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Glycosylphosphatidylinositol-Specific Phospholipase D in Nonalcoholic Fatty Liver Disease: A Preliminary Study

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Context: Recent studies demonstrated that *de novo* lipogenesis is increased in patients with nonalcoholic fatty liver disease (NAFLD). Patients with NAFLD also have plasma lipid abnormalities. These lipid abnormalities may in part be related to insulin resistance, which is common in patients with NAFLD. Insulin resistance is associated with alterations in proteins involved in lipid metabolism including glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD), which is involved in triglyceride metabolism.

Objective: The objective of the study was to determine whether alterations in serum and hepatic levels of GPI-PLD occur in patients with NAFLD.

Design and Patients: We examined the following: 1) levels of serum GPI-PLD in nondiabetics with nonalcoholic steatohepatitis, compared with matched controls; 2) hepatic expression of GPI-PLD mRNA in patients with normal liver or NAFLD; and 3) effect of overexpressing GPI-PLD *vs.* β-galactosidase (control) on global gene expression in a human hepatoma cell line.

Results: The serum levels of GPI-PLD were significantly higher in patients with nonalcoholic steatohepatitis than in matched controls (119 ± 24 *vs.*105 ± 15 μg/ml, $P = 0.047$). The hepatic expression of GPI-PLD mRNA was increased nearly 3-fold in NAFLD patients, compared with patients with normal liver (3.1 ± 2.6 *vs.* 1.1 ± 1.0 arbitrary units per microgram total RNA, $P = 0.026$). Finally, overexpressing GPI-PLD was associated with an increase in *de novo* lipogenesis genes.

Conclusions: Patients with NAFLD have elevated serum levels and hepatic expression of GPI-PLD, and its overexpression *in vitro* is associated with increased expression of *de novo* lipogenesis genes. These results suggest that GPI-PLD may play a role in the pathogenesis of NAFLD and/or its metabolic features and warrants further investigation. **(*J Clin Endocrinol Metab* 91: 2279–2285, 2006)**

NONALCOHOLIC FATTY LIVER disease (NAFLD) is the leading cause of abnormal liver functions in Western society. Histologically, NAFLD can range from fatty liver to nonalcoholic steatohepatitis (NASH) to cirrhosis and is associated with altered lipid metabolism both in the liver and plasma compartment (1, 2). Hepatic *de novo* lipogenesis is significantly increased in patients with NAFLD (3). Patients with NAFLD also have elevations in serum triglycerides and lower levels of high-density lipoproteins. The etiology of these lipid abnormalities may in part be related to insulin resistance, which is common in patients with NAFLD (4). Insulin resistance is associated with alterations in proteins involved in lipid metabolism including glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) (5).

GPI-PLD is an 815-amino acid protein expressed in numerous tissues and cells (6, 7). Liver has the highest level of GPI-PLD expression and is the primary organ contributing to GPI-PLD in the serum. GPI-PLD is abundant in serum (approximately 100 μg protein per milliliter) in which it associates with apolipoproteins AI and AIV (8–10). Increased serum GPI-PLD is associated with insulin resistance and elevated serum triglycerides (5), and overexpressing hepatic GPI-PLD in mice is associated with increases in fasting and postprandial triglycerides (11). This effect of GPI-PLD is mediated, at least in part, by reducing the catabolism of triglyceride-rich lipoproteins. Because patients with NAFLD frequently have insulin resistance and fasting and postprandial hypertriglyceridemia (4, 12), we hypothesized that subjects with NAFLD may have abnormal hepatic and serum levels of GPI-PLD as a contributor to the altered intra- and extrahepatic lipid metabolism.

To characterize GPI-PLD in patients with NAFLD, we conducted a study with three aims. First, we compared the serum levels of GPI-PLD in nondiabetic patients with NASH to age, gender, body mass index (BMI), and body fat-matched controls. Second, we measured the hepatic expression of GPI-PLD mRNA in patients with simple fatty liver and NASH, compared with normal hepatic histology. Third, to determine whether GPI-PLD may play a role in hepatic lipid metabolism, we examined the effect of overexpressing GPI-PLD *vs.* β-galactosidase (control) on global gene expression in a human hepatoma cell line (HepG2). We found that serum levels and hepatic GPI-PLD mRNA levels are significantly higher in patients with NAFLD. In addition, overexpression of GPI-PLD in HepG2 cells is associated with increases in *de novo* lipogenesis genes, suggesting that GPI-PLD

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Abbreviations: ACC, Acetyl coenzyme A carboxylase; ACLY, ATP citrate lyase; AdGPI-PLD, adenovirus expressing GPI-PLD; BMI, body mass index; DGAT, diacylglycerol acyltransferase; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; HADH, L-3-hydroxyacyl-coenzyme A dehydrogenase; HOMA, homeostasis model assessment; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SCD, stearoyl coenzyme-A desaturase; SREBP, sterol-response element binding protein.

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may have a role in the altered fatty acid and lipid metabolism in NAFLD.

Patients and Methods

Aim 1

Serum GPI-PLD levels were measured in 21 nondiabetic individuals with biopsy-proven NASH and 19 age-, gender-, BMI-, and body fat-matched nondiabetic individuals without liver disease. This cohort was studied previously, and it served as the basis for several papers related to NASH published by our group (13–17). These studies were conducted after these publications and in accordance with the guidelines in the Declaration of Helsinki. All patients provided informed consent to participate in these studies, all of which were approved by the Institutional Review Board and the Advisory Committee for the General Clinical Research Center of Indiana University School of Medicine. Fasting serum GPI-PLD levels were measured by ELISA from stored serum specimens (−80 C).

The details of clinical and histological criteria used to characterize the subjects with NASH and the controls in this cohort have been described previously (13–17). The anthropometric measurements available on this cohort of patients included height, weight, waist to hip ratio, body composition (measured by BODPOD method), and abdominal fat measurements (measured by single-slice computed tomography abdomen). Serum studies available for this analysis included liver biochemistries, fasting serum lipids, oxidized low-density lipoprotein (LDL), insulin, glucose, free fatty acids, leptin, and adiponectin. Insulin resistance was assessed by the homeostasis model assessment (HOMA) method, and individuals with HOMA greater than 3.5 were considered to have insulin resistance (18). Insulin resistance measured by HOMA correlates closely with euglycemic clamp techniques within our patient population, and in a previous study from this institution, HOMA greater than 3.5 was an accurate indicator of insulin resistance (18). The assay methods for these serum studies are described in previous publications (13–17).

Aim 2

The GPI-PLD mRNA was measured from the cell lysate of liver biopsy material of 13 subjects with NASH, 12 subjects with nonalcoholic steatosis, and 12 subjects with normal liver tissue (who had liver biopsies performed as part of donor evaluation for living related liver transplantation). These liver samples were obtained in the fasting state and collected as part of an institutional review board-approved protocol that allowed us to maintain a liver tissue bank. The majority of these liver samples were also used previously (13, 17). Serum samples were not available from this cohort. These subjects were not part of the cohort studied to address our first aim.

Total RNA was extracted and isolated from the core biopsy specimen using a RNeasy minikit (QIAGEN, Valencia, CA). DNase treatment was done by using DNA-free (Ambion, Austin, TX) to remove contaminating DNA in the extracted total RNA. Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) combined with the RNA 6000 Nano LabChip kit (Caliper Technologies Corp., Hopkinton, MA) was used to analyze the quality (28S to 18S ratio) and quantity of total RNA. RNA was stored at −80 C until used.

TaqMan real-time quantitative RT-PCR was used to quantitate GPI-PLD mRNA. The following sets of the primers and probes were designed using the Primer Express software (Applied Biosystems, Foster City, CA) to function according to the Taqman technology. GPI-PLD-specific primers and probes used for QPCR were: HumGPI-PLD forward, 5′-CCCAGCCTGAGCAACAAAG-3′, HumGPI-PLD reverse, 5′-TTC-CTCGCCTCTCACCGTC-3′, and HumGPI-PLD probe, 5′-FAM-AAACTGAACGTGGAGGCGGCCAAC-TEMRA-3′ synthesized from Integrated DNA Technologies (Coralville, IA). The amplicon was 720 bp and was verified by agarose gel electrophoresis. The RT-PCRs were performed in a Mx4000 multiplex quantitative PCR system (Stratagene, La Jolla, CA) using Brilliant Plus single-step quantitative RT-PCR core reagent kit (Stratagene) in a total reaction volume of 25 μl. The reaction contained 5 U StrataScript reverse transcriptase, 1.25 U SureStart *Taq* DNA polymerase, 0.2 mM GAUC mix, 5 mM $MgCl_2$, and 0.3 μM of each primer and probe for GPI-PLD. dNTP cycling conditions were as follows: 1 cycle for 90 min at 45 C, 1 cycle for 10 min at 95 C followed by 40 cycles of 15 sec denaturation at 95 C, and a 1-min annealing/extension at 55 C. Standard curves were generated for GPI-PLD from serial dilutions of human fetal liver total RNA (BD Biosciences CLONTECH, San Diego, CA). Unknown sample values were determined from standard curves, and data were expressed as arbitrary units normalized to total RNA as determined by Ribogreen (Molecular Probes, Portland, OR).

Aim 3

HepG2 cells, a human hepatoma cell line, were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM (GIBCO-BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) and maintained at 37 C in a humidified atmosphere (5% CO_2). For experiments, HepG2 cells were seeded in 100-mm culture plates (2.2×10^6 cells/plate) 2 d before viral transduction. Cells were transduced with recombinant adenovirus AdCMVGPI-PLD (AdGPI-PLD) or AdCMV-β-galactosidase (AdLacZ) (control) (19) with a multiplicity of infection of 0.56. Five hours after the addition of the virus, the media were switched to low-glucose DMEM supplemented with fatty-acid free BSA (1 mg/ml). The cells were harvested 6 and 12 h after viral transduction, and the total RNA was extracted using TriPure (Roche Diagnostics, Indianapolis, IN) followed by further purification with Absolutely RNA miniprep kit (Stratagene) and stored at −80 C until used for microarray analysis or Northern blotting. Four independent culture plates were used for each condition for a total of 16 microarrays.

The microarray procedure was done by the Indiana University Center for Medical Genomics following standard Affymetrix protocols (Affymetrix GeneChip expression analysis technical manual; Affymetrix, Santa Clara, CA). cRNA was hybridized to human genome U133A GeneChips for 17 h, followed by standard washing, staining, and scanning. Data were extracted using Affymetrix MicroArray Suite 5.0 software (MAS5; Affymetrix MicroArray Suite 5.0 user's guide, Santa Clara, CA). To confirm microarray observations, Northern blot analysis was performed on diacylglycerol acyltransferase (DGAT) 1 and DGAT2.

Northern blot analysis was performed to identify mRNA specific to DGAT1 and DGAT2. DGAT1- and DGAT2-specific probes were generated by PCR amplification from human liver QUICK-clone cDNA (BD Biosciences, San Diego, CA) using gene-specific primers. The primers used were: DGAT1 forward, 5′-CTTTCTGCTGCGACGGATCCTT-GAGATGCT-3′; DGAT1 reverse, 5′-CTATTGGCTGTCCGATGAT-GAGCGACAGC-3′; DGAT2 forward, 5′-AGAGGCCACAGAAGT-GAGCAAGAAG-3′; and DGAT2 reverse, 5′-CCCCAGGTGTCGGAGG-AGAAGAGGCCTCGACCA-3′. The PCRs were performed in a total volume of 50 μl containing 250 nM each of dCTP, dGTP, dATP, and dTTP, 250 nM forward and reverse primers and Advantage cDNA polymerase mix (BD Biosciences CLONTECH). Cycling conditions were as follows: 1 cycle of 5 min at 94 C for initial denaturation followed by 30 cycles of 1 min denaturation at 94 C, 1 min annealing at 58 C, and 2 min of extension at 72 C. PCR products were run on agarose gel and purified by QLA quick gel extraction kit (QIAGEN). Purified PCR products were cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol and were verified by sequencing (Biochemistry and Biotechnology Core Facilities, Indiana University, Indianapolis, IN). Probes for Northern blot analysis were generated using DECAprime TM II kit (Ambion) as per the manufacturer's instructions. Gene-specific bands were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and normalized to β-actin.

Statistical analysis

Statistical analyses were performed using StatView software (SAS Institute Inc., Cary, NC). Depending on the data distribution, comparisons were made between the two groups using the Student's t test or Mann-Whitney test. The association between continuous variables was tested using Spearman rank correlation. Data are presented as mean ± SD. $P < 0.05$ was considered statistically significant.

Microarray analyses were carried out using Microarray Data Portal, a proprietary analytical and informatics tool developed by the Center for Medical Genomics. To eliminate noisy data from probe sets that reflect background signals, we analyzed only those probe sets called present by MAS5 in at least half of the arrays for at least one of the conditions (20).

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TABLE 1. Demographic, anthropometric, and biochemical measurements in NASH and control groups[a]

	NASH (n = 21)	Controls (n = 19)	*P* value
Age, yr	41 ± 13	43 ± 14	0.6
Females, n	11	10	0.9
BMI, kg/m^2	33 ± 4.5	31 ± 4	0.15
Waist to hip ratio	0.95 ± 0.06	0.95 ± 0.05	0.8
Total body fat, %	40 ± 8	39 ± 10	0.3
Visceral fat, %	28 ± 12	22 ± 13	0.02
Fasting lipids			
Triglycerides, mmol/liter	3.53 ± 1.31	2.53 ± 1.15	0.01
Cholesterol, mmol/liter	5.35 ± 0.93	5.38 ± 1.29	0.9
HDL cholesterol, mmol/liter	0.93 ± 0.16	1.22 ± 0.36	0.003
LDL cholesterol, mmol/liter	3.49 ± 0.67	3.39 ± 1.06	0.7
Serum glucose, mmol/liter	5.16 ± 0.72	5.05 ± 0.61	0.2
Serum insulin, pmol/liter	170 ± 119	92 ± 68	<0.001
Serum C-peptide, mg/liter	6.0 ± 5.9	4.3 ± 3.6	0.3
HOMA	7 ± 5.4	3.2 ± 3.0	<0.001
AST, IU/liter	75 ± 56	26 ± 12	<0.001
ALT, IU/liter	99 ± 77	27 ± 8	<0.001
Leptin, ng/ml	22 ± 14	16 ± 10	0.3
Adiponectin, μg/ml	4.9±2.7	7.3 ± 3.5	0.02
Oxidized LDL, U/liter	56 ± 16	40 ± 12	<0.001
GPI-PLD, μg/ml	119 ± 24	105 ± 15	0.047

HDL, High-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
[a] Data represent mean ± SD unless specified otherwise.

Then we carried out an ANOVA with one factor for the gene transduced (GPI-PLD *vs.* LacZ) using log-transformed signal values. Multifactor ANOVA was carried out for the gene transduced, one for time, and their interaction. Genes that were significant for the gene transduced or their interaction at $P \leq 0.05$ were mapped onto Gene Ontology categories.

Results

Aim 1

The demographic, anthropometric, and serological characteristics of individuals with NASH and their controls are shown in Table 1. These results have been published previously (13–17) but are presented here again for the interpretation of the GPI-PLD levels. There were no demographic differences between the groups, and all participants were Caucasian. BMI, percent body fat, and waist to hip ratio were similar between the groups, but patients with NASH had significantly greater visceral fat mass than the controls (Table 1). As expected, subjects with NASH had higher fasting levels of insulin and HOMA values than the controls.

Serum GPI-PLD levels in patients with NASH were 119 ± 24 μg/ml and were significantly higher than the controls (105 ± 15 μg/ml, $P = 0.047$). There were no gender-specific differences in serum GPI-PLD levels in the total cohort (males 113 ± 16 *vs.* females 112 ± 26 mg/ml, $P = 0.4$). There was no statistically significant relationship between serum GPI-PLD levels and body weight, BMI, waist to hip ratio, percent body fat, percent visceral fat, or visceral fat mass. Serum GPI-PLD had a statistically significant negative correlation with age ($r = -0.45$, $P = 0.006$) as has been observed previously (21).

Aim 2

Hepatic GPI-PLD mRNA did not differ between patients with fatty liver or NASH; hence, the results from these two groups were combined (data not shown). Hepatic GPI-PLD mRNA was 3-fold higher in the combined group (NAFLD), compared with normal liver tissue (Fig. 1).

These results demonstrate that NAFLD is associated with elevations in liver expression of GPI-PLD mRNA. However, these results to do not distinguish whether increases in hepatic GPI-PLD expression are an epiphenomenon or may play a direct role in the pathogenesis of fatty liver or NASH.

Aim 3

To determine whether increased GPI-PLD expression may play an active role altering hepatic fatty acid and triglyceride metabolism, we overexpressed GPI-PLD in HepG2 cells, a human hepatoma cell line, and examined the effect on global gene expression. HepG2 cells were transduced with a control adenovirus expressing β-galactosidase (AdLacZ) or an adenovirus expressing GPI-PLD (AdGPI-PLD), and global gene

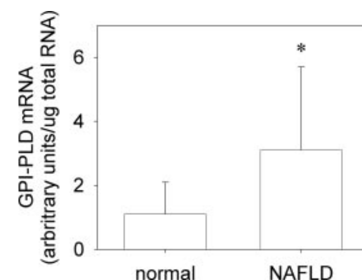

FIG. 1. Liver GPI-PLD mRNA expression is significantly increased in patients with NAFLD. Liver tissue was obtained from healthy volunteers (n = 12) undergoing liver biopsies as part of live liver donor evaluation or patients with NAFLD (n = 25) undergoing liver biopsy as part of their clinical care. The mean age was 41 ± 5 yr in the normal group and 39 ± 8 yr in the NAFLD group. The BMI was significantly higher in the NAFLD group (32 ± 3.5 kg/m^2), compared with the normal group (24 ± 3.2). Liver GPI-PLD mRNA was determined by real-time PCR as described in *Patients and Methods*. *, $P = 0.026$ by *t* test.

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TABLE 2. Gene ontology analysis of GPI-PLD-dependent gene expression

Ontology name	Gene ontology name	No. of genes	*P* value
Cellular component	Intracellular	249	1.1 E-5
	Chromosome	11	1.1E-4
	Chromatin	10	3.1E-4
	Nucleoplasm	16	0.012
	Cytoplasm	124	0.025
Molecular function	Nuclear mRNA splicing via spliceosome	15	4.9E-4
	Positive regulation of transcription, DNA dependent	7	0.002
	RNA binding	34	0.002
	Chromatin modification	10	0.006
	Transcription factor binding	20	0.006
	Transcription coactivator activity	13	0.008
Biological process	Metabolism	234	0.003
	Aromatic compound catabolism	2	0.005
	Polypyrimidine tract binding	2	0.012
	Peptidyl-serine phosphorylation	2	0.015
	Protein amino acid autophosphorylation	3	0.016

Genes that were significant for gene transduced at $P \leq 0.05$ were mapped onto gene ontology categories. The five gene ontologies with the lowest *P* value for cellular component, molecular function, and biological process are listed.

expression was determined using microarrays after 6 or 12 h. A total of 10,435 genes (47% of total gene probe set) were detected. Of these probe sets, 2340 differed at $P < 0.05$ (522 randomly expected) and 1270 genes at $P < 0.01$ (104 randomly expected).

Using a multifactor ANOVA, there were 2159 genes that were significantly different ($P < 0.05$) at 12 h, compared with 6 h, but were unrelated to the overexpression of GPI-PLD. Hence, the expression of these genes is related to time and/or cell cycle *per se* and was not analyzed further. Four hundred forty-one genes were significantly ($P < 0.05$) affected only by the overexpression of GPI-PLD independent of time. There were 773 genes that changed in a time- and GPI-PLD-dependent fashion. Analysis of the function of these genes using gene ontology demonstrated that the majority of these genes were intracellular and involved in metabolism (Tables 2 and 3). The molecular function of these genes varied. The highest numbers of genes are involved in RNA metabolism and regulation of transcription. Numerous genes involved in signal transduction including the MAPK pathway and cAMP signaling pathway were also altered with GPI-PLD overexpression. This manuscript will focus on the effect of GPI-PLD on lipid metabolism.

There were 32 genes involved in fatty acid and lipid metabolism that were affected by overexpressing GPI-PLD (Table 4). These genes are involved in *de novo* lipogenesis, triglyceride synthesis, and lipoprotein metabolism. The largest effect was on DGAT. DGAT1 increased nearly 3-fold with overexpressing GPI-PLD. To confirm the microarray results, Northern blot analysis of both isoforms of DGAT, DGAT1 and DGAT2, was performed. This confirmed that GPI-PLD overexpression increased DGAT1 but not DGAT2 mRNA levels (Fig. 2).

Discussion

The accumulation of triglycerides in liver results from an imbalance between the uptake of fatty acids, *de novo* lipogenesis, and export as very low-density lipoproteins or fatty acid oxidation. Numerous biochemical and hormonal defects

TABLE 3. Gene ontology analysis of time- and GPI-PLD-dependent gene expression

Ontology name	Gene ontology name	No. of genes	*P* value
Cellular component	Chromosome	21	4.0E-8
	Intracellular	423	1.2 E-6
	Chromatin	16	2.1E-5
	Ribonucleoprotein complex	39	6.3E-5
	Mitochondrion	47	1.6E-4
Molecular function	RNA binding	57	3.5E-4
	Nuclear mRNA splicing via splicosome	20	0.002
	Nucleosome dissembly	4	0.003
	Structural constituent of ribosome	20	0.003
	Protein targeting to mitochondrion	5	0.006
	Transcription coactivator activity	13	0.007
Biological process	RNA metabolism	57	7.7E-4
	Metabolism	401	0.001
	Electron transport activity	23	0.005
	RNA catabolism	7	0.005
	Oxidoreductase activity, acting on the CH-OH group of donors, NAD, or NADP as acceptor	11	0.006

Genes that were significant for gene transduced/time interaction at $P \leq 0.05$ were mapped onto gene ontology categories. The five gene ontologies with the lowest *P* value for cellular component, molecular function, and biological process are listed. CH-OH, Alcohol; NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate.

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TABLE 4. Effect of GPI-PLD overexpression on lipid gene expression

Gene symbol	Name	GenBank	Multifactor ANOVA	GPI-PLD *vs.* control fold change	
				6 h	12 h
DGAT1	Diacylglycerol *O*-acyltransferase homolog 1	AA978141	0.001	2.94	0.97
PCCB	Propionyl coenzyme A carboxylase, β-polypeptide	R15273	0.006	1.28	0.88
CLU	Apolipoprotein J	M25915.1	0.022	1.25	0.80
FDXR	Ferredoxin reductase	AI004037	0.041	1.23	0.87
SCD	Stearoyl coenzyme-A desaturase	AA678241	0.004	1.23	0.87
LYPLA2	Lysophospholipase II	AL031295	0.040	1.22	1.17
NR1H4	Bile acid receptor (Farnesoid X-activated receptor)	NM_005123.1	0.026	1.20	0.90
ELOVL1	Elongation of very long chain fatty acids protein 1	H93026	0.050	1.19	0.93
AZGP1	α2-Glycoprotein 1, zinc	AC004522	0.024	1.18	0.80
APOC3	Apolipoprotein CIII	AA909307	0.020	1.15	0.84
ZAP128	Peroxisomal acyl-coenzyme A thioester hydrolase 2a	NM_006821.1	0.009	1.15	0.84
SCARB1	Scavenger receptor class B type 1	NM_005505.1	0.036	1.15	0.96
CDS2	CDP-diacylglycerol synthase (phosphatidate cytidylyl-transferase) 2	AL568982	0.004	1.14	0.89
HADHSC	L-3-hydroxyacyl-coenzyme A dehydrogenase, short chain	AA902926	0.031	1.13	0.83
CD74	CD74 antigen	K01144.1	0.026	1.12	0.89
HADHA	Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase (trifunctional protein), α subunit	AA954955	0.027	1.11	0.89
ACACB	Acetyl-coenzyme A carboxylase β	AI057637	0.017	1.10	0.86
PPP2R1B	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), β-isoform	T79584	0.004	1.08	0.80
ACLY	ATP citrate lyase	NM_001096.1	0.018	1.08	0.91
SULT2A1	Sulfotransferase family 2A, dehydroepiandrosterone-preferring, member 1	AI028152	0.001	1.07	0.75
GGPS1	Geranylgeranyl diphosphate synthase 1	AA989390	0.001	1.06	0.80
HMGCR	3-Hydroxy-3-methylglutaryl-coenzyme A reductase	AI032963	0.044	1.04	0.90
CHKA	Choline kinase-α	AI991328	0.044	0.96	0.73
AKR1B10	Aldoketoreductase family 1, member B10 (aldose reductase)	AF044961	0.015	0.94	1.24
HEXB	Hexosaminidase B (β-polypeptide)	NM_000521.2	0.020	0.90	1.04
LIPA	Lipase A, lysosomal acid, cholesterol esterase	AA988640	0.032	0.90	0.97
SCP2	Sterol carrier protein 2	R19294	0.045	0.86	0.95
ACAA2	Acetyl-coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-coenzyme A thiolase)	AW072302	0.040	0.83	1.49
IMPA1	Inositol(myo)-1(or 4)-monophosphatase 1	NM_005536.2	0.012	0.79	1.19
WWOX	WW domain containing oxidoreductase	AA970231	0.021	0.78	1.19
OSBPL2	Oxysterol binding protein-like 2	AI753638	0.006	0.74	0.97
COLEC12	Collectin subfamily member 12	AA977789	0.018	0.53	1.27

Genes with $P \leq 0.05$ that are involved in lipid metabolism that were affected by overexpressing GPI-PLD in HepG2 cells were identified as described in *Patients and Methods*. Indentifying data, gene symbol, GenBank, and description were taken from Affymetrix annotations.

result in fatty liver (4). In this report, we demonstrate that GPI-PLD expression is elevated in patients with NAFLD and provide evidence that it may play a role in enhancing *de novo* lipogenesis in liver.

Donnelly *et al.* (3) demonstrated that the primary lipid defect in patients with NAFLD is a 5-fold increase in *de novo* lipogenesis. Consistent with this *in vivo* observation, a recent analysis of fatty acid synthesis and oxidation genes in patients with NAFLD demonstrated an increase in genes involved in *de novo* lipogenesis [acetyl coenzyme A carboxylase (ACC) 1 and 2] and triglyceride synthesis (DGAT1) as well as alterations in genes involved in β-oxidation of fatty acids [carnitine palmitoyltransferase 1a decreased, whereas long-chain acyl-coenzyme A dehydrogenase and long-chain L-3-hydroxyacyl-coenzyme A dehydrogenase (HADH)-α increased] (22). An increase in fatty oxidation genes may be a compensatory effect in response to the increase in *de novo* lipogenesis. The predicted net effect is an accumulation of intracellular fatty acids and triglycerides. Many of these same genes altered in NASH are similarly affected by overexpressing GPI-PLD *in vitro*. Genes affected by GPI-PLD overexpression that are involved in *de novo* lipogenesis and triglyceride synthesis include ATP citrate lyase (ACLY), ACC2, elongation of very long-chain fatty acids, stearoyl coenzyme-A desaturase (SCD), and DGAT1, whereas those involved in β-oxidation of fatty acids include short-chain HADH, HADHα, and acetyl-coenzyme A acyltransferase 2. These results are expected to result in a net increase in fatty acid synthesis, which may contribute to the increase in serum triglycerides observed when hepatic GPI-PLD expression is increased (11). This raises the possibility that the increase in hepatic GPI-PLD mRNA that occurs in patients with NAFLD may play an active role in the development of fatty liver. Many of these genes (ACC, SCD, ACLY, 3-hydroxy-3-methylglutaryl-coenzyme A reductase) are regulated by sterol-response element binding proteins (SREBP). The SREBP family of transcription factors regulates cholesterol and triglyceride synthesis. It is conceivable that overexpressing

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GPI-PLD alters the lipid composition of the endoplasmic reticulum resulting in activation of SREBPs. However, this was not examined in our study because sufficient liver tissue was not available.

In addition to altering genes involved in lipid metabolism, overexpressing GPI-PLD in HepG2 cells had a large effect on global gene expression. More than 100 genes involved in RNA metabolism and regulation of transcription were affected including 20 transcription factors and 18 corepressors/coactivators. Altering that many transcription factors likely results in many downstream effects. In addition, more than 30 growth factors or their receptors were altered including TGFβ1 (up 1.74-fold), IL-8 (up 1.6-fold), vascular endothelial growth factor (up 1.2-fold), IGF-I receptor (up 2.4-fold), and fibroblast growth factor receptor 1 (up 1.5-fold). Many of these growth factors have been implicated in the progression of fatty liver to the fibrosis and cirrhosis seen in NASH (23, 24). Clearly, overexpressing GPI-PLD affects many genes involved in a multitude of pathways and biological processes. Using global gene expression makes it difficult to determine which, if any, of these pathways or processes affected by GPI-PLD may contribute to the development of fatty liver and/or NASH. Our observation that liver GPI-PLD mRNA is elevated to the same extent in patients with fatty liver and NASH suggests that GPI-PLD is more related to the former rather than the latter. More direct experiments are needed to determine how and at what point GPI-PLD plays in the development and progression of NAFLD.

Our results are also the first to demonstrate acute regulation of DGAT1 expression. It is unclear how GPI-PLD activity may regulate DGAT1 and other genes. GPI-PLD-mediated cleavage of GPIs generates phosphatidic acid, which leads to an increase in diacylglycerol and the activation of protein kinase Cα (25). In addition, inositol glycans would also be released. Inositol glycans have been postulated to serve as insulin mediators and have been suggested to stimulate *de novo* lipogenesis and triglyceride synthesis (26, 27). Whether these signals are responsible for the effects of GPI-PLD on gene expression is unknown at this time.

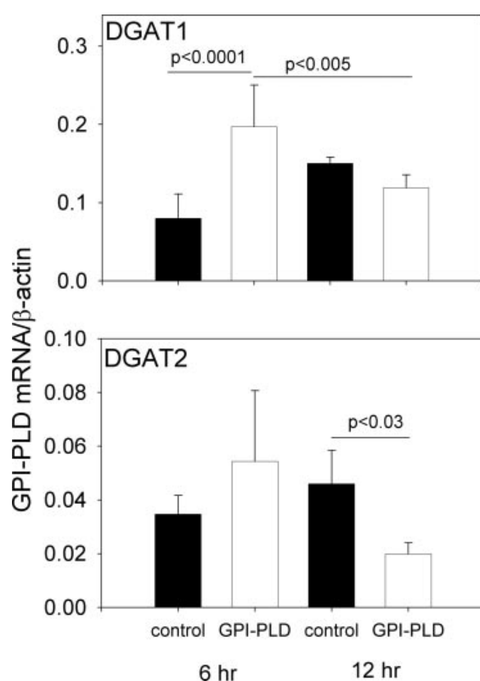

FIG. 2. GPI-PLD increases DGAT1 but not DGAT2 expression in HepG2 cells. HepG2 cells were transduced with AdLacZ or AdGPI-PLD and total RNA extracted after 6 or 12 h as described in *Materials and Methods*. DGAT1 and DGAT2 mRNA levels were determined by Northern blotting and normalized to β-actin mRNA. Results are from three independent experiments.

Why GPI-PLD expression is increased in NAFLD is unknown. Oxidative stress alters GPI-PLD expression in macrophage cell lines (28, 29). This might explain an alteration in NASH as increased oxidative stress is suggested to mediate the transition from fatty liver to NASH but does not explain the increase in patients with fatty liver. Glucose and insulin regulate GPI-PLD expression in pancreatic islet β-cells (30), raising the possibility that increased expression of hepatic GPI-PLD may be related to serum insulin levels in NAFLD patients. Increased expression of GPI-PLD not only will have effects within the hepatocyte but also will be expected to increase GPI-PLD secretion into the plasma. In mice, serum GPI-PLD is under genetic control (7, 31), and we have found that fasting serum GPI-PLD levels do correlate to levels of hepatic levels of GPI-PLD mRNA (32). However, serum levels of GPI-PLD also appear to be acutely regulated because serum GPI-PLD levels rise and fall during the postprandial state without changes in liver GPI-PLD mRNA levels (11). Hence, the increased serum levels of GPI-PLD in NASH patients may result from a combination of increased hepatic expression as well as the altered lipoprotein metabolism in the plasma compartment that occurs with insulin resistance.

Limitations of this study include use of stored samples [although serum GPI-PLD levels are stable frozen for at least 3 yr (Deeg, M. A., unpublished observation)], no data with respect to actual protein levels in the liver, and not being able to have serum and hepatic samples from the same cohort. Hence, we were not able to test whether there was a significant correlation between hepatic GPI-PLD mRNA and serum or hepatic GPI-PLD protein. In addition, overexpressing GPI-PLD in HepG2 cells may not entirely reflect the changes in gene expression that would occur in normal hepatocytes. In addition, the changes in gene expression after 6–12 h of overexpressing GPI-PLD may be different from that which occurs after prolonged increased expression of GPI-PLD. Nonetheless, these studies have yielded important preliminary findings to support GPI-PLD as a target for further evaluation.

In summary, patients with NAFLD have elevated serum levels and hepatic expression of GPI-PLD. Because increased expression of hepatic GPI-PLD is associated with increases in serum triglycerides and increased expression of *de novo* lipogenesis genes, GPI-PLD may play an active role in the pathogenesis of NAFLD or its metabolic features and warrants further investigation.

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