Increased expression of GPI-specific phospholipase D in mouse models of type 1 diabetes

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Increased expression of GPI-specific phospholipase D in mouse models of type 1 diabetes. Am J Physiol Endocrinol Metab 281: E147–E154, 2001.—Glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) is a high-density lipoprotein-associated protein. However, the tissue source(s) for circulating GPI-PLD and whether serum levels are regulated are unknown. Because the diabetic state alters lipoprotein metabolism, and liver and pancreatic islets are possible sources of GPI-PLD, we hypothesized that GPI-PLD levels would be altered in diabetes. GPI-PLD serum activity and liver mRNA were examined in two mouse models of type 1 diabetes, a nonobese diabetic (NOD) mouse model and low-dose streptozotocin-induced diabetes in CD-1 mice. With the onset of hyperglycemia (2- to 5-fold increase over nonobabetic levels), GPI-PLD serum activity and liver mRNA increased 2- to 4-fold in both models. Conversely, islet expression of GPI-PLD was absent as determined by immunofluorescence. Insulin may regulate GPI-PLD expression, because insulin treatment of diabetic NOD mice corrected the hyperglycemia along with reducing serum GPI-PLD activity and liver mRNA. Our data demonstrate that serum GPI-PLD levels are altered in the diabetic state and are consistent with liver as a contributor to circulating GPI-PLD.

Keywords: nonobese diabetic mouse; streptozotocin; glycosylphosphatidylinositol; apolipoproteins; pancreatic islets
HDL-associated proteins, including apoJ and paraoxonase, in the development of atherosclerosis has been appreciated (4, 12, 24, 25). Because serum GPI-PLD behaves as a minor HDL-associated protein (7, 10), its potential role in diabetes and atherosclerosis needs further investigation.

One goal in this report was to determine whether GPI-PLD expression is perturbed due to diabetes. We found that GPI-PLD expression was markedly increased in two mouse models for type 1 diabetes; these are nonobese diabetic (NOD) mice, known to develop spontaneous insulinitis, and low-dose streptozotocin (STZ)-induced diabetes in CD-1 mice. A role for regulation of GPI-PLD expression by insulin is proposed on the basis of these studies, combined with results of GPI-PLD expression in mice under differing nutritional status. A second goal was to determine whether islets contribute significantly to serum GPI-PLD levels. In the mouse models studied in the present investigation, GPI-PLD mRNA levels in the liver mirrored the changes in serum GPI-PLD. Overall, this is the first report showing that GPI-PLD levels can be regulated. Furthermore, our data are consistent with liver as a contributor to circulating GPI-PLD.

MATERIALS AND METHODS

Animals. CD-1 male mice (8 wk old) were purchased from Harlan Sprague Dawley (Indianapolis, IN). NOD mice (female) were from an inbred colony maintained at the University of Washington (NOD/RL). These mice were established from mating pairs originally provided by Dr. Pia Reich (Department of Medicine, Yale University) and are in their 23rd generation intercross. The frequency of diabetes is greater than 80% among female mice by 3 mo of age (13). Age-matched nondiabetic NOD mice were used as controls.

CD-1 mice were maintained in a temperature-controlled (25°C) facility with a strict 12:12-h light-dark cycle and were given free access to food and water. Mice were fed rodent chow pellets (Wayne Rodent BLOX 8604, Teklad, Madison, WI). NOD mice were maintained under specific pathogen-free conditions at 25°C with a strict 12:12-h light-dark cycle and had free access to food (autoclaved) and water (acid treated). Food was removed from the mice 4 h before the collection of blood from the retroorbital sinus (15). Serum was used immediately or stored at −70°C before analysis. Mice were killed by cervical dislocation. This project was approved by the Animal Care and Use Committees of Indiana University and the University of Washington.

Drug treatment. STZ was dissolved in sterile citrate buffer (50 mM sodium citrate, pH 4.5) and injected intraperitoneally into CD-1 mice (40 mg/kg, ~20 μl) within 5 min of preparation. STZ or citrate buffer alone was administered for 5 consecutive days during the 1st wk of this study. These mice were not treated with insulin.

NOD mice were monitored for diabetes by weekly weighing and urinary glucose testing (Diastix, Eli Lilly, Indianapolis, IN). Minimal insulin replacement therapy (Regular Iletin II and bovine insulin (1/50 final dilution; Sigma)) was initiated after a positive urine glucose test. Insulin dosage was determined on the basis of urinary glucose output and body weight and was designed to maintain body weight comparable to that of nondiabetic littermates. Insulin doses ranged from 20 to 600 U/kg every other day. Nondiabetic littermates were not treated with insulin at any time during this study.

**GPI-PLD enzymatic activity.** GPI-PLD activity was determined using the [3H]myristate-labeled membrane form of variant glycoprotein as the substrate (9). Phosphatidic acid was confirmed as the only reaction product by thin-layer chromatography (data not shown, but see examples in Refs. 5, 9, and 18). One unit of activity was arbitrarily designated as the amount of enzyme converting 1% of substrate per minute.

**Analytical procedures.** Cholesterol, total triglyceride (which includes glycerol), and glucose concentrations were determined using commercially available kits (catalog numbers: 352–20, 339–10, and 315–100, respectively, Sigma, St. Louis, MO). Insulin and glucagon assays were performed by the Indiana University Diabetes Research and Training Center Immunoassay Core with commercially available kits (Linco Research, St. Charles, MO).

**Fast performance liquid chromatography.** Plasma lipoproteins were separated by high-performance liquid chromatography (HPLC) size-exclusion chromatography using a Superose 6 column (Pharmacia, Piscataway, NJ). One hundred microliters of pooled serum (from 2 or 3 mice) were loaded onto the column and eluted with buffer consisting of 150 mM NaCl, 20 mM HEPES (pH 7.4), and 0.02% NaN3 at a flow rate of 0.25 ml/min at 4°C. Sixty fractions, 500 μl each, were collected and assayed for cholesterol, triglyceride, and GPI-PLD activity.

**Western blot analysis.** GPI-PLD and apolipoprotein (apo) A-I were detected by immunoblotting after SDS-PAGE using 7 or 12% acrylamide gels, respectively. Nitrocellulose membranes (Schleicher and Schuell, Keene, NH) were used for the protein transfer, and specific proteins were visualized by chemiluminescence using commercial reagents (SuperSignal Pierce, Rockford, IL). Band intensities were compared by densitometry using a Bio-Rad GS-700 Imaging Densitometer and Molecular Analyst software (Bio-Rad, Hercules, CA). GPI-PLD immunoreactivity was identified using a GPI-PLD antibody by Western blotting, and changes relative to nondiabetic samples were determined.

**GPI-PLD was identified using anti-GPI-PLD**771, a peptide-specific rabbit polyclonal antibody generated against a peptide sequence corresponding to amino acids 771–790 in human serum GPI-PLD (M. Deeg, unpublished observations). ApoA-I was identified using a polyclonal rabbit anti-mouse antibody (BioDesign International, Kennebunk, ME).

**Insulinitis.** Pancreatic tissues were isolated from NOD or CD-1 mice and fixed in 4% paraformaldehyde for 4 h. Tissues were embedded in paraffin blocks, and 5-μm sections were mounted on polylysine-coated slides. The degree of insulinitis was determined from multiple nonsequential slides from individual mice. Approximately 5–10 islets/section were scored. Each islet was assigned a score, as previously described (6): 0 = no lymphocytic infiltration; 1 = peri-insulitis; 2 = <50% islet infiltration; and 3 = >50% islet infiltration. The sum of all scores for each mouse was divided by the number of islets analyzed to derive a final insulinitis score.

**Immunofluorescence.** To determine GPI-PLD expression in islets, paraffin sections (see above) were deparaffinized and rehydrated with xylene and graded ethanol. GPI-PLD, insulin, and glucagon were identified as previously described (9) using anti-GPI-PLD771 sera (1/50 dilution), guinea pig anti-bovine insulin (1/50 final dilution; Sigma), and a murine monoclonal anti-glucagon (1/100 final dilution; Sigma). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA): fluorescein-conjugated goat anti-rabbit antiserum at a final concentration of 150 μg protein/ml; Cy5-conjugated goat anti-guinea pig antiserum at a final concentration of 150 μg protein/ml; and...
Table 1. Summary of metabolic, hormone, and lipid values in nondiabetic, diabetic, and insulin-treated diabetic NOD mice

<table>
<thead>
<tr>
<th></th>
<th>Glucose, mg/dl</th>
<th>Insulin, ng/ml</th>
<th>Glucagon, pg/ml</th>
<th>Total Cholesterol, mg/dl</th>
<th>Total Triglycerides, mg/dl</th>
<th>apoA-I, mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>nonDM (5)</td>
<td>173 ± 26</td>
<td>1.03 ± 0.02</td>
<td>52.3 ± 9.8</td>
<td>90 ± 9</td>
<td>115 ± 32</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>DM (5)</td>
<td>860 ± 176*</td>
<td>6.56†</td>
<td>ND</td>
<td>144 ± 43*</td>
<td>160 ± 56</td>
<td>4.7 ± 0.8</td>
</tr>
<tr>
<td>DM + insulin (7)</td>
<td>154 ± 149</td>
<td>24.1, 48.3</td>
<td>51.4 ± 1.7</td>
<td>107 ± 26</td>
<td>189 ± 86*</td>
<td>4.5 ± 0.5</td>
</tr>
</tbody>
</table>

Blood was collected after a 4-h fast from nondiabetic (nonDM), diabetic (DM), and insulin-treated diabetic (DM + insulin) nonobese diabetic (NOD) mice. apoA-I, apo-lipoprotein A-I. Metabolic, hormone, and lipid assays were determined as described in MATERIALS AND METHODS. Values are individual or means ± SD for no. of animals in parentheses. ND, not determined. *P < 0.05 vs. nonDM. †Pool from 3 animals.

Texas Red-conjugated sheep anti-mouse antiserum at a final concentration of 140 μg protein/ml.

The immunostained samples were examined with a Bio-Rad MRC 1024 laser-scanning confocal microscope (Hercules, CA) mounted on a Nikon Diaphot 300 platform (Renal Imaging Facility, Indiana University, Indianapolis, IN). The fluorescent tags were individually excited at 488, 522, or 588 nm for fluorescein, Texas Red, and Cy5, respectively, and monitored at emission wavelengths of 522, 588, or 680, respectively. Islets were graded as positive or negative for insulin and GPI-PLD immunoreactivity. All emission wavelengths were monitored at each excitation wavelength to ensure that the observed fluorescence did not result from the wrong fluorescent tag.

mRNA quantification. Total RNA was extracted from liver using a commercial kit (PolyAtract mRNA isolation system IV, Promega, Madison, WI). mRNA specific for GPI-PLD was identified by Northern blotting by use of a full-length GPI-IV, Promega, Madison, WI). mRNA specific for GPI-PLD was identified by Northern blotting by use of a full-length GPI-PLD cDNA (3.4 kb) obtained from a library generated in pBluescript II SK+ (Stratagene) from a mouse glucagonoma cell line αTC6 (14). Three major transcripts are seen for GPI-PLD. The major band is 3.9 kb in length with variable minor bands at 5.4 and 8.0 kb. Only the 3.9-kb transcript was quantitated; β-actin mRNA was used to normalize loading.

Statistics. Values are reported as means ± SD, with the number of animals (n) in parentheses. Statistical differences between groups were determined by one-way ANOVA. P < 0.05 was accepted as statistically significant.

RESULTS

Diabetic phenotypes in NOD and CD-1 mice. NOD mice develop a spontaneous autoimmune destruction of β-cells within islets that is similar in character to type 1 diabetes in humans (29). This results in hyperglycemia and hypoinsulinemia, as shown in Table 1. Diabetic NOD mice had a fivefold increase in glucose and an ∼50% reduction in insulin levels compared with nondiabetic mice. Plasma cholesterol and triglyceride concentrations were increased by 1.4-fold and 1.6-fold, respectively. Diabetic mice treated with insulin maintained reduced plasma glucose, cholesterol, and glucagon levels, although triglyceride levels remained elevated compared with nondiabetic mice. This may reflect the every-other-day dosing of insulin and the variation in glycemic control (Table 1). The triglyceride levels may also be influenced by the individual variation in nutritional status. Levels of plasma apoA-I, the primary protein found on HDL, remained unchanged.

STZ treatment also results in hyperglycemia and hypoinsulinemia and inflammatory processes that are targeted primarily to the pancreas (16). Thus this route to diabetes offers another model in which to study GPI-PLD expression. For CD-1 mice, changes in diabetic parameters at 0, 7, 14, and 28 days after STZ treatment were monitored, where day 0 is the first STZ treatment day (Fig. 1). Plasma insulin levels decreased ~30% by day 7 and were ninefold lower than on day 0 by day 28. Concomitant hyperglycemia was observed at day 10 (not shown) and by day 14 was increased over twofold from day 0. Glucagon levels were significantly elevated at days 14 and 28. By 7 days, STZ-treated CD-1 mice showed extensive insulitis, consistent with the deterioration of diabetic parameters. Insulitis was evident as early as day 1 (not shown).

Diabetes in NOD and CD-1 mice results in increased GPI-PLD expression. Diabetes onset in NOD mice resulted in significant increases in GPI-PLD expression (Fig. 2). Serum enzyme activity and immunoreactivity were elevated 2- and 3.5-fold, respectively, in diabetic

![Image](http://ajpendo.physiology.org/DownloadedFrom/MaterialsAndMethods)
mice compared with nondiabetic mice. Hence, there is a decrease in the specific activity of GPI-PLD in the diabetic mice. This decrease in specific activity is likely secondary to the increase in serum lipids, which interfere with the GPI-PLD assay (data not shown). Therefore, measuring GPI-PLD immunoreactivity more accurately reflects changes in serum GPI-PLD concentrations, although we cannot eliminate the possibility that changes in GPI-PLD immunoreactivity may also occur in the diabetic state. Hepatic mRNA levels for GPI-PLD were increased fourfold as a result of diabetes. Treatment of diabetic mice with insulin reversed these changes, reducing GPI-PLD levels to those of nondiabetic mice. For the three groups of NOD mice, Western blot analysis of serum for GPI-PLD showed the presence of the expected 115-kDa protein only (data not shown).

For CD-1 mice, serum GPI-PLD immunoreactivity and liver mRNA (Fig. 3) mirrored increases as seen for glucose and glucagon. There were significant increases for serum GPI-PLD immunoreactivity and liver mRNA levels of ~1.5-fold at day 14 and ~2-fold at day 28. Overall, GPI-PLD expression was elevated by hypoinsulinemic phenotypes.

**Tissue sources of GPI-PLD.** The origin of circulating GPI-PLD is controversial and has not been previously examined in diabetic mice. Two likely sources for serum GPI-PLD are liver and pancreatic islets. Destruction of pancreatic β-cells by autoimmune disease or STZ treatment would be expected to result in decreased amounts of GPI-PLD from this tissue source.

Pancreatic sections from nondiabetic and diabetic NOD mice were triple-stained for GPI-PLD, glucagon, and insulin. For nondiabetic mice, glucagon and insulin were readily identified in pancreatic islets (Fig. 4). GPI-PLD immunoreactivity was also identified and colocalized with insulin, suggesting that GPI-PLD is expressed primarily in β-cells. In diabetic NOD mice,
glucagon was readily identified, but GPI-PLD and insulin expression were below the level of detection in nearly all islets (Fig. 4 and Table 2). Faint staining for insulin in the islet from the diabetic animal shown (Fig. 4) has the same distribution as glucagon, suggesting that this image derives from the glucagon probe.

Quantification of islet numbers showing immunoreactivity for insulin and GPI-PLD in islets from diabetic and nondiabetic NOD mice compared with normal islets from CD-1 mice substantiate a reduction in islet production of both insulin and GPI-PLD due to diabetes (Table 2). For CD-1 mice, the majority of islets examined contained insulin and/or GPI-PLD compared with 50–65% for nondiabetic NOD and 5% for diabetic NOD mice. The reduction in staining seen for nondiabetic NOD vs. CD-1 mice may reflect ongoing insulitis, which occurs before onset of frank diabetes (3). The main point is that, because islet GPI-PLD expression is lost upon frank diabetes onset but serum GPI-PLD is increased in diabetic NOD and STZ-treated CD-1 mice (Figs. 2 and 3), the liver may likely be the primary source for GPI-PLD at least in the diabetic state.

**GPL-PLD expression may be controlled by insulin.** Diabetes onset in NOD and CD-1 mice is associated with a myriad of metabolic changes, including hyperglycemia, hypoinsulinemia, alterations in circulating lipids, and potentially other parameters that may regulate GPI-PLD expression. It is tempting to speculate that hypoinsulinemia is the driving factor in the increased expression of GPI-PLD seen for diabetic NOD and CD-1 mice. Because severe starvation can induce many of the same metabolic and hormonal changes as diabetes except for hyperglycemia, we used nutritional status as a tool to test for a potential role of insulin in GPI-PLD expression.

Serum GPI-PLD levels, liver GPI-PLD mRNA levels, and various hormonal markers were determined for CD-1 mice that were fasted for 4 h (fed) and 72 h (severe starvation). As expected, there was no difference in serum glucose, but insulin levels decreased markedly (Table 3). Severe starvation resulted in a small increase in serum GPI-PLD activity, a 50% increase in serum GPI-PLD immunoreactivity, and a greater than twofold increase in liver GPI-PLD mRNA levels.

**GPI-PLD is primarily associated with HDL in nondiabetic and diabetic states.** In normal serum, GPI-PLD behaves as an apolipoprotein, forming a complex with apoA-I (7, 10). Because the concentration and composition of many lipoproteins are altered in the diabetic state, it is possible that GPI-PLD may redistribute among circulating lipoprotein fractions in our diabetic mice.

**GPI-PLD associations with lipoproteins were examined using serum taken from nondiabetic, diabetic, and insulin-treated diabetic NOD mice.** Lipoprotein profiles obtained by FPLC showed marked differences among the groups (Fig. 5). For all groups of mice, the majority of cholesterol eluted as HDL (fractions 28–35) and overall levels were increased in diabetic mice, consistent with the 40% elevation in total cholesterol level (Table 1). Conversely, triglycerides, levels of which were elevated in diabetic and insulin-treated mice (Table 1), were nearly exclusively associated with very low-density lipoprotein (VLDL) particles eluting...
near the column void volume (fractions 10–20). The majority (97%) of GPI-PLD activity eluted just before HDL (fractions 21–27). This relationship of GPI-PLD complexes migrating before HDL is a consistent finding and is also seen in human serum (7). Overall, the lipoprotein patterns are consistent with total lipid measurements and suggest that GPI-PLD is associated with a specific subfraction of HDL in both diabetic and nondiabetic states.

DISCUSSION

There were two main findings in this report. First, hypoinsulinemic states led to increases in liver expression of GPI-PLD. Both hepatic mRNA levels and serum immunoreactivity and enzymatic activity were increased. The finding that levels of GPI-PLD can be regulated by altered physiological states of diabetes and fasting constitutes the first evidence that GPI-PLD expression is not merely constitutive. It is clear from our previous studies and this report that the regulation of the GPI-PLD gene is complex and involves genetic factors (14), nutritional status, and extent and type of diabetes. Second, our data are consistent with the possibility that the liver is the main source of circulating GPI-PLD. Overall, changes in liver mRNA levels paralleled changes in serum immunoreactivity in all cases studied here. Although a variety of cell types can produce GPI-PLD, secretion into plasma appears proportionately greater from liver.

In the mouse, the liver is the most abundant source of mRNA for GPI-PLD (14). However, in other species, the source of circulating GPI-PLD is less clear. Immunohistochemical detection of this protein has been found in a variety of cell types (20, 30). Furthermore, a confusing observation in studies of human GPI-PLD is that the amino acid sequence reported for serum GPI-PLD matches that predicted from human pancreatic and not liver GPI-PLD cDNA (10, 28). This latter difference could be explained by genetic polymorphisms that may exist between the individuals from whom liver and pancreas samples were taken for cloning or mistakes in sequencing (14). Future studies are needed in which serum, liver, and pancreas samples can be derived from individual subjects for final determinations of GPI-PLD sources in humans.

It is tempting to speculate that insulin regulates GPI-PLD expression. Circumstantial evidence for this includes the increased mRNA levels seen in models of hypoinsulinemia studied here. Treatment of diabetic NOD mice with insulin led to a rapid and complete return of GPI-PLD liver mRNA and serum levels to those of nondiabetic mice. Because of the complex metabolic alterations induced by diabetes and fasting, we

Table 3. Effect of fasting on GPI-PLD serum levels and liver mRNA levels

<table>
<thead>
<tr>
<th></th>
<th>Activity, U/ml</th>
<th>Immunoreactivity, degree of increase</th>
<th>Liver mRNA, degree of increase</th>
<th>Glucose, mg/dl</th>
<th>Insulin, ng/ml</th>
<th>Glucagon, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed (2)</td>
<td>37, 39</td>
<td>1.0</td>
<td>1.0</td>
<td>168, 183</td>
<td>1.62, 0.58</td>
<td>43.14*</td>
</tr>
<tr>
<td>Fast (3)</td>
<td>56 ± 1.3</td>
<td>1.50 ± 0.10</td>
<td>2.65 ± 0.88</td>
<td>152 ± 39</td>
<td>0.20 ± 0.13</td>
<td>58, 57</td>
</tr>
</tbody>
</table>

CD-1 mice were fasted 4 (fed) or 72 h (fast), and serum hormone levels and GPI-PLD activity and immunoreactivity, as well as liver GPI-PLD mRNA levels, were determined as described in MATERIALS AND METHODS. Nos. of animals used are shown in parentheses, and results are individual values or means ± SD. *Serum pooled from 2 animals.

Fig. 5. Gel filtration of lipoproteins and GPI-PLD complexes from nondiabetic, diabetic, and insulin-treated NOD mice. Lipoproteins in serum obtained from nondiabetic, diabetic, and insulin-treated diabetic NOD mice were separated by gel filtration, and cholesterol, triglyceride, and GPI-PLD activity was determined in each fraction as described in MATERIALS AND METHODS. Serum was pooled from 2 or 3 mice. Results are representative of 2 separate fast performance liquid chromatography runs.
cannot rule out other modulators of GPI-PLD expression. It is unclear from this work whether GPI-PLD is regulated at the level of transcription and/or whether posttranscriptional processes are involved in controlling mRNA levels for GPI-PLD. Further studies of the direct effects of insulin on GPI-PLD in hepatic cell systems, changes due to nutritional status in other mouse models, and the mode of regulation are needed. In addition, sequencing of the promoter regions of GPI-PLD is needed to establish whether insulin response elements exist.

Altered turnover of GPI-PLD in serum could also affect its circulating concentrations. Our studies of NOD mice showed that both serum HDL and GPI-PLD levels were elevated by diabetes onset and reduced after insulin administration. Because GPI-PLD associates with HDL, one interpretation of this concentration data is that serum GPI-PLD was elevated by diabetes because of the increase in its binding pool. Thus GPI-PLD concentration merely reflects HDL concentration. There are two arguments against this idea. First, in a previous study (14), HDL and GPI-PLD activity levels did not correlate. For C57BL/6 mice fed an atherogenic diet, HDL levels were decreased twofold, yet GPI-PLD activities remained the same. Second, apoA-I concentrations were essentially unchanged among the groups of NOD mice (Table 1). Diabetic NOD mice showed increased HDL cholesterol levels, suggesting that although particle composition was altered, the number of HDL particles did not change. In humans, GPI-PLD is associated with a small, discrete HDL-containing apoA-I and apoA-IV that accounts for no more than 0.2% of the total apoA-I (7). (Note: Preliminary data suggest that the mouse GPI-PLD complexes have similar properties to those of humans.) Hence, it is conceivable that this GPI-PLD-containing subclass of HDL may change without affecting total HDL. Further work is needed to confirm whether HDL turnover, or turnover of specific HDL subfractions, contributes to determining GPI-PLD levels.

Although we still do not know the primary function of serum GPI-PLD, the observations reported here provide some important fundamental information regarding liver as a source of serum GPI-PLD. In addition, our results suggest that serum GPI-PLD levels may be regulated and altered in pathologic conditions. Finally, serum GPI-PLD behaves very much like an apolipoprotein and joins the growing list of minor, but often important, metabolically active proteins associated with HDL particles.

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