reticulum (GPAT3), and two located in the outer mitochondrial membrane (GPAT1 and GPAT2) [3, 5, 26, 183]. Only GPAT1 and GPAT2 have been cloned. GPAT2 and GPAT3 activities are sensitive to *N*-ethylmaleimide (NEM), whereas GPAT1 is not. GPAT1 activity accounts for 30-50% of total liver GPAT activity, but is only 10% of total GPAT activity in other tissues [29]. Unlike GPAT2 and 3, GPAT1 is transcriptionally regulated by carbohydrate re-feeding via carbohydrate-responsive elements and by insulin via SREBP-1c, suggesting that glycerolipid metabolism can be regulated through changes in GPAT1 expression in response to altered nutrient availability [41, 47, 49].

Recent studies in primary hepatocytes demonstrated that overexpression of GPAT1 primarily directs exogenous fatty acids away from β-oxidation and toward DAG and TAG rather than

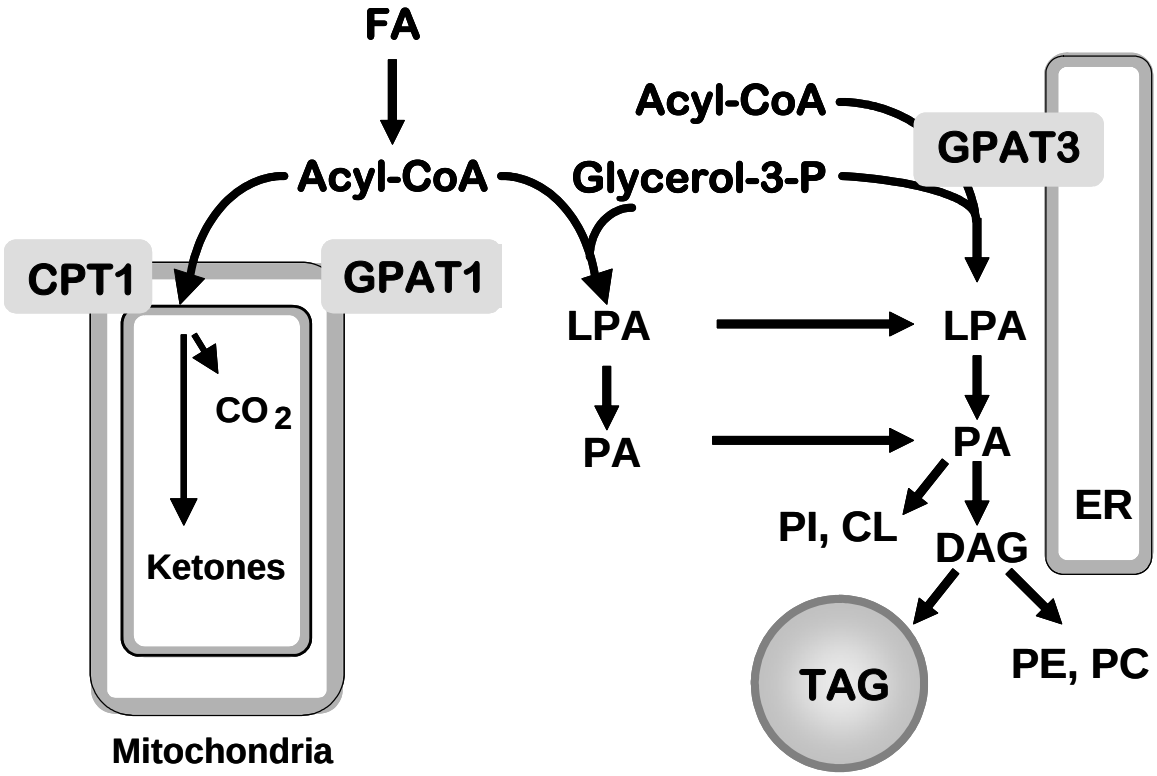

Figure 3.1: Pathway of *de novo* glycerolipid synthesis. GPAT isoforms located in mitochondria (GPAT1) and endoplasmic reticulum (GPAT3) catalyze the initial step in the synthesis of triacylglycerol (TAG). Acyl-CoA use for β-oxidation or TAG synthesis is regulated reciprocally by carnitine palmitoyl transferase-1 (CPT-1) and GPAT1. Lysophosphatidic acid (LPA), phosphatidic acid (PA), and diacylglycerol (DAG), triacylglycerol (TAG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and fatty acid (FA).

phospholipid synthesis. [36, 37]. Conversely, when GPAT1 is absent, mice are protected from high-fat-diet-induced hepatic steatosis and insulin resistance [2, 14]. These studies suggest that GPAT1 plays an important role in the regulation of hepatic TAG accumulation, and that the synthesis of this TAG pool can affect hepatic insulin sensitivity.

To determine whether an increase in hepatic TAG and the synthesis of its glycerolipid precursors leads to hepatic and systemic insulin resistance in the absence of obesity, we targeted the overexpression of GPAT1 to the liver using an adenoviral-GPAT1 construct (Ad-GPAT1). Because GPAT1 is a key enzyme in the pathway of *de novo* TAG synthesis, we expected to induce hepatic steatosis without the need to feed a lipogenic diet. Also, because GPAT1 specific activity is elevated in livers from mice with diet-induced obesity and from *ob/ob* mice with leptin-deficiency, overexpression of GPAT1 is a realistic model for the hepatic steatosis observed in insulin resistant animals [36]. The accumulation or increased flux of lipid metabolites in the glycerolipid synthetic pathway, including acyl-CoAs, LPA, and DAG, have been implicated in the development of insulin resistance [118, 184-189]. We hypothesized that hepatic overexpression of GPAT1 would cause both lipid metabolites and TAG to accumulate and increase hepatic insulin resistance in the absence of obesity or high-fat feeding.

3.3 METHODS

Recombinant adenoviruses. The construction and generation of recombinant GPAT1-Flag adenovirus and Ad-EGFP have been described previously [37]. These viruses were plaque purified and then further amplified and purified for injection into rats by previously described methods [103, 190].

Animal experiments. All procedures involving animals were approved by the Duke University or Vanderbilt University Institutional Animal Care and Use Committees. Male Wistar rats (300-350 g;

Charles River) were housed in individual cages with a 12 hour light cycle and given free access to standard chow (Harlan Teklad 7001, Harlan Teklad Laboratories). Rats received a single dose (1.0 x 10^{12} or 2.0 x 10^{12} particles/mL per 300 g body weight) of Ad-GPAT1 or Ad-EGFP adenoviruses by tail-vein injection. Rats were given a dose (15 mg/kg body weight) of cyclosporine the day before and the day of the virus administration to minimize the immune response. Food consumption and body weight were monitored daily. Five to 7 days after virus injection, food was withdrawn 4 h before collection of blood by heart puncture of anesthetized animals. Tissues were collected by clamp freezing and stored at -80º C.

Hyperinsulinemic-euglycemic clamp experiments. Hyperinsulinemic-euglycemic clamp studies were performed as described previously with the following modifications (32). Male Wistar rats (300 g body weight) were anesthetized with sodium pentobarbital (50 mg/kg), and catheters were implanted in the carotid artery, external jugular vein, and ileal vein. After surgery, the rats recovered for 2 wk, then, Ad-GPAT1 or Ad-EGFP virus was injected through the tail vein at 1.0-2.0 x 10^{12} particles/mL 7 d before the clamp study. At -150 min a bolus of [3-^{3}H] glucose (15 μ Ci/kg) was administered followed by a constant infusion of labeled glucose (0.15 μCi/kg/min). Somatostatin was infused at 3 μg/kg/min, and glucagon and insulin were infused at 2.6 ng/kg/min and 3 mU/kg/min, respectively, to maintain plasma glucagon and insulin levels at ~ 40 pg/mL (arterial plasma) and ~3 ng/mL (arterial plasma), respectively. 2-deoxy-[^{14}C]-glucose (50 μCi/rat) was administered through the carotid arterial catheter at 120 min during the clamp. During the clamp, blood glucose was monitored every 10 min via carotid arterial catheter samples Plasma glucose, [^{3}H]H_2O, [3-^{3}H] glucose, and 2-deoxy-[^{14}C]-glucose were measured to determine glucose disposal rate, hepatic glucose output, and the glycolytic rate. Liver and skeletal muscle glycogen content and [^{3}H]radioactivity in glycogen was measured to analyze glycogen synthesis. Skeletal muscle 2-deoxy-[^{14}C]-glucose-6-phosphatase was measured to determine glucose uptake.

Measurement of plasma metabolic variables. Plasma TAG concentrations were measured using an enzymatic colorimetric assay kit (Stanbio Laboratory). Plasma cholesterol levels were measured using the Cholesterol CII kit (Wako Chemicals). Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, and β-hydroxybutyrate concentrations were measured using kits from Stanbio Laboratory. Fatty acid concentrations were measured using a kit from Roche. Plasma insulin and leptin levels were measured by radioimmunoassay (Linco). Very low density and high density plasma lipoprotein fractions were isolated from 100 μL total plasma using fast protein liquid chromatography with a Superose 6 HR10/30 column (Pharmacia Biotech Inc). Triglyceride and cholesterol contents of plasma fractions were determined using the colorimetric kits described above.

Membrane isolation and measurement of GPAT1 activity. Liver tissue was homogenized in Medium I (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM DTT) with 10 up-and-down strokes in a Teflon-glass motor-driven homogenizer. Homogenates were centrifuged at 100,000 x g for 1h to obtain the total membrane fraction. The membrane pellet was re-homogenized in Medium I and stored in 100 μL aliquots at -80º C for the enzyme assay. GPAT1 specific activity was assayed at RT in a 200 μL reaction mixture containing 75 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mg/mL bovine serum albumin (essentially FA-free), 1 mM DTT, 8 mM NaF, 800 μM [^{3}H]glycerol-3-phosphate, and 80 μM palmitoyl-CoA [183]. The reaction was initiated by adding 10-30 μg of total membrane protein to the assay mix after incubating the membrane protein on ice for 15 min in the presence or absence of 1 mM NEM. GPAT1 activity is calculated as the activity that is uninhibited by NEM.

Western blot. GPAT1-FLAG and EGFP expression were determined in liver (and muscle) total particulate fractions by Western blot using a mouse monoclonal anti-FLAG antibody (Clone M2, Sigma) and a monoclonal anti-EGFP antibody (ab3277, Abcam Inc.). PEPCK protein expression was determined in rat liver samples using a rabbit polyclonal anti-PEPCK (Santa Cruz) antibody.

Tissue triglyceride and glycogen content. Liver, muscle, or heart tissue (100 mg) was homogenized in water and lipids were extracted into $CHCl_3$ [191] dried in a SpeedVac and resuspended in 1 mL $CHCl_3$. A fraction of the original lipid extract (50 µL for liver and 200 µL for heart or muscle) was dried and resuspended in 100 µL isopropanol, 1% Triton X-100 at room temperature for 1 h. The TAG content of 10-30 µL of the lipid sample was determined using an enzymatic colorimetric method (Stanbio Laboratory). Liver glycogen content was measured using an amyloglycosidase-based assay [192].

Mass spectrometric analysis of liver lipid contents. The extraction procedures for acyl-CoA, LPA, and DAG species were performed as described previously [14]. To extract acyl-CoA, approximately 100 mg of liver was homogenized with a 17:0-CoA internal standard. Acyl-CoAs were purified using Oligonucleotide Purification Cartridges (Applied Biosystems) and were slowly eluted with 60% acetonitrile. For LPA measurements, approximately 100 mg of liver was homogenized in chloroform/methanol (1:1; v/v) with 1 nM C17:0-lysophosphatidic acid as the internal standard. Samples were homogenized and centrifuged twice, each time with chloroform/methanol (1:1; v/v). The supernatants were combined and mixed with dH_2O to obtain an aqueous phase. The aqueous phase was then mixed with dH_2O and applied to conditioned Waters Oasis MAX extraction cartridges (Waters Corporations). After a washing step, LPA and phosphatidic acid species were eluted with methanol for LC/MS/MS analysis. Different LPA species were separated with an HPLC Betasil C18 Dash HTS column (Thermo Electron Corporation) using varying gradients of solutions A (95 % dH_2O, 5 % acetonitrile, 2 mM ammonium acetate) and B (95% acetonitrile, 5% dH_2O, 2 mM ammonium acetate).

For DAG, approximately 100 mg of liver tissue was homogenized in ice-cold $CHCl_3$/methanol (2:1; v/v) containing 0.01% butylated hydroxytoluene and the internal standards 1,3-

dipentadecanoin and 1,2,3-triheptadecanoate. Organic and aqueous phases were separated by adding $CHCl_3$ and H_2O. After centrifugation, the organic layer was collected, dried under nitrogen flow, and reconstituted with hexane/methylene chloride/ether (89/10/1; v/v). DAG was separated from TAG with preconditioned columns (Waters Sep Pak Cartridge WAT020845) and eluted with hexane/ethyl acetate (85/15, v/v) under a low negative pressure.

Lipid metabolite extracts were subjected to LC/MS/MS analysis. A turbo ionspray source was interfaced with an API 3000 tandem mass spectrometer (Applied Biosystems) in conjunction with two Perkin Elmer 200 Series micro pumps and a 200 Series autosampler (Perkin Elmer). Total acyl-CoA, LPA, phosphatidic acid, and DAG content were expressed as the sum of individual species.

Quantitative RT-PCR. RNA was extracted from 0.02 g of frozen liver tissue using the RNeasy Mini spin column kit (Qiagen Inc) in combination with DNase digest treatment. Quantitative real-time reverse transcriptase PCR for IL-1β, PEPCK, G6Pase, and TNF-α were performed using an ABI PRISM 7500 Sequence Detection System instrument and Taq-Man® Universal PCR Master Mix (Applied Biosystems, Inc.) with Invitrogen MML-V reverse transcriptase. Reactions were performed in triplicate. Target gene expression was normalized to β-actin expression. All data were quantified using the relative standard curve method as described in Applied Biosystems User Bulletin No. 2 (P/N 4303859).

NF-κB activity. Liver (100 mg) was homogenized in 400 μL Dignam A Buffer (10 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, Sigma Protease Inhibitor Cocktail 100 μL /10 mL buffer) in a glass Dounce homogenizer. Homogenates were left on ice for 20 min, then 40 μL of 10% NP-40 was added, and homogenates were vortexed for 15 s. Cytoplasmic extracts were collected after the homogenates were centrifuged at 4500 x g for 3 min at 4º C. The pellets were washed twice

in Dignam A Buffer. Nuclear membranes were disrupted with 100 µL of Dignam C Buffer (10 mM HEPES pH 7.5, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, and Sigma Protease Inhibitor Cocktail 100 µL/10 mL buffer) on ice for 15 min. Nuclear extracts were collected after centrifugation at 14,000 rpm for 5 min to pellet the DNA. NF-κB binding activity was determined using the TransAM^{TM} NF-κB p65 Transcription Factor Assay ELISA kit (Active Motif). 25 µg of liver nuclear extract was used in each well.

PKCε activity. PKCε membrane translocation assays were performed according to methods previously described (Qu et al., 1999; Donnelly et al., 1994). Briefly, 50 µg of crude membrane and cytosol protein extracts were resolved by SDS-PAGE using an 8 % gel and electrotransferred onto PVDF. Membranes were immunoblotted with a rabbit anti-peptide antibody against PKCε (Santa Cruz Biotechnology), diluted 1:100 in rinsing solution. PKCε translocation was expressed as the ratio of arbitrary units of membrane bands over cytosol bands. Protein bands were quantified using a Bio-Rad Chemidoc SRX and Quantity One Software (BioRad).

Statistics -- Data were analyzed by Student's *t* test or Kruskal-Wallis ANOVA with Dunn multiple comparison post-test. Data are shown as mean ± S.E.

3.4 RESULTS

Liver-specific overexpression of GPAT1 -- To examine the effects of GPAT1 overexpression on hepatic lipid metabolism, chow-fed Wistar rats were studied 5-7 days after treatment with 1.0 - 2.0 x 10^{12} particles/mL of Ad-GPAT1 or Ad-EGFP virus. The recombinant adenovirus employed directs expression of a Flag-tagged version of GPAT, allowing specific detection of the overexpressed protein in virus-transduced tissues. Rats treated with Ad-GPAT1 had 2.7-fold higher liver NEM-resistant GPAT1 activity than rats treated with Ad-EGFP virus (**Figure 3.2A**). No increase in NEM-

resistant GPAT1 activity was detected in epididymal fat from Ad-GPAT1-treated rats (Ad-EGFP 0.36 ± 0.1 and Ad-GPAT1 0.41 ± 0.1 nmol/min/mg protein; n = 6). GPAT1-Flag expression was detected in liver but not in skeletal muscle or heart from Ad-GPAT1-treated rats and no GPAT1-Flag was detected in liver from Ad-EGFP-treated rats (**Figure 3.2B**). EGFP expression was detected in liver by Western blot in all Ad-EGFP-treated rats (data not shown). Plasma ALT levels were within the normal range in both Ad-GPAT1- and Ad-EGFP-treated rats, and ALT values did not differ between groups, indicating that the viral treatments did not cause significant cytotoxic effects (**Table 3.1**).

Hepatic overexpression of GPAT1 causes hepatic steatosis, hypertriglyceridemia, and muscle TAG accumulation – Overexpression of GPAT1 increased hepatic TAG content 2.7-fold (**Figure 3.3A**). Thus, GPAT1 appeared to increase hepatic glycerolipid synthesis. The hepatic steatosis observed in Ad-GPAT1-treated rats was accompanied by a 2.6-fold increase in total plasma TAG concentration (**Table 3.1**). TAG content in plasma VLDL fractions was 4-fold higher in Ad-GPAT1-treated rats (**Figure 3.3C**). Although total plasma cholesterol content did not change with Ad-GPAT1-treatment (Table 1), VLDL cholesterol was 2.5-fold higher in Ad-GPAT1-treated rats, whereas the LDL cholesterol concentration was slightly lower (**Figure 3.3D**). These data suggested that GPAT1 directs the synthesis of a TAG pool that is available for both cytosolic storage and secretion in VLDL. To determine whether the increase in plasma TAG in Ad-GPAT-1-treated rats was due to an increase in secretion of VLDL particles or to an increase in TAG incorporation into VLDL particles, VLDL ApoB100 and ApoB48 levels were determined by SDS-PAGE analysis of VLDL lipoprotein fractions. No differences in ApoB100 or ApoB48 protein levels were observed (data not shown), suggesting that the increase in VLDL TAG was due to an increased amount of TAG incorporated into each particle rather than an increase in the number of VLDL particles secreted. These results are similar to those observed in mice treated with Ad-GPAT1, which had increased TAG secretion with no increases in plasma ApoB concentration (34). In addition to increased TAG content in liver and plasma, gastrocnemius muscle in Ad-GPAT1-treated rats contained more TAG (**Figure 3.3B**), and

soleus muscle (EGFP 94.6 ± 37.6; GPAT 185.7 ± 54.0 ng/ug protein, p = 0.25) and heart muscle (EGFP 14.5 ± 1.4; GPAT 21.2 ± 2.4 ng/ug protein, p = 0.07) also had a trend towards higher TAG content in Ad-GPAT1-treated rats. Elevated plasma TAG and fatty acid concentrations may have contributed to this peripheral TAG accumulation (**Table 3.1**). The high circulating plasma TAG concentration did not appear to affect adipose tissue, as body weight and fat pad weights of Ad-GPAT1 and Ad-EGFP-treated rats did not differ (**Table 3.1**). Additionally, there were no differences in food intake or plasma leptin concentrations between groups, suggesting that hepatic GPAT1 overexpression did not alter satiety or adipokine production (**Table 3.1**).

Ad-GPAT1-treated rats were insulin resistant –

Since hepatic steatosis, hypertriglyceridemia, and peripheral TAG accumulation often accompany insulin resistance, we proposed that Ad-GPAT1-treated rats would be less insulin sensitive than Ad-EGFP-treated rats. Although insulin to glucose ratios between the two groups did not differ, Ad-GPAT1-treated rats had a lower liver glycogen content after a 4 h fast, suggesting that Ad-GPAT1-treated rats might have hepatic insulin resistance (**Table 3.1**). To measure insulin sensitivity, we performed hyperinsulinemic-euglycemic clamp studies. Ad-GPAT1-treated rats maintained euglycemia with a 40% lower rate of glucose infusion during clamp experiments compared to Ad-EGFP-treated rats (**Figure 3.4A**), confirming whole-body insulin resistance.

Basal hepatic glucose output was slightly elevated in Ad-GPAT1-treated rats (**Figure 3.4B**). During the clamp procedure, insulin suppressed hepatic glucose output 77% in Ad-EGFP-treated rats, but only 37% in Ad-GPAT-treated rats, indicating that the livers of Ad-GPAT1-treated rats were insulin resistant (**Figure 3.4B**). Although the amount of glucose incorporated into liver glycogen was 22% lower in Ad-GPAT1-treated rats, the difference was not significant **(Figure 3.4C)**. To confirm that the increase in glucose production was due to increased hepatic gluconeogenesis, we examined

Table 3.1: Physiologic and plasma measurements. [a]

		EGFP		GPAT
Physiologic parameters after 4 h fast				
Body weight, g		342 ± 8		329 ± 6
Food intake/day		26.5 ± 1.7		24.1 ± 1.2
Liver weight, g		15.8 ± .05		16.5 ± 0.7
Liver weight, % of BW		4.6 ± 0.1		5.0 ± 0.1
Liver glycogen, μg/mg protein		23 ± 2		17 ± 3 **
Epididymal fat weight, g		3.6 ± 0.2		3.3 ± 0.1
Epididymal fat wt, % of BW		1.0 ± 0.1		1.0 ± 0.1
Plasma parameters after 4 h fast				
ALT, U/L		67 ± 19		47 ± 7
TAG, mg/dL		179 ± 28		472 ± 97 **
Cholesterol, mg/dL		91 ± 7		103 ± 7
Fatty acid, μM		120 ± 0.0		190 ± 30 *
β-Hydroxybutyrate, mg/dL		2.0 ± 0.2		2.1 ± 0.1
Blood glucose, mg/dL[b]		128 ± 4		118 ± 3 *
Insulin, ng/mL		4.5 ± 0.4		3.4 ± 0.5
Insulin/glucose ratio		0.035 ± 0.004		0.029 ± 0.004
Leptin, ng/mL		3.4 ± 0.6		2.4 ± 0.5
TNF-α, pg/mL		77 ± 14		50 ± 14
IL-6, pg/mL		27 ± 11		38 ± 9
IL-1β, pg/mL		15 ± 4		24 ± 4

[a]Rats were treated with 1.0-2.0x 10^{12} particles/mL of Ad-GPAT1 or Ad-EGFP virus for 5-7 days and fasted for 4 h before samples were collected. Results are expressed as mean ± SE. * $p < 0.05$ ** $p < 0.01$ Triacylglycerol (TAG), , alanine aminotransferase (ALT), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6). 4 h fast (Ad-EGFP, n = 9; Ad-GPAT, n = 11).

[b] After an overnight fast, the glucose values were 127.4 ± 4.5 (Ad-EGFP; n=8) and 126.2 ± 4.7 (Ad-GPAT1; n=8), and the insulin values were 0.76 ± 019 (Ad-EGFP; n=8) and 0.93 ± 0.1 (Ad-GPAT; n=8).

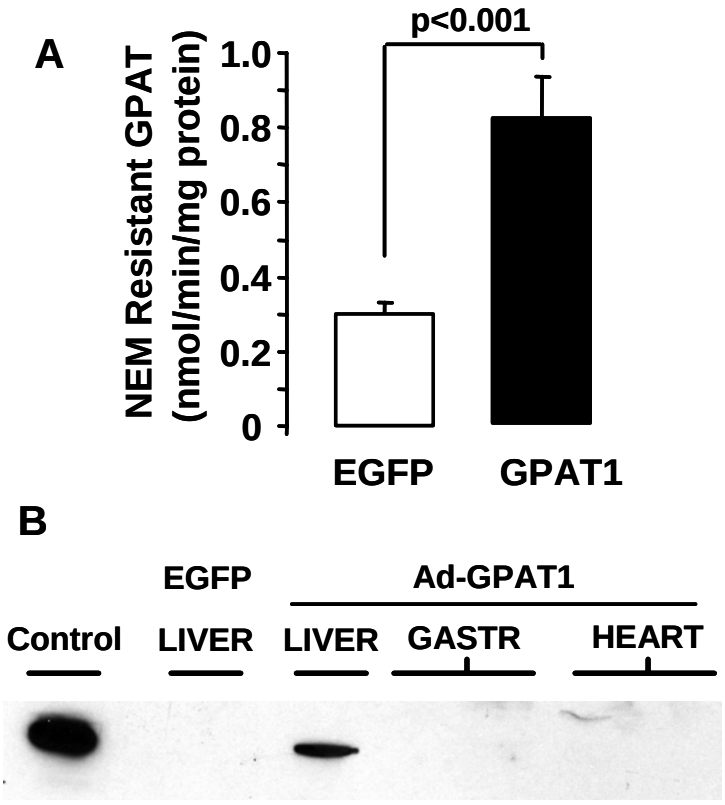

Figure 3.2: GPAT1 activity and expression in rat liver.
Rats were treated with 1.0-2.0 x 10^{12} particles/mL of Ad-GPAT1 or Ad-EGFP virus for 5-7 d. (Ad-EGFP, n = 9; Ad-GPAT1, n =11). **A)** GPAT1 enzyme activity was determined in liver total membranes after inhibiting endoplasmic reticulum GPAT with NEM. Results are expressed as mean ± SE. **B)** Anti-FLAG western blot of total membranes from primary hepatocytes infected with Ad-GPAT1 (Control), from liver of a rat infected with Ad-EGFP (EGFP), and from liver, gastrocnemius muscle (2 lanes), and heart (2 lanes) of rats infected with Ad-GPAT.

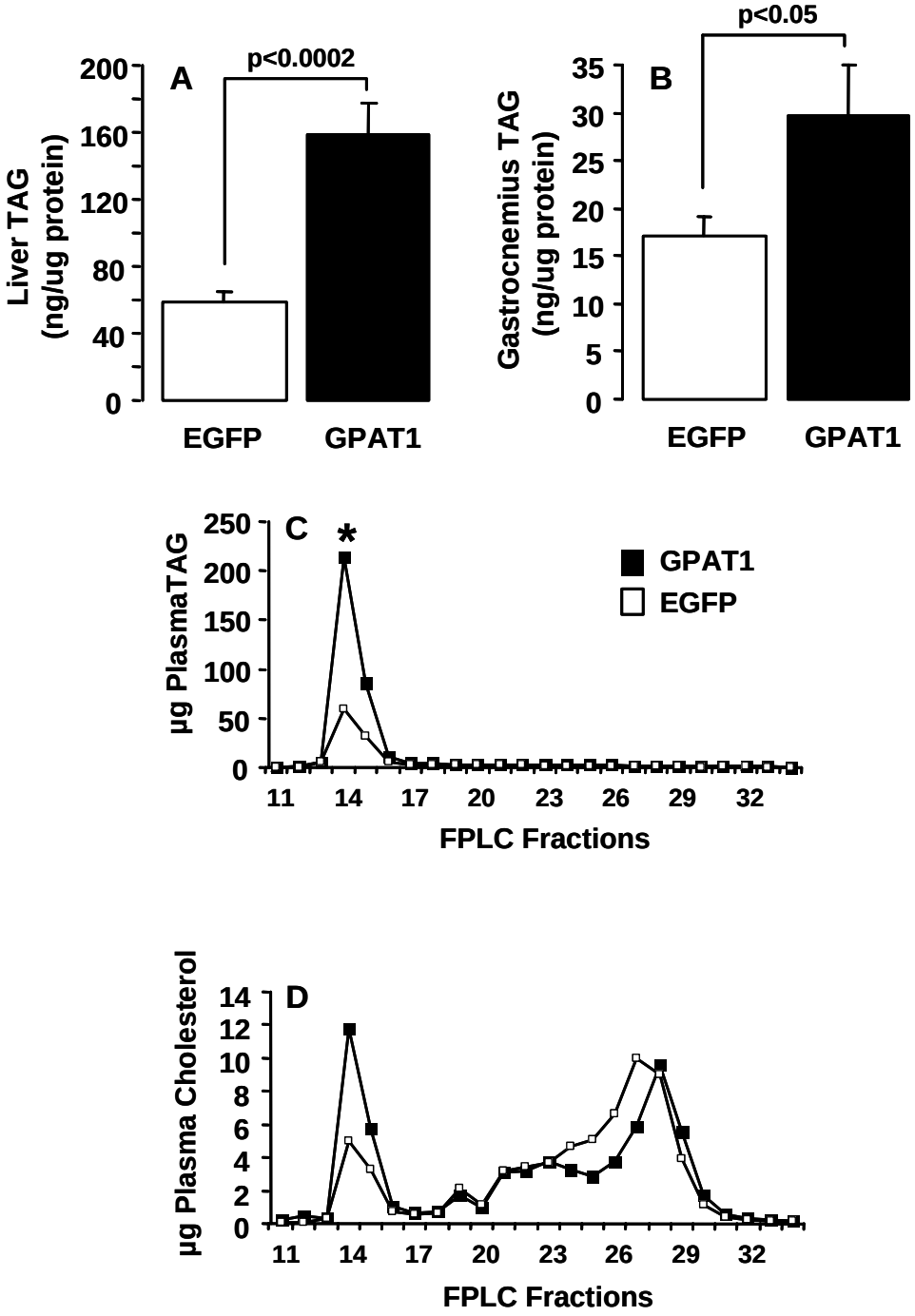

Figure 3.3: Liver, skeletal muscle, and VLDL triacylglycerol (TAG) content was increased in Ad-GPAT1-treated rats. Rats were treated with 1.0-2.0 x 10^{12} particles/mL of Ad-GPAT1 or Ad-EGFP virus for 5-7 days and fasted for 4 h before tissue collection. (Ad-EGFP, n = 9 and Ad-GPAT1, n = 11 for liver and gastrocnemius). Results are expressed as mean ± SE. **A)** Liver TAG**. B)** Gastrocnemius muscle TAG. Plasma lipoprotein fractions were separated by FPLC. Triglyceride and cholesterol are reported as averages (EGFP, n = 4; GPAT, n = 6). **C)** VLDL TAG (fraction 14; * $p<0.05$). **D)** VLDL cholesterol (fraction 14; $p< 0.06$).

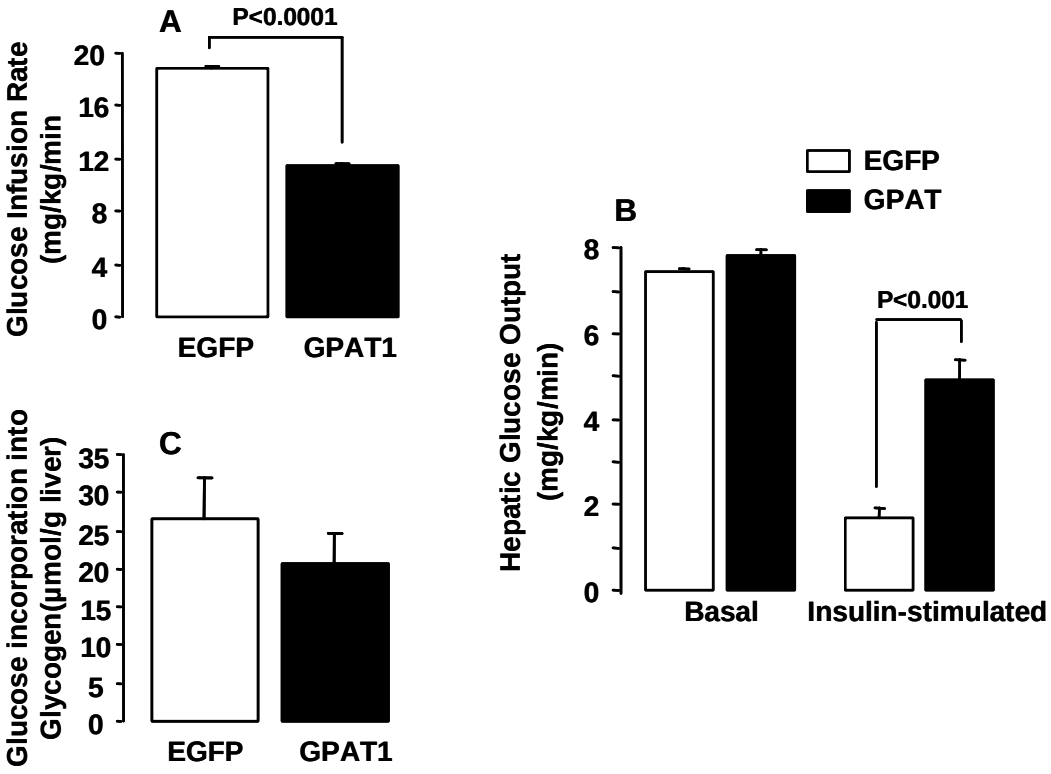

Figure 3.4A-C: Ad-GPAT1-treated rats were insulin resistant. Rats were treated with 1.0-2.0 x 10^{12} particles/mL of Ad-GPAT1 or Ad-EGFP virus for 7 d and food-deprived for 24 h before hyperinsulinemic-euglycemic clamp experiments (Ad-EGFP, n = 8; Ad-GPAT1, n = 8). **A)** Glucose infusion rate. **B)** Basal and insulin-stimulated hepatic glucose output. **C)** Glucose incorporation into liver glycogen (p = 0.36).

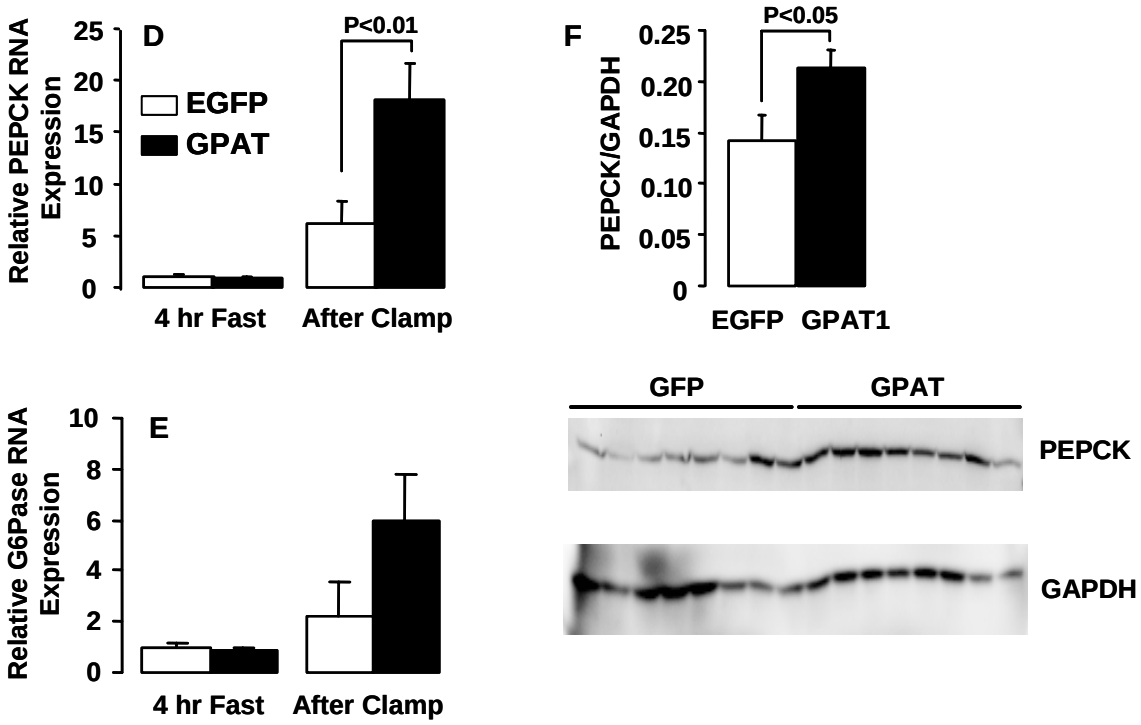

Figure 3.4 D-F: Ad-GPAT1-treated rats were insulin resistant D) PEPCK and **E)** G6Pase mRNA expression were measured in liver from Ad-GPAT1-treated rats fasted for 4 h or fasted for 24 h followed by hyperinsulinemic-euglycemic clamps for 3 h. PEPCK mRNA expression after clamp **(**n = 6). G6Pase mRNA expression after clamp (p = 0.33) (n = 6). **F)** PEPCK protein expression after clamp (n = 8).

hepatic phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) expression after the clamp procedure. Liver PEPCK mRNA and protein levels were 3-fold and 1.6-fold higher, respectively, in Ad-GPAT1-treated rats (**Figure 3.4D-F**). Although hepatic G6Pase mRNA expression was 2.7-fold higher in Ad-GPAT1-treated rats after the clamp, the difference was not significant (**Figure 3.4E**).

Basal glucose disposal rates were similar in Ad-GPAT1- and Ad-EGFP-treated rats (**Figure 3.5A**). During the clamp, insulin elevated the glucose disposal rate 3-fold in Ad-EGFP-treated rats, but only 2-fold in Ad-GPAT1-treated rats, indicating that hepatic overexpression of GPAT1 reduced peripheral insulin sensitivity. Hepatic overexpression of GPAT1 also lowered 2-deoxyglucose uptake into gastrocnemius muscle by 40%, suggesting reduced uptake of glucose by skeletal muscle (**Figure 3.5A, B**). The 175% increase in gastrocnemius TAG content observed in Ad-GPAT1-treated rats may have contributed to the altered insulin sensitivity **(Figure 3.3B).** In other muscles examined, the differences in 2-deoxy-glucose uptake did not reach significance, but uptake into Ad-GPAT soleus muscle was reduced 30 % (EGFP 809 ± 121; GPAT 563 ± 84, $p = 0.12$), and glucose incorporation into Ad-GPAT vastus lateralis muscle glycogen was reduced 58 % (EGFP 27.4 ± 8.1; GPAT 11.6 ± 1.7, $p = 0.07$). These data, taken together, with the marked reduction in gastrocnemius uptake of glucose, suggest that hepatic overexpression of GPAT1 reduces glucose metabolism and insulin sensitivity in skeletal muscle.

Elevated hepatic DAG contributes to hepatic insulin resistance through activation of PKCε – Hepatic steatosis is often present in animals with insulin resistance, but the link between increased hepatic TAG accumulation and reduced hepatic insulin sensitivity is not completely understood. It has been hypothesized that lipid metabolites accumulate in the liver in conjunction with increased TAG synthesis and act in signaling pathways to disrupt insulin responses. For example, DAG and acyl-CoA accumulation accompany insulin resistance in muscle and liver [118, 184-189]. GPAT1$^{-/-}$ mice

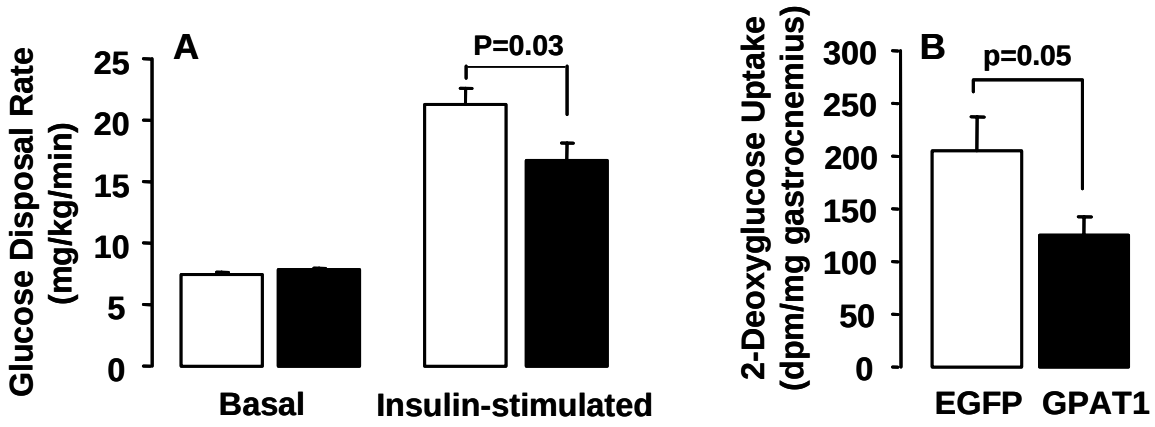

Figure 3.5: Skeletal muscle glucose metabolism was impaired in Ad-GPAT1-treated rats. Rats were food-deprived for 24 h before hyperinsulinemic-euglycemic clamp experiments. **A)** Basal and insulin-stimulated hepatic glucose disposal rates. **B)** 2-Deoxyglucose uptake in gastrocnemius muscle. Ad-EGFP, n = 8; Ad-GPAT1, n = 8.

fed a high-fat safflower-oil diet for 3 weeks were protected from diet-induced insulin resistance and had an increased hepatic content of acyl-CoA, decreased content of DAG, and less activation of protein kinase Cε (PKCε) [14]. To determine whether overexpression of GPAT1 would lead to the opposite changes in hepatic lipid intermediates, we measured the content of LPA, DAG and acyl-CoA in livers from Ad-GPAT1- and Ad-EGFP-treated rats. Total hepatic LPA content was elevated 4-fold in Ad-GPAT1-treated rats, 16:0-LPA was elevated 5-fold, and other hepatic LPA species (14:0-, 18:1-, and 16:1- LPA species) were elevated to a lesser extent compared to Ad-EGFP-treated rats **(Figure 3.6A)**. Total hepatic acyl-CoA content, however, did not differ between Ad-GPAT1 and Ad-EGFP-treated rats, although the hepatic content of 18:0-CoA was lower (**Figure 3.6B**). Hepatic DAG content was elevated 2-fold in Ad-GPAT1-treated rats (**Figure 3.6C**). The most prominent elevations were in DAG species that contained palmitate (16:0-18:1, 16:0-16:0, and 16:0-18:2), but several DAG species containing oleate and stearate were also elevated compared to Ad-EGFP-treated rats (**Figure 3.6C**). Despite elevated amounts of DAG species containing 18:1, hepatic stearoyl-CoA desaturase-1 (SCD-1) expression was similar in Ad-GPAT1- and Ad-EGFP-treated rats (**Table 3.2**).

In liver from insulin resistant rodents DAG accumulation has been associated with increased PKCε activity and defects in insulin stimulation of IRS-2 tyrosine phosphorylation [188]. Because high-fat fed GPAT1$^{-/-}$ mice had lower hepatic PKCε activity [14], we measured PKCε activity in liver from Ad-GPAT1 and Ad-EGFP-treated rats to determine whether elevated DAG could be causing insulin resistance in Ad-GPAT1-treated rats through this mechanism. Membrane-associated activated PKCε was 30% higher in liver from Ad-GPAT1-treated rats (**Figure 3.7**), suggesting that PKCε activation could have contributed to the insulin resistance observed in Ad-GPAT1-treated rats.

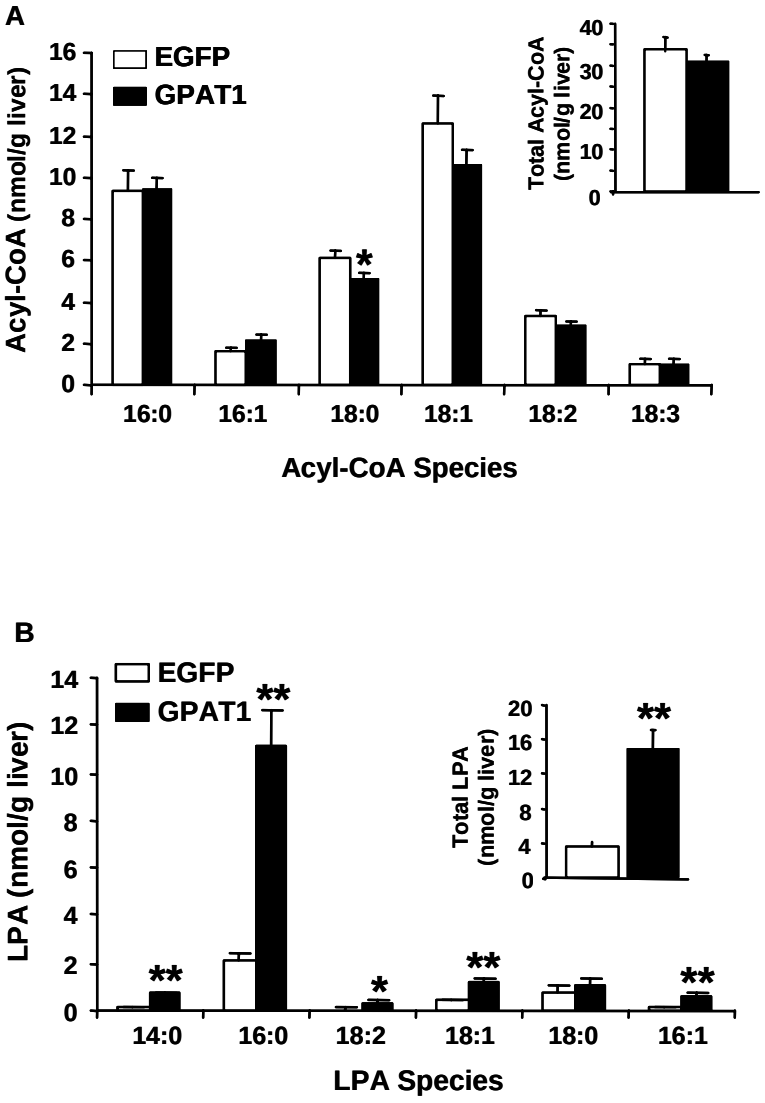

Figure 3.6A-B: Hepatic lipid metabolites were altered in Ad-GPAT1-treated rats. Rats were treated with 1.0-2.0 x 10^{12} particles/mL Ad-GPAT1 or Ad-EGFP for 5-7 days and food-deprived 4 h before tissues were collected. Liver lipid metabolites were measured by mass spectrometry. **A)** Total acyl-CoA content (inset) and acyl-CoA species (18:0-CoA *$p<0.03$). **B)** Total LPA content (inset) (**$p< 0.001$) and LPA species (* $p<0.01$; **$p<0.001$).

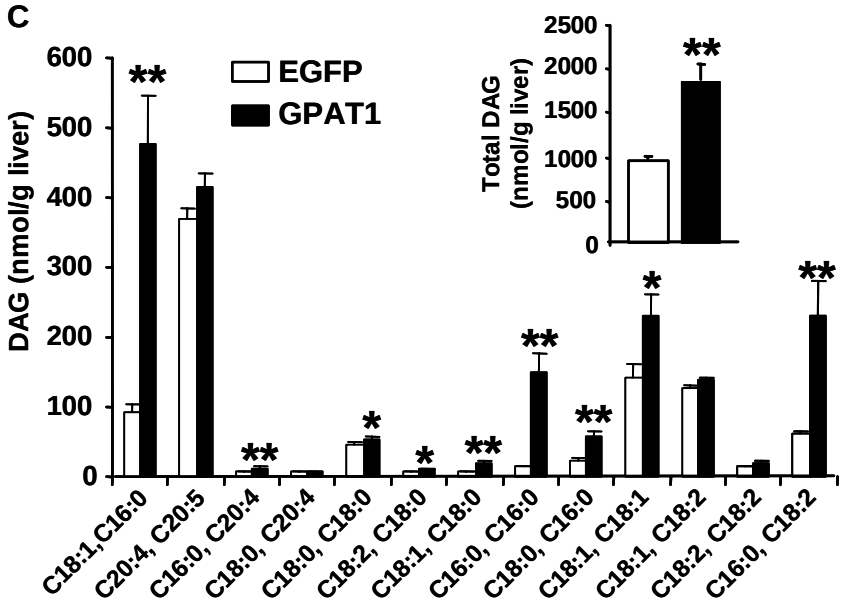

Figure 3.6C: Hepatic lipid metabolites were altered in Ad-GPAT1-treated rats. Rats were treated with 1.0-2.0 x 10^{12} particles/mL Ad-GPAT1 or Ad-EGFP for 5-7 days and food-deprived 4 h before tissues were collected. Liver lipid metabolites were measured by mass spectrometry. **C)** Total DAG (inset) (**p<0.001) and DAG species (* p< 0.05) (** p<0.001). Ad-EGFP n = 9; Ad-GPAT1 n = 11.

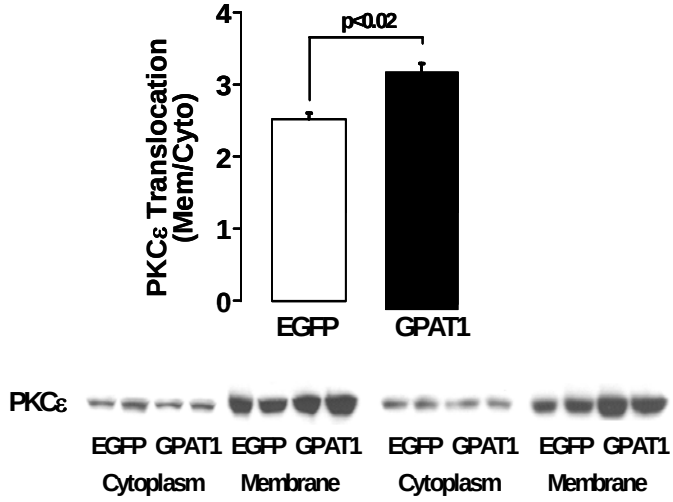

Figure 3.7: Hepatic PKCε activity was elevated in Ad-GPAT1-treated rats. Rats were treated with 1.0-2.0 x 10^{12} particles/mL Ad-GPAT1 or Ad-EGFP for 5-7 days and fasted for 4 h before tissues were collected. Liver cytosolic and membrane fractions were isolated, and the amount of PKCε in each fraction was determined by Western Blot. Ad-EGFP, n = 4; Ad-GPAT1, n = 4.

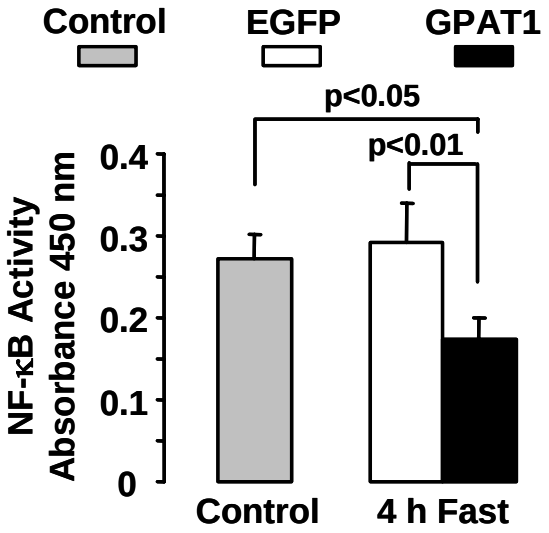

Figure 3.8: NF-κB activity was lower in Ad-GPAT1-treated rats. Rats were treated with 1.0-2.0 x 10^{12} particles/mL Ad-GPAT1 or Ad-EGFP for 5-7 days. Tissues were collected after a 4 h fast and NF-κB activity was measured in rat liver nuclear extracts with an ELISA kit. Control (non-viral), n = 4; After 4 h fast Ad-EGFP n = 19; Ad-GPAT1 n = 21. Results are presented as mean ± SE.

Table 3.2: Hepatic gene expression.

Gene expression after 4 h fast		**EGFP**		**GPAT**
SCD-1		1.0 ± 0.2		0.8 ± 0.1
TNF-α		1.0 ± 0.2		0.9 ± 0.2
IL-1β		1.0 ± 0.2		1.0 ± 0.3

Rats were treated with 1.0-2.0x 10^{12} particles/mL of Ad-GPAT1 or Ad-EGFP virus for 5-7 days and fasted for 4 h before livers were collected. Results expressed as mean ± SE. Stearoyl-CoA desaturase-1 (SCD-1), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β)
(EGFP, n = 5; GPAT n = 5).

Hepatic inflammation did not contribute to insulin resistance in Ad-GPAT1-treated rats – Because recent studies of insulin resistance have focused on the role of the immune system in the development of hepatic insulin resistance, we measured plasma cytokine levels and hepatic NF-κB activity. Plasma IL-1β, IL-6, and TNFα concentrations did not differ between Ad-GPAT1-treated and Ad-EGFP-treated rats (**Table 3.1**), and hepatic expression of TNF-α and IL-β did not differ between groups (**Table 3.2**). Additionally, hepatic NF-κB activity was 50% lower in Ad-GPAT1-treated rats compared to Ad-EGFP-treated rats and untreated controls, indicating that hepatic overexpression of GPAT1 caused insulin resistance in the absence of increased hepatic inflammation (**Figure3.8**)

3.5 DISCUSSION

The major finding of this study was that overexpressing GPAT1 in liver caused hepatic steatosis and hepatic insulin resistance in the absence of obesity or high-fat feeding. Surprisingly, TAG also accumulated in gastrocnemius muscle, in concert with development of insulin resistance in that tissue, possibly secondary to the increase in circulating VLDL-TAG and FFA. GPAT is the committed step in glycerolipid synthesis, and GPAT1, normally up regulated by SREBP-1c under conditions in which lipogenesis is enhanced, appears to control the amount of TAG synthesized, even when food intake or composition is not altered.

Hyperinsulinemic-euglycemic clamp studies confirmed that insulin resistance had developed in Ad-GPAT1-treated rats within 5-7 d of adenovirus treatment. In the Ad-GPAT1-treated rats, forty percent less glucose was required to maintain euglycemia than in the control rats, and hepatic glucose output was 2.7-fold higher during the clamp, consistent with severely reduced hepatic insulin sensitivity. The increased gluconeogenesis in Ad-GPAT1-treated rats was associated with elevated PEPCK mRNA and protein expression. Although hepatic glycogen content was significantly lower in Ad-GPAT1-treated rats after a 4 h fast, hepatic glycogen synthesis was not suppressed during the

clamp. These results suggest that GPAT1 overexpression impaired insulin suppression of gluconeogenesis but had little effect on insulin-stimulated glycogen synthesis.

In addition to causing profound insulin resistance in liver, GPAT1 overexpression in liver also resulted in a lower insulin-stimulated systemic glucose disposal rate and less uptake of glucose into gastrocnemius muscle. These data are consistent with a decreased rate of muscle glucose metabolism and suggest that hepatic GPAT1 overexpression increased peripheral insulin resistance. Although the uptake of 2-deoxyglucose was not statistically different in two other muscles, it is possible that hepatic overexpression of GPAT1 for a longer period than 7 days might lead to more severe peripheral insulin resistance. Peripheral insulin resistance was associated with increases in the TAG content of skeletal and heart muscle. Since Ad-GPAT1 was not expressed in these tissues, it is likely that the increase in intramuscular TAG content resulted from the elevated plasma VLDL-TAG and fatty acid available for muscle uptake and storage. Although the uptake of 2-deoxyglucose was not statistically different in two other muscles, it is possible that hepatic overexpression of GPAT1 for a longer period than 7 days might lead to more severe peripheral insulin resistance. Elevated plasma lipids can lead to TAG accumulation and insulin resistance in skeletal muscle [8, 193-195]. Ad-GPAT1-treated rats ate the same amount of food, gained the same amount of weight as Ad-EGFP-treated controls, and did not show differences in epididymal fat pad mass or plasma leptin concentrations. Thus, alterations in body fat mass did not contribute to the systemic insulin resistance observed in Ad-GPAT1-treated rats.

What caused hepatic insulin resistance when GPAT1 was overexpressed? The frequent association of insulin resistance with TAG accumulation in liver and other tissues suggests that TAG or a related lipid metabolite antagonizes pathways of insulin signaling. Consistent with this idea, experimental lowering of liver TAG content in other rodent models results in amelioration of hepatic and peripheral insulin resistance [14, 103]. Hepatic overexpression of GPAT1 increased liver TAG

content 2.7-fold, and dramatically increased the intracellular content of immediate and downstream products of GPAT1, LPA and DAG. In primary rat hepatocytes treated with Ad-GPAT1 and in livers from mice treated with Ad-GPAT1, we and others reported an increase in fatty acid incorporation into DAG and an increase in DAG content [15, 36, 37]. In the present study, the enrichment of 16:0 species in both LPA and DAG reflects the preferred 16:0-CoA substrate of GPAT1 [29] and strongly suggests that both metabolites were derived from *de novo* synthesis rather than from the hydrolysis of membrane phospholipids.

LPA is well-recognized as a ligand for G-protein coupled LPA receptors that stimulate cell growth [196], and extracellular LPA, acting via LPA receptors on adipocytes, inactivates glycogen synthase kinase 3 (GSK3) in a PI_3K independent manner [197]. Thus, extracellular LPA signaling could enhance glycogen synthesis independent of insulin signaling, but we observed a decrease rather than an increase in hepatic glycogen content in Ad-GPAT treated animals in the current study. Moreover, there are no known inhibitory effects of extracellular or intracellular LPA on insulin signaling pathways. DAG accumulation, however, and its concomitant activation of PKC isoforms has been implicated in the development of insulin resistance in skeletal muscle and liver [117, 198], and the activation of PKCε and PKCδ has been implicated in hepatic insulin resistance [188, 199, 200]. Further, mice deficient in GPAT1, have a lower hepatic DAG content than wild type controls and are protected via a PKCε-mediated mechanism from high-fat-diet-induced insulin resistance [14]. In the current study, PKCε activity was 30% higher in the livers of Ad-GPAT1-treated rats compared to control livers. PKCε interacts with the insulin receptor and prevents tyrosine phosphorylation of IRS-2, thereby blocking insulin signaling upstream of IRS-2 [200]. High-fat feeding of wild type mice disrupts insulin stimulation of IRS-2-associated PI3K activity, but this pathway was maintained in $GPAT1^{-/-}$ mice, as was insulin-stimulated suppression of hepatic gluconeogenesis [14]. Thus, the

failure of insulin to suppress hepatic PEPCK gene expression and gluconeogenesis in Ad-GPAT1-treated rats may be due to PKCε-mediated inhibition of IRS-2-associated PI_3K activity.

SCD1 catalyzes the desaturation of 18:0 to 18:1, and reduced expression of SCD1, which was observed in $GPAT1^{-/-}$ mice [13, 15], is associated with decreased hepatic TAG synthesis [201, 202]. In diet-induced obesity, SCD1 expression may be required for the development of hepatic insulin resistance [203]. Although low compared to 16:0 species, 18:1-species of LPA and DAG were 40% higher in Ad-GPAT1-treated rats, similar to a report of Ad-GPAT overexpression in mice in which SCD1 mRNA expression increased 10-fold [15]. In our study, however, no difference was observed in SCD-1 mRNA expression in Ad-GPAT1 and Ad-EGFP rats, indicating that SCD1 had not influenced insulin resistance.

Although overexpression of GPAT1 in primary hepatocytes did not increase the amount of [^{14}C]fatty acid that was incorporated into media TAG (19, 20), the amount of TAG in plasma did increase when GPAT1 was overexpressed in mice or rats. In mice treated with Ad-GPAT1, the rate of TAG secretion increased 86% [15], and, similarly, we found that in Ad-GPAT1-treated rats plasma TAG was 2.6-fold higher than in controls, and that there was a large increase of TAG in the VLDL fraction. These data suggest that the pool of TAG synthesized by GPAT1 is available both for storage and for incorporation into VLDL. Because the amount of apoB in Ad-GPAT-treated mouse plasma [15] or in VLDL from Ad-GPAT-treated rats did not differ from controls, it is likely that GPAT1 overexpression increased the amount of TAG incorporated into VLDL but not the number of VLDL particles secreted. Although total plasma cholesterol content was not elevated in Ad-GPAT1-treated rats, plasma cholesterol was redistributed away from HDL and into the VLDL fraction.

Several recent studies have implicated hepatic NF-κB activity, hepatocellular inflammatory pathways, and elevated hepatic expression of IL-1β, TNF-α, and IL-6 in the development of hepatic

insulin resistance [204, 205]. However, compared to Ad-EGFP-treated rats, hepatic NF-κB activity was not elevated in Ad-GPAT1-treated rats, and neither hepatic expression of IL-1β and TNF-α nor plasma cytokine concentrations differed. These data show that, at least over the short time frame of the current study (7 days), the fatty liver induced by GPAT1 overexpression did not cause hepatic inflammation and inflammatory cytokines did not contribute to insulin resistance in this model.

The accumulation of glycerolipid metabolites has been implicated in the pathogenesis of hepatic insulin resistance, but a causal relationship has not been clearly established. Interpretation of most animal models of hepatic steatosis and insulin resistance is complicated by underlying obesity, genetically-induced over-feeding, lipodystrophy, or alterations in adipokine production. In our study, GPAT1 overexpression enhanced *de novo* glycerolipid synthesis in rat liver and caused hepatic steatosis, hypertriglyceridemia, and both hepatic and peripheral insulin resistance in the absence of obesity, high-fat feeding, or elevated inflammatory markers. These data support the idea that liver lipid metabolism itself can play a central role in the development of both hepatic and systemic insulin resistance. Hepatic insulin resistance was associated with an elevated hepatic content of LPA, DAG, and TAG, as well as increased PKCε activation, whereas peripheral insulin resistance was associated with excess TAG storage in muscle. Further study of the regulation of hepatic *de novo* glycerolipid synthesis and of the role of glycerolipid intermediates as intracellular signaling molecules promises a better understanding of the mechanisms involved in lipid-mediated hepatic insulin resistance. Enzymes in the pathway of glycerolipid biosynthesis may prove to be therapeutic targets for hepatic insulin resistance and type 2 diabetes.

3.6 Acknowledgements

This work was supported by grants from the NIH: DK56598 (RAC), DK-58398 (CBN), DK60667 (MS), DK-40936 (GIS), U24 DK59635, and P30 DK34987, and by an award from the American Heart Association, MidAtlantic Affiliate (CAN). GIS is an Investigator of the Howard Hughes MedicalInstitute.

CHAPTER IV. IDENTIFICATION OF A NOVEL GPAT, GPAT4

Acyl-glycerol-3-phosphate acyltransferase-6 (AGPAT6) is a newly cloned enzyme with conserved acyltransferase motifs shared by known GPAT and AGPAT isoforms. AGPAT6 knock-out mice have reduced liver and adipose tissue TAG content, a phenotype consistant with a deficiency in either GPAT or AGPAT activity. However, the specific acyltransferase activity of AGPAT6 had not been determined. This chapter describes our work to identify AGPAT6 as a novel NEM-sensitive GPAT, GPAT4.

Manuscript #2: **Identification of a Novel *sn*-Glycerol-3-Phosphate Acyltransferase Isoform, GPAT4, in *Agpat6*-Deficient Mice**

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4.1 ABSTRACT:

Elucidation of the metabolic pathways of triacylglycerol synthesis is critical to the understanding of chronic metabolic disorders such as obesity, cardiovascular disease, and diabetes. Glycerol-*sn*-3-phosphate acyltransferase (GPAT) and *sn*-1-acylglycerol-3-phosphate acyltransferase (AGPAT) catalyze the first and second steps in *de novo* triacylglycerol synthesis. AGPAT6 is one of eight AGPAT isoforms identified through sequence homology, but the enzyme activity for AGPAT6 has not been confirmed. *Agpat6* deficient mice have a subdermal lipodystrophy, reduced liver and adipose tissue triacylglycerol content, and resistance to high fat diet-induced obesity. We found that in liver and brown adipose tissue from *Agpat6*$^{-/-}$ mice NEM-sensitive GPAT specific activity was 60% lower than in tissues from wild type mice, but that AGPAT specific activity was similar in wild type and *Agpat6*$^{-/-}$ mice. Over-expression of *Agpat6* in Cos-7 cells increased an NEM-sensitive GPAT specific activity that used saturated and unsaturated 16 and 18 carbon

acyl-CoA substrates similarly, and increased GPAT's lysophosphatidic acid product. AGPAT specific activity was not increased in the AGPAT6-expressing cells. Thus, "*Agpat6*$^{-/-}$ mice" are actually deficient in a novel NEM-sensitive GPAT, GPAT4, and should be called *Gpat4*$^{-/-}$ mice.

4.2 INTRODUCTION:

The regulation of triacylglycerol (TAG) synthesis and metabolism plays an important role in whole body energy homeostasis in mammals, and dysregulation of TAG synthesis and oxidation pathways have been implicated in the pathogenesis of obesity, lipodystrophy, cardiovascular disease, insulin resistance, and type 2 diabetes. *De novo* triacylglycerol synthesis begins with the formation of lysophosphatidic acid (LPA) through the acylation of glycerol-3-phosphate at the *sn*-1 position by *sn*-1 glycerol-3-phosphate acyltransferase (GPAT) (**Figure 4.1**). The second enzyme in *de novo* triacylglycerol synthesis, 1-acylglycerol-3-phosphate acyltransferase (AGPAT), acylates lysophosphatidic acid (LPA) to form phosphatidic acid (PA). Lipin (phosphatidic acid phosphohydrolase) converts PA to diacylglycerol (DAG), and diacylglycerol acyltransferase acylates DAG to form TAG. PA and DAG are also precursors for glycerophospholipids.

Three mammalian GPAT isoforms have been cloned [33, 34, 41]. GPAT1 and GPAT2 are located in the outer mitochondrial membrane, whereas GPAT3 is located in the endoplasmic reticulum. GPAT1 is resistant to inactivation by sulfhydryl agents like *N*-ethylmaleimide (NEM), prefers palmitoyl-CoA as a substrate compared to oleoyl-CoA, and plays a regulatory role in liver TAG synthesis [29]. GPAT2 is NEM-sensitive and has no

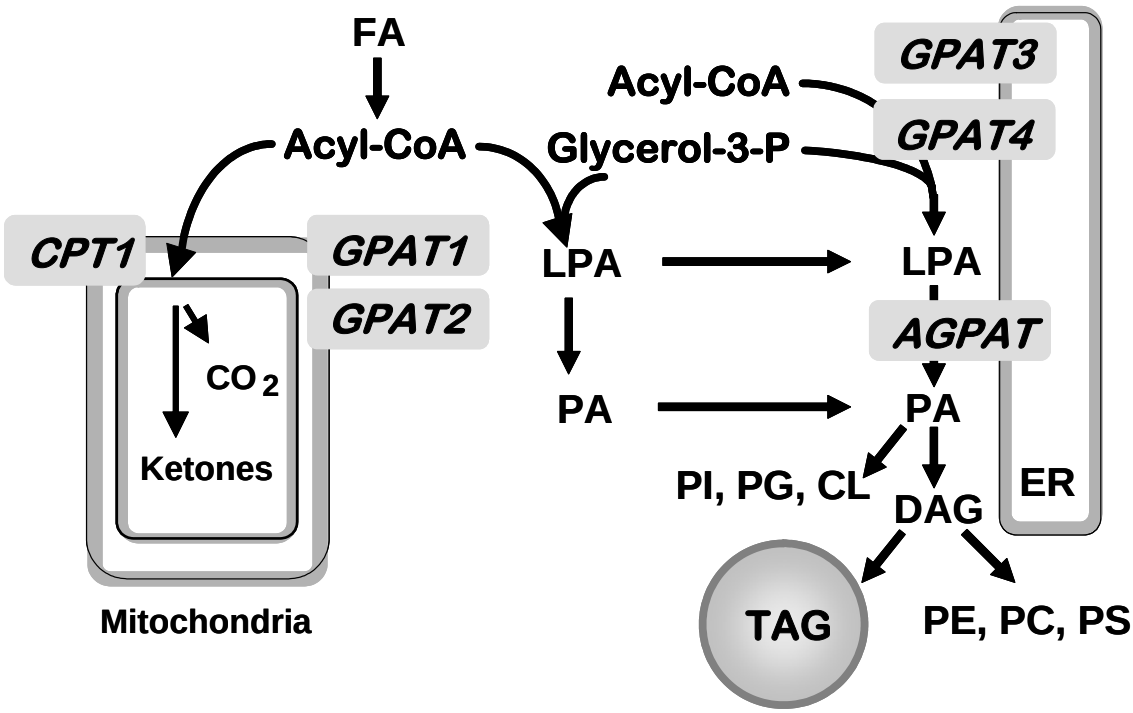

Figure 4.1. Pathway of *de novo* glycerolipid synthesis. GPAT isoforms located in mitochondria (GPAT1 and 2) and endoplasmic reticulum (GPAT3 and 4) catalyze the initial step in the synthesis of glycerophospholipids and triacylglycerol (TAG). AGPAT isoforms catalyze the second step in this pathway by acylating lysophosphatidic acid to form phosphatidic acid (PA). Acyl-CoA used for □-oxidation or TAG synthesis is regulated reciprocally by carnitine palmitoyl transferase-1 (CPT-1) and GPAT1 [13]. Cardiolipin (CL), diacylglycerol (DAG), triacylglycerol (TAG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylserine (PS), and fatty acid (FA).

preference for palmitoyl-CoA [3]. Recently, the gene product previously called AGPAT8 in the NCBI database was shown to have NEM-sensitive GPAT activity and to lack AGPAT activity; this gene product was re-named GPAT3 [34]. Because siRNA knockdown of *Gpat3* in 3T3-L1 adipocytes reduces total GPAT activity by ~60% [4, 34], it is likely that GPAT3 is important for TAG synthesis in WAT. However, GPAT3 is unlikely to play a major role in TAG synthesis in liver where its mRNA expression is very low [4, 34]. In liver, GPAT1 accounts for 30-50% of the total GPAT activity [29]. The remaining hepatic GPAT activity is NEM-sensitive and microsomal, suggesting that one or more additional microsomal NEM-sensitive GPAT isoforms must exist.

Eight genes with predicted amino acid sequence similarities to GPAT1 have been named AGPAT isoforms 1-8. AGPAT1 and 2 are well characterized and have high AGPAT activity [60, 61]. Mutations in AGPAT2 cause generalized human congenital lipodystrophy, with absent abdominal and subcutaneous adipose tissue, systemic insulin resistance, and hypertriglyceridemia [67]. Compared to AGPAT1 and 2, the AGPAT activities reported for AGPAT3-5 are extremely low [206]; no enzyme activity has been reported for AGPAT6 or 7.

We previously characterized mice with a targeted deletion in *Agpat6* (*Agpat6*$^{-/-}$ mice; NP_848934) [4, 35]. These mice have a subdermal lipodystrophy, resistance to high-fat diet induced obesity, and a 50% reduction of TAG content in liver, adipose tissue and mammary epithelium, suggesting an important role for AGPAT6 in TAG synthesis in these tissues. AGPAT6 shares amino acid sequence homology with other glycerolipid acyltransferases at

Table 4.I: Acyltransferase motifs in *E. coli* and mouse AGPAT and GPAT isoforms

	Motif I[a]	Motif II[a]	Motif III[a]	Motif IV[a]	Motif V[f]
Function[a]	**Catalysis**	**G-3-P binding**	**G-3-P binding**	**Catalysis**	
AGPAT1 (NP_061350)	104 **H**QSSL**D**	140 GIIFID**R**	173 **F**P**EG**TRNH	200 VPII**P**IVNSSY	231 **V**LPPVSTEGLTPD**D**
AGPAT2 (NP_080488)	98 **H**QSLI**D**	137 GVYFIN**R**	170 YP**EG**TRND	197 VPII**P**VVYSSF	227 **V**LDAVPTNGLTDA**D**
AGPAT6 (GPAT4[e]) (NP_061213)	248 **H**TSPI**D**	286 PHVWFE**R**	320 **F**P**EG**T***C***IN	343 ATVY**P**VAIK**YD**	385 **V**WYLPPMTREKDE**D**
AGPAT8 (GPAT3[d]) (NP_766303)	229 **H**RTRV**D**	268 VHIFIH**R**	301 **F**P**EG**T***C***LT	324 GTIY**P**VAIK**Y**N	366 **V**WYMPPMTREEGE**D**
***E. coli* GPAT** (plsB) (BAE78043)	306 **H**RSHM**D**	349 GAFFIR**R**	383 **F**V**EG**GRSR	417 ITLI**P**IYIG**Y**E	
GPAT1[b] (NP_032175)	230 **H**RSHI**D**	272 GGFFIR**R**	313 **F**L**EG**TRSR	347 ILVI**P**VGIS**YD**	
GPAT2[c] (NP_001074558)	202 **H**KSLL**D**	231 T***C***SPAL**R** or 235 ALRALL**R** or 247 LGGFLPP[33]	291 GSP**G**RLSA or 285 **F**L**E**EPPGS[33]	320 ATLV**P**VAIA**YD**	

[a]Acyltransferase motifs as we described in [30]. Bolded residues are highly conserved across these GPAT family members. Underlined amino acids are important for GPAT1 activity as determined by mutagenesis [29, 30]. *Cysteines* that may be targets for *N*-ethylmaleimide are highlighted and italicized. [b]Previously called mtGPAT or mtGAT. [c]Also identified as xGPAT1 [33]. [d]Identified in GenBank as AGPAT8, but has only GPAT activity [34]. [e]Identified in GenBank as AGPAT6, but has only GPAT activity as reported here. [f]Motif V as described in [35].

conserved motifs I-V (**Table 4.1**), and is closely related to GPAT1 and 2 in motifs I-IV, which were identified as important for acyltransferase catalysis and glycerol-3-phosphate binding domains [30]. AGPAT6 shares 66% amino acid homology with "AGPAT8", recently identified as GPAT3 [34], and confocal microscopy shows that AGPAT6 localizes to the endoplasmic reticulum [35].

In previous studies of AGPAT6, we were unable to detect AGPAT or GPAT activity in insect or Cos-7 cells transfected with *Agpat6* constructs [35]. Convinced, however, that AGPAT6 must have either AGPAT or GPAT activity, we re-cloned *Agpat6* with a C-terminal Flag epitope tag into pcDNA3.1, transfected Cos-7 cells, and measured AGPAT and GPAT activities. We also measured these enzyme activities in liver and adipose tissue from *Agpat6*$^{-/-}$ mice. Our studies now identify *Agpat6* as a gene encoding the primary microsomal NEM-sensitive GPAT activity in liver, GPAT4.

4.3 METHODS:

Agpat6$^{-/-}$ mice.

Agpat6 $^{-/-}$ *m*ice were generated from a gene-trap cell line identified in BayGenomics, a gene-trapping resource [4, 35]. This strain was backcrossed to C57BL/6J for at least four generations. The mice were housed in a barrier facility with a 12 h light/12 h dark cycle, and fed a chow diet containing 4.5% fat (Ralston Purina, St. Louis, MO). All animal protocols were approved by the institutional animal care and use committee. Tissues were harvested from 6 month old mice, snap-frozen in liquid nitrogen, and stored at -80°C. A previous

characterization of the *Agpat6*$^{-/-}$ mice revealed a similar phenotype in adipose tissue and liver of males and females [4, 35], and both sexes were used in the studies described here.

Cloning of *Agpat6*.

Agpat6 was amplified by PCR from mouse liver RNA at 57.5 ˚C with Pfx50 (Invitrogen) using a forward primer (5'cacc**ggatcc**acgtgcgtcctccaccat) containing a BamHI restriction site (**bold**) and a reverse primer (5'ccc**tctaga**cta<u>cttgtcgtcatcgtccttgtagtcgg</u>accggctgcggtcct) containing a Flag epitope sequence (<u>underlined</u>) and an XbaI restriction site (**bold**). The amplification product was digested with BamHI and XbaI and ligated into the similarly digested pcDNA3.1 mammalian expression vector (Invitrogen).

Nucleofection of Cos-7 cells with *Agpat6*.

Cos-7 cells were grown in DMEM high glucose (GIBCO) with 10% FBS and 1% penicillin/streptomycin. The cells were grown to confluence and passaged one day before nucleofection. Cells were harvested and 4.0 x 10^6 cells were nucleofected with 12 μg *Agpat6* or empty vector pcDNA3.1 plasmid DNA in 100 μL of Nucleofector™ Kit V Solution using the Nucleofector™ system (AMAXA Biosystems). Cells were then transferred to 100 mm cell culture dishes containing RPMI and 10 % FBS and 1% penicillin/streptomycin, and were harvested 18 h later.

Membrane isolation and measurement of GPAT activity.

Liver or adipose tissue was homogenized in Medium I + DTT (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM DTT) with 10 up-and-down strokes in a Teflon-

glass motor-driven homogenizer. Homogenates were centrifuged at 100,000 x g for 1 h to obtain total membrane fractions. The membrane pellet was re-homogenized in Medium I + DTT and stored in 100 μL aliquots at -80º C. GPAT specific activity was assayed at 23 ^{o}C for 10 min in a 200 μL reaction mixture containing 75 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 2 mg/mL bovine serum albumin (essentially FA-free), 1 mM DTT, 8 mM NaF, 300 μM [^{3}H]glycerol-3-phosphate, and 80 μM palmitoyl-CoA [183]. The reaction was initiated by adding 2.5-7.5 μg (WAT), 5-15 μg (BAT), or 10-30 μg (liver) total membrane protein to the assay mixture after incubating the membrane protein on ice for 15 min in the presence or absence of 1 mM NEM. These assays measured initial rates. The reaction was stopped by adding 1% perchloric acid and the products were extracted into chloroform [191]. NEM-sensitive activity is calculated as the total GPAT activity minus the GPAT activity that is not inhibited by NEM. For nucleofected Cos-7 cells, culture dishes were scraped with Medium I + DTT and the samples were homogenized and centrifuged as described above to obtain total membrane fractions. GPAT specific activity was measured as above with 5-15 μg of total Cos-7 membrane protein. To identify the products from the GPAT assay, the chloroform extracts were spotted on a silica H thin layer chromatography plate (Analtech) together with authentic standards (Avanti) and developed in $CHCl_3$/pyridine/formic acid (88%) (50/30/7; v/v). The plates were exposed to iodine vapor, and the bands corresponding to LPA, PA, and DAG were scraped into scintillation vials containing Cytoscint (ICN) and counted. To determine acyl-CoA preference, 60 μM of each of several acyl-CoA species and 10-20 μg protein were used in the GPAT assay.

AGPAT assay.

AGPAT specific activity was assayed at 37˚ C in a 100 µl mixture containing 100 mM HEPES-NaOH pH 7.5, 200 mM NaCl, 10 mM EDTA, 8 mM NaF, 1 mg/ml bovine serum albumin (fatty acid free), 1 mM DTT, 5% (w/v) glycerol, 40 µM oleoyl-CoA, and 20 µM 1-[^{3}H]oleoyl-LPA (1 µCi, Perkin-Elmer NET-1100). The reaction was initiated by adding 5 µg of total membrane protein to the assay mixture. Fifteen µl of the reaction mixture was removed at indicated time points up to 10 min and spotted directly onto a Silica H plate together with authentic standards. The radio labeled PA product was separated from the radio labeled LPA substrate in chloroform:pyridine:formic acid (50/30/7; v/v). Authentic lipid standards for LPA and PA were visualized by exposure to iodine vapor. Spots corresponding to PA were scraped into 500 µl methanol:water (1:1), Cytocint (ICN) was added and radioactivity was quantified.

Immunoblotting.

Cos-7 cell total membrane proteins were separated on an 8% poly-acrylamide gel containing 1% SDS, transferred to a PVDF membrane (Bio-Rad), and incubated with mono-clonal antibody against the FLAG epitope (Clone M2, Sigma). For chemiluminescent detection, the immunoreactive bands were visualized by incubating the membrane with horseradish peroxidase-conjugated goat anti-mouse IgG and PicoWest reagents (Pierce) according to the manufacturer's instructions.

Gene expression analyses.

Total RNA was extracted from mouse tissues with TRIzol (Invitrogen). Two μg of RNA were reverse-transcribed with oligo dT and random primers (Invitrogen). Five percent of the resulting cDNA was used for each real-time RT-PCR reaction. Real-time RT-PCR was performed in duplicate with Quantitect SYBR Green PCR mix (QIAGEN) in an iCycler Realtime Detection System (BioRad) [4, 207]. Data presented were derived from starting quantity (SQ) values normalized by the square root of the product of values obtained for the housekeeping genes beta-2-microglobulin (β2m) and TATA box binding protein (Tbp), or to 18S rRNA. Primer sequences used in this study have been described previously [207] or are shown in **Table 4.2**. Primers for *Gpat3* were previously cited as *Agpat8* [4].

Table 4.2. Primer sequences used in real-time PCR experiments

Gene	Forward	Reverse
β2m	5'cagcatggctcgctcggtgac	5'cgtagcagttcagtatgttcg
Gpat1	5'agcaagtcctgcgctatcat	5'ctcgtgtgggtgattgtgac
Gpat2	5'aagaaagaggtacagcgtatcc	5'gtggagagccctcctgcacag
Gpat3	5'gtacatgcctcccatgactag	5'gatccgttgcccacgatcatc
18S rRNA	5'accgcagctaggaataatgga	5'gcctcagttccgaaaacca

Statistics. Data are shown as mean ± S.E. Significance was established using Student's *t* test. Differences were considered significant at $p < 0.05$.

4.4 RESULTS:

Agpat6$^{-/-}$ mouse tissues have lower NEM-sensitive GPAT activity

To determine whether tissues from *Agpat6*$^{-/-}$ mice have lower GPAT activity, we measured GPAT activity in total membrane fractions from liver, BAT, and gonadal WAT in the presence and absence of NEM. Total GPAT activity was 49% lower in liver from *Agpat6*$^{-/-}$ mice ($p < 0.001$) (**Figure 4.2A**). NEM-resistant activity (GPAT1) was similar in liver from knockout and wild-type mice (**Figure 4.2B**), but the NEM-sensitive activity was reduced 65% ($p < 0.0001$) (**Figure 4.2C**). AGPAT activity was unchanged in *Agpat6*$^{-/-}$ mouse liver (**Figure 4.2D**). In *Agpat6*$^{-/-}$ BAT, total GPAT specific activity was reduced 50% ($p < 0.01$) and NEM-sensitive specific activity was reduced 65% ($p < 0.001$) (**Figure 4.3**). However, GPAT specific activities were identical in gonadal WAT from wild type and *Agpat6*$^{-/-}$ mice (**Figure 4.3**). Thus, liver and BAT tissue from *Agpat6*$^{-/-}$ mice exhibit normal AGPAT activity, but reduced NEM-sensitive microsomal GPAT activity.

Expression of *Gpat1*-3 mRNA transcripts in *Agpat6*$^{-/-}$ mice

BAT from *Agpat6*$^{-/-}$ mice does not show altered expression of mRNA for *Agpat1-5*, *7* or *8* [4]. To determine whether the deficiency of microsomal GPAT activity altered the mRNA expression of other GPAT isoforms, we measured the mRNA expression of *Gpat1*, *2*, and *3* in liver, BAT, and inguinal WAT from *Agpat6*$^{-/-}$ mice. *Gpat1* expression was 2-fold higher in liver of *Agpat6*$^{-/-}$ mice (**Table 4.3**), but this increase in mRNA expression did not correspond to an increase in GPAT1 (NEM-resistant) activity (**Figure 4.2C**). Agpat6$^{-/-}$ and wild-type mice had similar expression levels for *Gpat2* and *Gpat3* in liver and for *Gpat1, Gpat2,* and *Gpat3* in BAT and WAT.

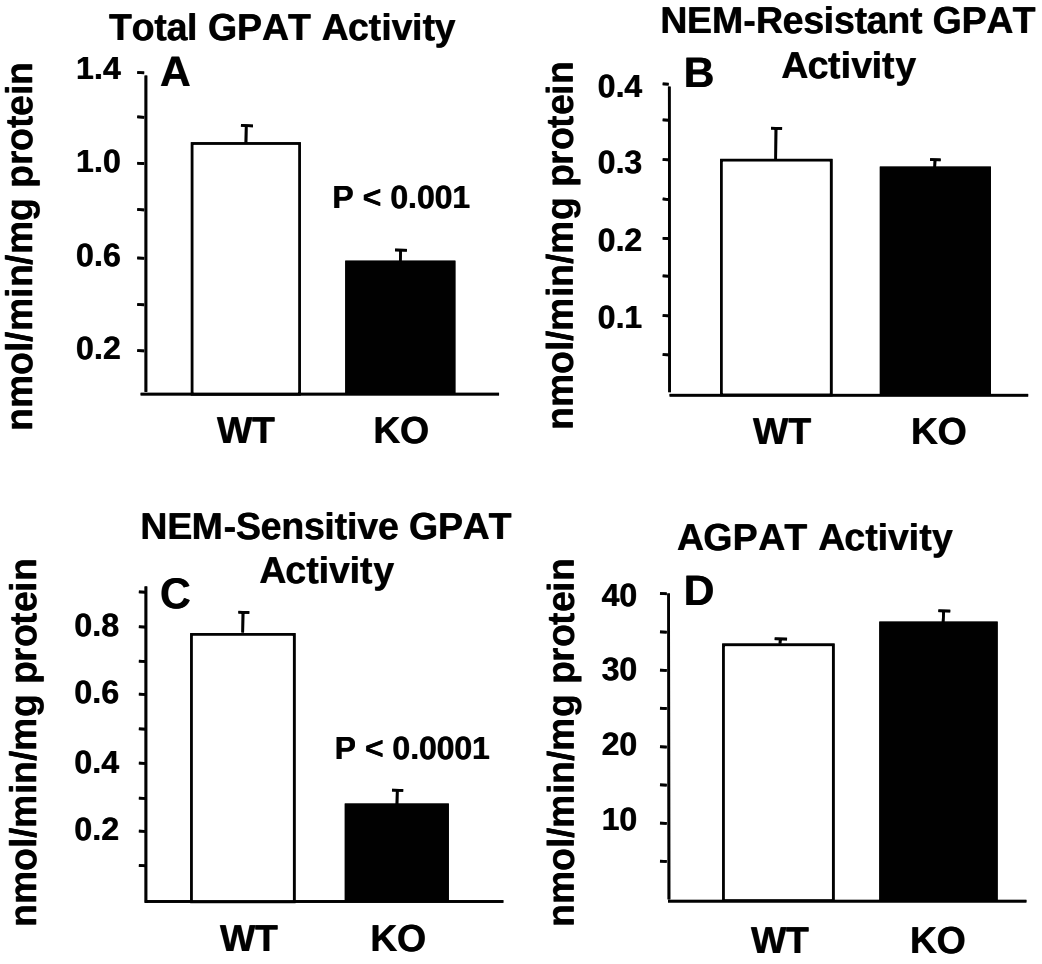

Figure 4.2. NEM-sensitive GPAT specific activity was reduced in liver from *Agpat6*$^{-/-}$ mice. In female mice GPAT specific activity was measured in liver total particulate preparations from female mice as described in Methods, (A), total activity; (B), NEM-sensitive activity, and (C), NEM-resistant activity. AGPAT specific activity (D) in total particulate preparations was measured as described in Methods. (n =6)

Table 4.3. mRNA levels of GPAT enzymes in wild-type and *Agpat6*$^{-/-}$ mouse tissues.

*, $p < 0.05$ *vs*. wild-type (n=4).

	Liver		Brown adipose tissue		Inguinal white adipose tissue	
	Wild-type	*Agpat6*$^{-/-}$	Wild-type	*Agpat6*$^{-/-}$	Wild-type	*Agpat6*$^{-/-}$
Gpat1	0.20 ± 0.02	0.37 ± 0.1 *	0.73 ± 0.16	0.84 ± 0.23	0.07 ± 0.03	0.06 ± 0.05
Gpat2	0.10 ± 0.04	0.08 ± 0.02	0.05 ± 0.002	0.03 ± 0.03	0.08 ± 0.08	0.11 ± 0.17
Gpat3	0.64 ± 0.13	0.80 ± 0.19	1.41 ± 0.64	1.00 ± 0.19	2.05 ± 0.62	1.58 ± 1.24

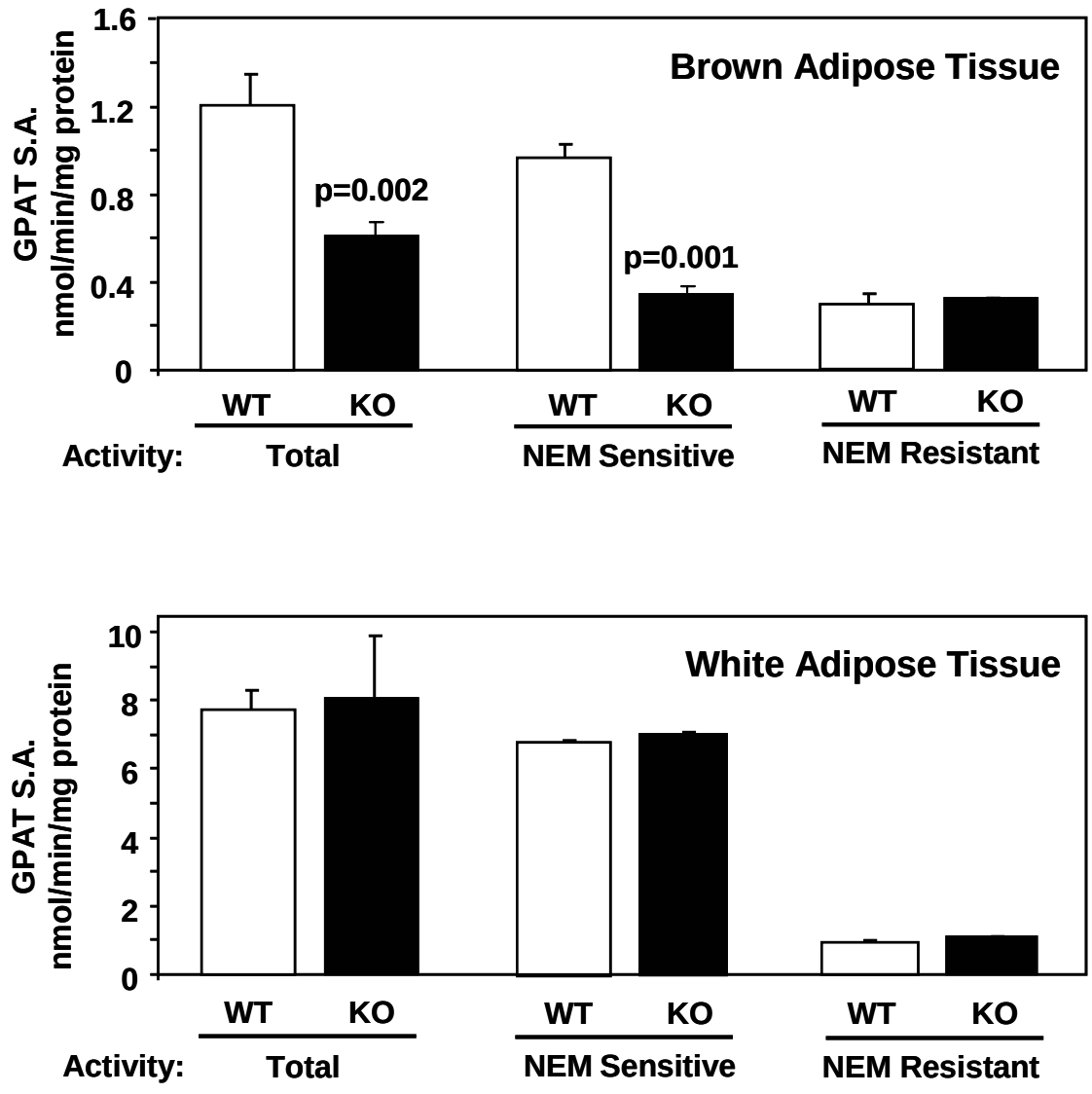

Figure 4.3. NEM-sensitive GPAT specific activity is reduced in brown adipose tissue (BAT) but not in gonadal adipose tissue (WAT). Total, NEM- sensitive, and NEM-resistant GPAT specific activity was measured in BAT from male mice and in gonadal WAT from female mice., WT=wild type and KO=*Agpat6*$^{-/-}$ mice. (n = 6)

Overexpression of *Agpat6/Gpat4* in Cos-7 cells increases NEM-sensitive GPAT activity

To further investigate the enzyme activity encoded by *Agpat6*/*Gpat4* in a heterologous cell system, we expressed *Gpat4* cDNA tagged with a FLAG epitope in Cos-7 cells. Protein expression levels were monitored by Western blot using an anti-FLAG antibody (**Figure 4.4A**). Cells transfected with the *Gpat4* expression construct exhibited 60% higher NEM-sensitive GPAT activity than cells transfected with vector ($p < 0.01$), whereas AGPAT activity was similar between the two (**Figure 4.4 B, C**). The products of the GPAT assay were analyzed by thin layer chromatography and showed a 2-fold increase in labeled LPA and a 70% increase in labeled PA in samples from *Gpat4*-transfected cells **(Figure 4.4D).** The relative distribution of LPA, PA, and DAG products of GPAT assays was similar in cells transfected with empty vector or *Gpat4*; there was no relative increase in PA, which might be expected with AGPAT activity **(Figure 4.4E).** Thus, overexpression of *Gpat4* increased both NEM-sensitive GPAT activity and flux through the *de novo* glycerolipid synthetic pathway.

To determine whether GPAT4 has a preference for a specific acyl-CoA substrate, we measured GPAT activity in Cos-7 cell total membranes from *Agpat6*-transfected cells in the presence of acyl-CoA substrates of varying chain length and saturation. Although able to use C12:0-CoA, GPAT4 preferred to use acyl-CoA substrates with 16 or 18 carbons as compared to C12:0- or C20:4-CoA **(Figure 4.4F).**

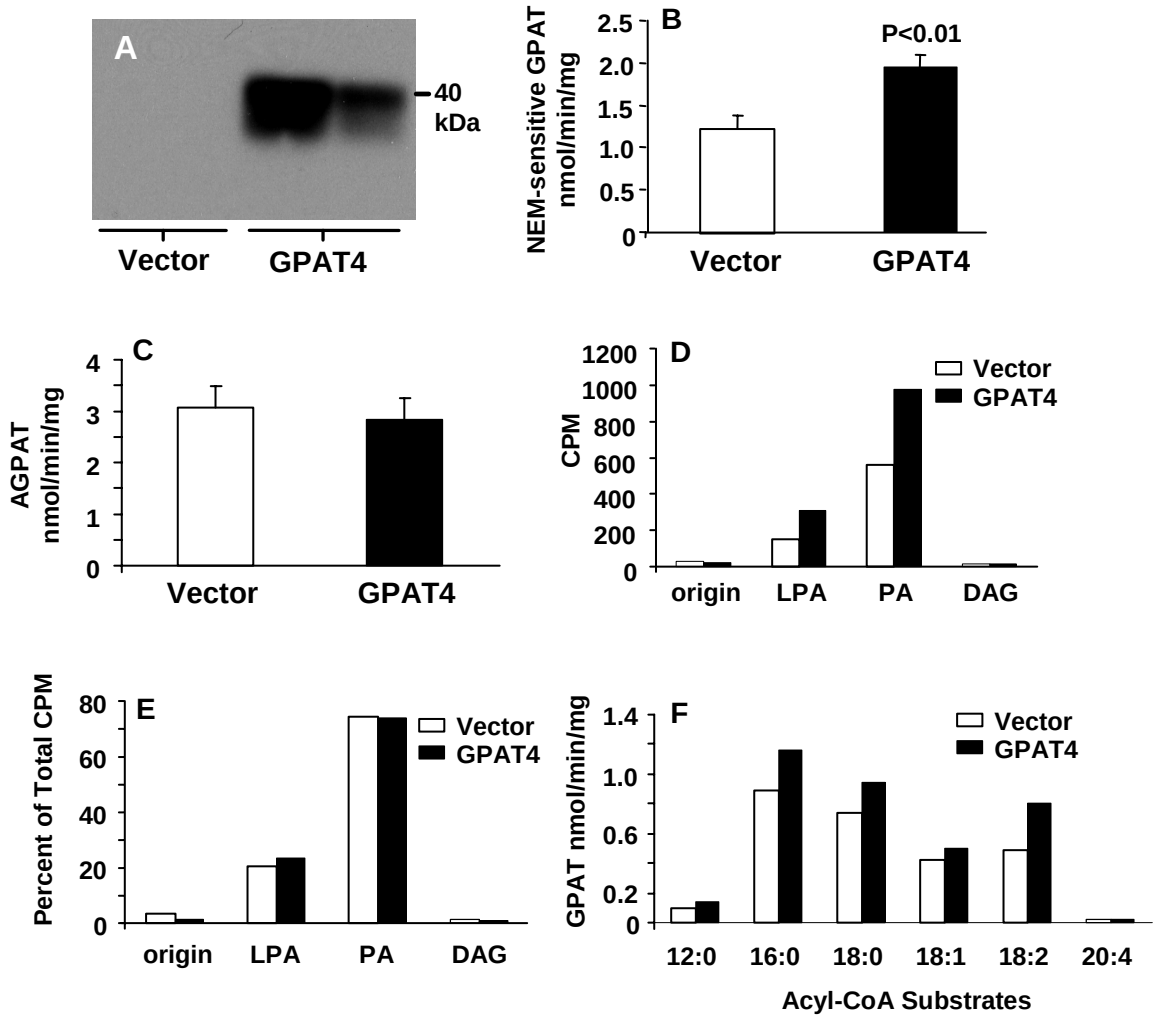

Figure 4.4. Overexpression of GPAT4 in Cos-7 cells increases NEM-sensitive GPAT activity. Cos-7 cells were transfected with pcDNA3.1 or with *Gpat4*. A) Immunoblot of Cos-7 cell total membrane fractions against the Flag epitope (2 independent transfections); B) NEM-sensitive GPAT activity in Cos-7 total membrane fraction (n = 6, $p<0.01$); C) AGPAT specific activity in Cos-7 cell total membrane fraction (n =4); D) Radio labeled glycerolipid products from GPAT assays of Cos-7 cell total membranes were separated by TLC and quantified as described in Methods; E) Distribution of radio labeled glycerolipid products from GPAT assay as a percentage of total counts (average of 2 independent experiments); F) Total GPAT activity in Cos-7 total membrane preparations with different acyl-CoA substrates (60 μM) (average of 2 independent experiments).

4.5 DISCUSSION:

Agpat6$^{-/-}$ mice have a subdermal lipodystrophy and reduced TAG content in BAT, WAT, liver, and mammary epithelium, suggesting that AGPAT6 plays an important role in TAG synthesis in these tissues [4, 35]. Because AGPAT catalyzes the second step in *de novo* TAG synthesis, the phenotype of the *Agpat6*$^{-/-}$ mice was consistent with the idea that AGPAT6 catalyzes that second step. Based on amino acid sequence homology, mouse AGPAT6 appeared to be an AGPAT, but as shown here, acts as a GPAT instead. When expressed in Cos-7 cells, we observed a 60% increase in NEM-sensitive GPAT activity, but no increase in AGPAT activity. It is unclear why previous expression constructs for this enzyme, which contained C-terminal V5 or V5-His epitope tags, failed to exhibit GPAT or AGPAT activity when expressed in insect or Cos-7 cells [35]; it may be that these epitope tags interfered with enzyme action, while the Flag epitope used here did not. Consistent with the true identity of AGPAT6 as a GPAT enzyme, the previously characterized *Agpat6*-deficient mice have 65% lower NEM-sensitive GPAT specific activity in liver and BAT, but no change in AGPAT specific activity. Thus, *Agpat6* has been misidentified and actually encodes an NEM-sensitive GPAT activity. As the fourth described GPAT, we have renamed it GPAT4. Further, since the *Agpat6*$^{-/-}$ mice are deficient in GPAT and not in AGPAT activity, they should be called *Gpat4*$^{-/-}$ mice.

Gpat4 mRNA is highly expressed in liver [35] and accounts for almost 50% of the total GPAT activity in wild-type mouse liver, as demonstrated by the 49% reduction in *Gpat4*$^{-/-}$ mice. This loss in hepatic GPAT activity may be responsible for the 40-50% decrease in liver TAG reported for *Gpat4*$^{-/-}$ mice [4]. GPAT4 represented 65% of liver

NEM-sensitive activity GPAT specific activity, suggesting that GPAT4 is the major NEM-sensitive isoform in liver, but that one or more additional NEM-sensitive GPAT isoforms is also present. To determine whether the mRNA expression of other GPAT isoforms increased in *Gpat4*$^{-/-}$ mouse liver to compensate for the loss of GPAT4, we measured the expression of *Gpat1*, *2*, and *3*. Neither *Gpat2* nor *Gpat3* mRNA expression was elevated in liver from the *Gpat4*$^{-/-}$ mice, but *Gpat1* mRNA was elevated 2-fold, although this increase did not correspond to an increase in NEM-resistant (GPAT1) GPAT specific activity. Gene expression does not invariably correspond to protein expression, and GPAT1 enzyme activity may be regulated by posttranslational mechanisms [32]. We previously reported that the mRNAs for *Agpat1-5, 7,* and *8* did not change in BAT from the *Gpat4*$^{-/-}$ mice [4].

GPAT4 also appears to be the primary NEM-sensitive GPAT in BAT, as 65% of the NEM-sensitive GPAT activity was lost in BAT from male *Gpat4*$^{-/-}$ mice. NEM-resistant activity in *Gpat4*$^{-/-}$ BAT was unaltered, and none of the known GPAT isoforms was up regulated at the transcriptional level to compensate for the loss of GPAT4. Interestingly, total and NEM-sensitive GPAT activities were not reduced in gonadal WAT from *Gpat4*$^{-/-}$ mice, despite the previous demonstration that the TAG content of WAT was reduced in chow-fed male *Gpat4*$^{-/-}$ mice [4]. It may be that additional, unknown GPAT isoforms are responsible for activity in white adipose tissue, or that a compensatory increase in activity of known GPATs occurs at a post-transcriptional level. It is also possible that differences exist in GPAT4 expression and activity in WAT of male and female mice, as *Gpat4* mRNA expression is lower in parametrial fat than in epididymal fat [4].

Analysis of GPAT assay products from transfected Cos-7 cells showed that LPA was elevated 2-fold as a result of GPAT4 overexpression, consistent with a GPAT activity. Labeled PA was also increased 70% in samples from GPAT4-transfected cells, indicating that the LPA produced by GPAT4 can be used as a substrate for down-stream AGPAT enzymes in *de novo* glycerolipid synthesis. The lack of a specific increase in PA only in samples from cells transfected with GPAT4 *vs*. vector is inconsistent with elevated AGPAT activity. Subsequent formation of DAG did not occur, probably because the NaF in the assay mixture inhibits phosphatases and because lipin (phosphatidic acid phosphohydrolase) is primarily resident in the cytosol and would therefore be largely absent in the membrane preparation.

The identification of two novel NEM-sensitive GPAT isoforms, GPAT3 [34] and GPAT4 (this paper), with different tissue specific expression patterns has revealed the complexity of *de novo* glycerolipid synthesis regulation. More studies are needed to fully characterize the roles of GPAT3 and GPAT4 in liver and adipose tissue triacylglycerol synthesis, the mechanisms by which these isoforms are regulated during different physiologic states, and the effects of their glycerolipid synthetic products on downstream signaling pathways [208]. Importantly, it is unknown whether additional GPAT isoforms remain to be identified. The so-called AGPATs 3, 4, 5, and 7 might also actually be GPAT isoforms. Further studies are needed to confirm the specific acyltransferase activities of these enzymes.

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CHAPTER V SYNTHESIS

This dissertation describes two independent projects that examine the roles of GPAT isoforms in *de novo* triacylglycerol synthesis. The results of these studies confirm the importance of GPAT isoforms in the regulation of TAG synthesis, and also implicate glycerolipid intermediates as important lipid signaling molecules involved in the regulation of insulin signaling pathways. This chapter will discuss the key findings from these two projects and the future implications of these findings for the understanding and treatment of type 2 diabetes.

5.1 Increased hepatic *de novo* glycerolipid synthesis causes insulin resistance

Hepatic TAG accumulation has been associated with insulin resistance, but it is not clear whether insulin resistance causes fatty liver or whether fatty liver causes insulin resistance. Most models of insulin resistance are not appropriate for isolating the role of liver TAG accumulation in the development of insulin resistance because high-fat feeding and genetic manipulation of insulin or leptin signaling pathways affect multiple tissues. To determine whether a liver-specific increase in TAG synthesis could cause hepatic and systemic insulin resistance, we overexpressed GPAT1 in rat liver with an adenoviral expression vector. Overexpression of GPAT1 increased hepatic LPA, DAG, and TAG content 2-fold above Ad-GFP control liver lipid levels. This increase in liver glycerolipid

mass was associated with hepatic and systemic insulin resistance in Ad-GPAT1-treated rats. Ad-GPAT-1-treatment did not increase GPAT1 expression in adipose tissue, and Ad-GPAT1 and Ad-EGFP-treated rats did not differ in body weight or fat pad mass, indicating that the primary disturbance in lipid metabolism occurred in liver, not in adipose tissue. Elevated plasma VLDL-TAG levels in Ad-GPAT1-treated rats may have contributed to elevated skeletal muscle TAG content and, subsequently, to the decrease in insulin-mediated glucose uptake by skeletal muscle. PKCε translocation to the plasma membrane was elevated in liver from Ad-GPAT1-treated rats, implicating DAG-mediated activation of PKCε in the mechanism of hepatic insulin resistance caused by GPAT1 overexpression. Immune mediators of insulin resistance, such as NFκB, IL-6, and TNFα, were not elevated in plasma or liver from Ad-GPAT1-treated rats, suggesting that the mechanism of insulin resistance was not immune-mediated.

The most important finding from this study was that systemic insulin resistance can develop when hepatic *de novo* glycerolipid synthesis is elevated, suggesting that hepatic lipid accumulation and the subsequent development of hepatic insulin resistance could initiate the development of systemic insulin resistance. The second most important finding of this study is that a glycerolipid metabolite plays an important role in the mechanism of hepatic insulin resistance. Our finding that hepatic insulin resistance was associated with elevated hepatic DAG content and increased PKCε translocation supports earlier findings in GPAT1$^{-/-}$ mice that increased hepatic insulin sensitivity is associated with decreased hepatic DAG content and decreased PKCε translocation (Neschen, 2005). These results implicate the DAG pool

generated during *de novo* glycerolipid synthesis as a key player in the lipid-mediated mechanism of hepatic insulin resistance.

Our study did not, however, explore the specific actions of PKCε that lead to inhibition of the insulin signaling pathway, nor did it prove that DAG accumulation and PKCε translocation are necessary for the mechanism of hepatic insulin resistance in Ad-GPAT-treated rats. Other studies have associated increased DAG accumulation and PKCε translocation, with reductions in IRS-1/2 associated PI3K activity and Akt2 activity, but these are only associations (Neschen, 2005; Samuel, 2003). One way to show that PKCε activation is necessary for GPAT1-mediated hepatic insulin resistance would be to simultaneously treat rats with adenoviruses for GPAT1 and for a shRNA against PKCε. If knock-down of PKCε could prevent the hepatic insulin resistance caused by GPAT1 overexpression, then we would know that PKCε activation is involved in the inhibition of insulin signaling. A similar experiment has been done in rats fed high-fat safflower oil diets in which knock-down of PKCε prevents diet-induced hepatic insulin resistance [129]. In this study, a reduction in PKCε prevents diet-induced inactivation of insulin signaling through IRS-2, and evidence for a direct interaction between PKCε and the incsulin receptor are presented. Taken together, these studies strongly suggest that accumulation of hepatic DAG activates PKCε, which inhibits insulin receptor signaling by preventing the activation of IRS-2.

In addition to confirming the role of PKCε in the mechanism of hepatic insulin resistance in Ad-GPAT1-treated rats, it is also important to confirm that DAG derived from

de novo glycerolipid synthesis is responsible for activation of PKCε and subsequent inactivation of insulin signaling. DAG can also be synthesized from membrane phospholipids by PLC and PLD, and label incorporation into PC was 30% reduced in CHO cells that stably overexpressed GPAT1 [38]. Increased PLC or PLD activity could explain this reduction in PC labeling with GPAT1 overexpression, and the DAG produced from these phospholipase reactions could contribute to the DAG that accumulates in hepatocytes. Could overexpression of GPAT1 cause increased PLC and/or PLD activity, leading to increased DAG that could be used for intracellular signaling, such as activation of PKCε? To determine whether DAG accumulation is involved in the mechanism of hepatic insulin resistance and to determine which DAG synthesis pathway is involved, I would treat rats simultaneously with adenoviruses expressing GPAT1 and either shRNA against one of the phosphatidic acid phosphohydrolases, or shRNA against PLC or PLD. These experiments would determine which pools of DAG might be involved in the mechanism of insulin resistance in Ad-GPAT-1 treated rats. If DAG is involved, then knock-down of one or more of the enzymes that synthesizes DAG should reduce that DAG pool and prevent Ad-GPAT1-treated rats from developing hepatic insulin resistance.

Recently, another research group has attempted to confirm that TAG is not the lipid mediator responsible for hepatic insulin resistance. These investigators treated high-fat safflower oil fed rats with antisense oligonucleotides (ASO) to DGAT1 or DGAT2, the enzymes which convert DAG to TAG [209]. The authors hypothesized that a reduction in hepatic TAG content due to knock-down of DGAT would fail to protect rats from diet-induced hepatic insulin resistance because DAG content would increase in rats treated with

DGAT ASOs. As expected, DGAT1 ASO treatment did not protect rats from high-fat-diet induced insulin resistance. However, knock-down of DGAT1 did not affect hepatic TAG content, despite a significant decrease in hepatic DGAT1 activity. DGAT1 is not the predominant DGAT isoform in liver, which may explain why knock-down of DGAT-1 did not alter the hepatic TAG content. Surprisingly, however, rats treated with DGAT2 ASO were protected from diet-induced insulin resistance, and hepatic DAG content was reduced, not increased. This reduction in hepatic DAG was associated with reduced PKCε translocation and increased IRS-2 associated PI3K activity. These results support the hypothesis that hepatic DAG is involved in the mechanism of hepatic insulin resistance, but because both hepatic DAG and TAG content were reduced, they do not rule out TAG as a lipid mediator of insulin resistance. Additionally, this study is complicated by simultaneous knock-down of DGAT2 in both liver and adipose tissue, and the authors did not adequately explore the role of DGAT2 knock-down in adipose tissue on the protective effects of the ASO treatment. Further studies are needed to determine conclusively whether DAG is an important lipid mediator of hepatic insulin resistance.

In addition to DAG, LPA and PA produced during *de novo* triacylglycerol synthesis could act as lipid mediators of insulin resistance. Both PA and LPA are known to have important functions in intracellular signaling pathways, and LPA does accumulate in the livers of Ad-GPAT1-treated rats. Although the literature does not report a mechanism for LPA-mediated insulin resistance, it is possible that LPA signaling could be involved. Simultaneous adenovirus-mediated overexpression of GPAT1 and AGPAT1 or 2 might be expected to reduce the LPA accumulation seen in liver from Ad-GPAT1-treated rats. If

reducing hepatic LPA protected Ad-GPAT1-treated rats from hepatic insulin resistance, then one would conclude that LPA must be involved in the mechanism of hepatic insulin resistance. Alternately, if reducing hepatic LPA content did not protect Ad-GPAT1-treated rats from developing hepatic insulin resistance, then glycerolipid intermediates such as PA or DAG may be involved. Adenovirus-mediated knock-down and overexpression of AGPAT isoforms might shed light on the role of PA as a lipid mediator of insulin resistance. Studies like these are needed to tease-out the roles of individual glycerolipid metabolites in the mechanism of hepatic insulin resistance.

5.2 Sphingolipids and Insulin Resistance in Ad-GPAT1-treated Rats and GPAT1$^{-/-}$ Mice

One of the weaknesses of our proposed mechanism for insulin resistance in Ad-GPAT1-treated rats is that we did not show a definite cause-effect relationship between the accumulation of DAG and activation of PKCε or between activation of PKCε and insulin resistance. Although preventing the activation of PKCε in rats fed a high safflower oil diet (primarily linoleate) prevented the development of hepatic insulin resistance (Samuel, 2007), there is still no definitive proof that DAG accumulation activates PKCε. Another lipid that activates PKC isoforms is ceramide, and recent bioinformatics data associate ceramide and DAG with hepatic steatosis and insulin resistance in *ob/ob* mice [210]. Thus, ceramide may be an important mediator of hepatic insulin resistance.

Ceramide can be synthesized from palmitoyl-CoA and serine by serine palmitoyl transferase (SPT) and ceramide synthase, and from sphingomyelin by sphingomyelinase (SMAse) at several intracellular locations (Figure 2.5). The regulation of *de novo* ceramide

synthesis appears to depend largely on the availability of palmitoyl-CoA, and inhibiting CPT-1 increases ceramide production in the presence of palmitoyl-CoA [138, 211]. Increased TAG synthesis and storage has also been associated with increased ceramide production [136]. Because GPAT1 has a substrate preference for palmitoyl-CoA and because overexpression of GPAT1 in primary hepatocytes results in a reduction in beta-oxidation, it is possible that over-expression of GPAT1 could increase *de novo* ceramide production in hepatocytes. Decreased oxidation of palmitoyl-CoA due to increased production of malonyl-CoA during *de novo* lipogenesis might result in increased synthesis of TAG and ceramide, leading to insulin resistance (Figure 2.6). On the other hand, overexpression of GPAT1 could reduce the palmitoyl-CoA available for *de novo* ceramide synthesis and prevent the accumulation of ceramide (Figure 2.7). If ceramide accumulation is altered in liver from Ad-GPAT1-treated rats, then DAG accumulation may not be the only lipid causing insulin resistance in this model.

Recently, an important publication in *Cell Metabolism* demonstrated that intravenous infusion of a soybean-oil-based diet (primarily linoleate) causes insulin resistance that is associated with increased hepatic DAG accumulation, whereas intravenous infusion of a lard-oil diet (primarily palmitate and stearate) causes insulin resistance that is associated with hepatic increases in ceramide and DAG [104]. Furthermore, hepatic insulin resistance was prevented by myriocin, an inhibitor of serine palmitoyl transferase (SPT), in the lard-oil group but not in the soybean oil group [104]. This protective effect of myriocin was attributed to a reduction in hepatic ceramide content only in the lard oil group. DAG content was not affected by myriocin in either diet group.

As I explained in section 2.9, the affects of GPAT1 deficiency on ceramide and DAG accumulation may explain why GPAT1$^{-/-}$ mice fed a high-fat safflower oil diet were protected from insulin resistance, whereas GPAT1$^{-/-}$ mice fed a high-saturated/high-sucrose diet were more insulin resistant than their wild-type controls. The following statements are a review of that hypothesis. If one assumes that in mice fed the high-fat safflower oil diet, the mechanism of insulin resistance is primarily mediated by DAG, then reducing DAG would protect these mice from developing insulin resistance. Thus, GPAT1$^{-/-}$ mice fed the high-fat safflower oil diet were protected from insulin resistance because they had less hepatic DAG accumulation and less PKCε activation. [14]. However, GPAT1$^{-/-}$ mice fed the high fat/high sucrose diet (HH) were not protected from hepatic insulin resistance because this diet provided palmitate (40 g/ 5777 kg) from the high fat content and from *de novo* lipogenesis (increased by the high sucrose diet). In fact, GPAT$^{-/-}$ mice were more insulin resistant than their wild-type controls, perhaps because palmitate was more readily available for ceramide synthesis. Increased ceramide synthesis might occur for several reasons. First, the use of palmitoyl-CoA for glyceorlipid synthesis was reduced due to knock-out of GPAT1. Secondly, the enzymes that perform β-oxidation were inundated by fatty acids from the high-fat diet, and CPT1 was inhibited by elevated *de novo* lipogenesis, thus blocking the oxidation of palmitate. To determine whether these ideas are correct, we would have to measure hepatic ceramide content in GPAT1$^{-/-}$ mice fed either the high-fat safflower oil diet or the HH diet. If ceramide is elevated in livers from GPAT1$^{-/-}$ mice on the HH diet, we could inhibit ceramide synthesis with myriocin or an ASO against SPT to determine whether reducing ceramide would restore insulin sensitivity.

With the exception of our Ad-GPAT1 study, all of the studies that have suggested a DAG-PKCε or a DAG-PKCδ mechanism for the development of hepatic insulin resistance have used a linoleate-based diet to cause insulin resistance, [14, 119, 120, 129, 209]. Thus, the proposed role of PKCε and PKCδ in the development of high-fat-diet-induced hepatic insulin resistance may only be relevant to diets high in linoleate or other polyunsaturated fatty acids. Because humans eat diets high in both saturated and polyunsaturated fatty acids, models of diet-induced insulin resistance that are high in one type of fatty acid may not be relevant to human insulin resistance. Further studies are needed to determine whether PKCε is required for the development of insulin resistance in other models of insulin resistance. ASO-mediated knock-down of PKCε in *ob/ob* mice and in mice fed diets in lard or high in sucrose might answer this question.

5.3 De novo glycerolipid synthesis and modulation of the hepatic acute immune response

Although many recent studies of hepatic and systemic insulin resistance have implicated the activation of the acute immune response as an important step in the mechanism of insulin resistance, we did not find any evidence for an elevated immune response in Ad-GPAT1-treated rats. In fact, we found that GPAT1 overexpression consistently suppresses NFκB activity. NFκB activity was suppressed in primary hepatocytes treated with Ad-GPAT1, and the stable expression of GPAT1 in CHO cells suppressed the activity of the NFκB luciferase reporter (unpublished data, C.A.N and Cliona Stapelton). These experiments suggest that changes in GPAT1 activity can modulate the

binding of NFκB to its DNA binding sequence. One explanation for this observation is that overexpression of GPAT1 reduces reactive oxygen species (ROS) produced as a byproduct of β-oxidation. NFκB is re-dox sensitive, and can be activated by oxidative stress. Because livers from GPAT1$^{-/-}$ mice have increased markers of oxidative stress, it is plausible that reduced β-oxidation and ROS production in the livers of Ad-GPAT1-treated rats reduces normal levels of active NFκB [178]. Another possible explanation for the GPAT1-mediated reduction in NFκB activity is that LPA produced by GPAT activates PPARγ, which can inhibit NFκB activity. It is well known that activation of PPARγ can inhibit NFκB activity [17], and LPA may be an endogenous ligand for PPARγ [74]. Additionally, stable overexpression of GPAT1 in CHO cells activated PPARγ DNA binding to a PPRE luciferase reporter construct (unpublished data, Cliona Stapleton). Additional experiments designed to examine the role of GPAT1-derived LPA-mediated activation of PPARγ would clarify the mechanism of reduced NFκB activity observed when GPAT1 activity is elevated. These studies might include overexpression of GPAT1 in PPARγ deficient hepatocytes. If GPAT1 overexpression fails to suppress NFκB activity in primary hepatocyes that lack PPARγ, then the suppression must occur via a PPARγ-mediated mechanism.

5.4 The Future of Insulin Resistance Research – Putting it All Together

In the past several years there have been numerous reports of “essential” inflammatory and lipid mediators of insulin resistance. Knock-down studies have implicated NFκB, TNFα, IL-6, DAG, and ceramide as "essential" mediators of insulin resistance in high-fat fed or genetically insulin resistant animals, but how can all of these mediators be essential? Is there one silver bullet in a long sequence of events that lead to systemic insulin

resistance? Where does this sequence to insulin resistance begin, and what is the final step? Is the sequence really linear, or is it somewhat circular? Future studies that can answer questions about this sequence of events are needed so that the most effective pharmalogical treatments and preventative strategies can be developed.

5.5 GPAT3 (AGPAT6) is the hepatic erGPAT isoform

AGPAT6 is a novel acyltransferase that was classified as an AGPAT based on amino acid sequence similarity to AGPAT1 and AGPAT2. However, AGPAT6 also shares sequence homology with GPAT isoforms at the same conserved acyltransferase motifs, and no specific acyltransferase activity for AGPAT6 was measured [4]. AGPAT6 $^{-/-}$ mice are deficient in TAG in liver and adipose tissue, a phenotype that could be explained by a deficiency in either GPAT or AGPAT activity. The recent discovery that GPAT3 had been misclassified as AGPAT8, and the 66% amino acid sequence similarity of GPAT3 and AGPAT6 prompted us to measure both AGPAT and GPAT activities in AGPAT6$^{-/-}$ mouse tissues. We found that NEM sensitive GPAT activity was decreased 65% in liver and brown adipose tissue from AGPAT6$^{-/-}$ mice. AGPAT activity did not differ in those tissues. To confirm that AGPAT6 catalyzes GPAT activity, we overexpressed AGPAT6 in Cos-7 cells. Cos-7 cells transfected with AGPAT6 had increased NEM-sensitive GPAT activity, and AGPAT activity did not differ in total membrane preparations from AGPAT6 and vector control transfected cells. Thus, we have identified a novel NEM-sensitive GPAT, GPAT4, located in the endoplasmic reticulum of liver and brown adipose tissue. Importantly, identifying the primary erGPAT isoform in liver allows further study of the regulation of

hepatic *de novo* glycerolipid synthesis that was not possible before GPAT4 was cloned and identified.

5.6 Functional role of GPAT4 in hepatocytes:

Now that there are 4 known GPAT isoforms, there is a lot of work to be done in characterizing their individual roles in regulating glycerolipid metabolism in different tissues and under different physiologic conditions. In particular, GPAT4 is the primary erGPAT isoform in liver, and its activity accounts for ~33 % of total liver GPAT activity. How does the role of GPAT4 differ from that of GPAT1? One way to begin to answer this question is to examine the incorporation of labeled fatty acids into glycerolipids in hepatocytes that either overexpress or are deficient in GPAT4. These experiments would tell us what proportion of labeled fatty acid is incorporated into PL, CE, and TAG. It would also be interesting to determine whether altering GPAT4 activity would cause reciprocal regulation of fatty acid oxidation similar to that seen with changes in GPAT1 activity. Because GPAT4 is located on the ER and not on mitochondria, I would suspect that overexpression of GPAT4 would not decrease β-oxidation in the same way as overexpression of GPAT1 does. Because the role of GPAT4 in TAG synthesis might be different under different nutritional conditions, it would be interesting to determine what happens to fatty acid label incorporation under conditions of high and low media fatty acid content, high and low glucose content, and combinations of high/low glucose and high and low/fatty acids.

5.7 Transcriptional regulation of GPAT4 by hormones:

One of the important questions that can be addressed now that GPAT4 has been identified is how it is transcriptionally regulated in liver and other tissues. GPAT1 expression and activity are regulated by insulin, suggesting both transcriptional and post-translational regulatory mechanisms. Insulin, however, does not appear to regulate GPAT4 in the same manner as GPAT1, since hepatic NEM-sensitive activity does not change in rats that are fasted and re-fed [29]**.** However, regulation of GPAT4 activity by insulin could be hidden by reciprocal changes in the activity of other NEM-sensitive GPAT isoforms present in liver, such as GPAT3.

If GPAT4 is not regulated by nutritional status, then how is it regulated? The fact that GPAT4$^{-/-}$ mice were unable to produce enough lipid for proper lactation suggests that GPAT4 is necessary for milk production. During pregnancy, estrogen and progesterone stimulate the breasts to lay down fat in preparation for milk production, and prolactin and growth hormone stimulate the proliferation and differentiation of mammary epithelial cells through activation of STAT 5 [212]. Thus, GPAT4 may be transcriptionally regulated by estrogen, progesterone, prolactin, or growth hormone. To determine whether one of these hormones regulates GPAT4 expression in hepatocytes, I would measure GPAT4 mRNA expression and activity in untreated and hormone treated primary rat hepatocytes.

Another possible transcriptional activator of GPAT4 might be PPARγ, which is known to activate a large number of lipogenic genes, especially those related to adipocyte differentiation. Since GPAT4 is expressed in adipose tissue, and its expression increases

during early pre-adipocyte differentiation (unpublished data L. Vergnes), it is possible that it is regulated by PPARγ. Even though PPARγ does not play a primary role in regulating hepatic lipogenic gene expression under normal physiologic conditions, PPARγ is up regulated in many animal models of fatty liver (Schadinger, 2005), and PPARγ agonists, which are commonly used to treat diabetes, can cause an increase in hepatic lipid accumulation [213]. It would be interesting to know whether GPAT4 is partially responsible for the increase in hepatic lipids caused by PPARγ agonists. To test this hypothesis, I would treat primary hepatocytes with a PPARγ agonist, such as rosiglitizone, and measure GPAT4 mRNA expression in untreated and treated cells. A better understanding of the regulation of GPAT4 in hepatocytes would improve our knowledge of hepatic glycerolipid synthesis during different physiologic conditions.

5.7 Does GPAT4 modulate insulin sensitivity?

Hepatic overexpression of GPAT1 causes insulin resistance, possibly through elevation in hepatic LPA or DAG content. Although selective knockout of either GPAT1 or GPAT4 reduces hepatic TAG content, suggesting that they both regulate TAG synthesis, it is not clear whether the two isoforms channel glycerolipid products into separate functional pools. This idea is particularly important for LPA, PA, or DAG, which may have functions in intracellular signaling pathways. If GPAT1 and GPAT4 synthesize different pools of DAG, then it is possible that one pool would affect PKC activation and insulin signaling and the other pool would not. To address those questions, future studies could investigate the effects of knockdown or overexpression of GPAT4 in liver on hepatic and systemic insulin

sensitivity in relation to the amount of LPC, PA, and DAG that accumulate in the liver tissue under those conditions.

5.8 Public Health Significance

The prevalence of obesity and its co morbidities, including nonalcoholic fatty liver disease, cardiovascular disease and type 2 diabetes, are increasing at a rapid rate in the US and world-wide, affecting both adult and child populations. These chronic diseases are characterized by disturbances in the normal regulation of lipid and carbohydrate metabolism. Thus, a solid understanding of the regulation of lipid metabolism is vital to our ability to treat and prevent chronic disease. GPAT and AGPAT isoforms are important enzymes that regulate glycerolipid metabolism and intracellular signaling by glycerolipid metabolites. Work reported in this dissertation has improved our knowledge of the role of GPAT1 in regulating diacylglyerol synthesis for intracellular signaling pathways that can inhibit insulin signaling and cause hepatic insulin resistance. Additionally, increased hepatic GPAT1 activity caused hyperlipidemia, which is a risk factor for cardiovascular disease. Further studies might explore the possibility of developing a GPAT1 inhibitor to treat type 2 diabetes and hyperlipidemia. GPAT3, which was recently identified as an important regulator of TAG synthesis in white adipose tissue, may also affect insulin sensitivity in adipose tissue and the development of obesity. Further studies might explore the possibility of developing GPAT3 specific inhibitors as treatments for obesity and diabetes.

In addition to their role in energy regulation, GPAT isoforms are important for the regulation of glycerolipid synthesis in reproductive tissues, such as mammary tissue and

testis. GPAT4$^{-/-}$ mice are unable to make enough lipid-rich milk to feed their pups, indicating a vital role for GPAT4 in milk production. Additionally, GPAT4$^{-/-}$ mice had fewer and smaller alveoli than wild-type mice, suggesting that glycerolipid signaling could be involved in the proliferation of mammary epithelia. Further studies might explore the role of aberrant GPAT4 activity in the proliferation and differentiation of mammary epithelium and the development of mammary epithelial tumors.

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