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Glycosylphosphatidylinositol-specific phospholipase D improves glucose tolerance

Nandita S. Raikwar[a], Rosario F. Bowen-Deeg[a], X. Sean Du[b], Martin G. Low[b], Mark A. Deeg[a,c,*]

[a]*Department of Medicine and of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA*
[b]*Department of Physiology and Cellular Biophysics, Columbia University, New York, NY 10032, USA*
[c]*Department of Veterans Affairs, Richard L. Roudebush VAMC, Indianapolis, IN 46202, USA*

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Abstract

Insulin regulation of energy metabolism is complex and involves numerous signaling cascades. Insulin has been suggested to stimulate a phospholipase that cleaves glycosylphosphatidylinositols resulting in the generation of an inositol glycan that serves as an insulin mediator. To determine if glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) may play a role in glucose metabolism, we examined the effect of overexpressing GPI-PLD using adenovirus-mediated gene transfer in C57BL/6 mice. Overexpressing GPI-PLD was associated with a decrease in fasting glucose as well as an improvement in glucose tolerance as determined by an intraperitoneal glucose tolerance test. This effect to improve glucose tolerance does not result from an increase in insulin sensitivity, as overexpressing GPI-PLD does not alter the response to insulin. In contrast, the insulin response during the glucose tolerance test in GPI-PLD–overexpressing mice was increased. Overexpressing GPI-PLD in an insulinoma cell line enhanced glucose-stimulated insulin secretion, suggesting that enhanced insulin secretion in vivo may have contributed to the improved glucose tolerance.
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1. Introduction

Regulation of the intake, utilization, and storage of energy is a highly integrated process involving the interaction of numerous organs, genes, hormones, and metabolites [1,2]. Disruption of these processes can lead to various diseases including obesity, atherosclerosis, and type 2 diabetes mellitus. Insulin is a major regulator of blood glucose levels, and considerable effort has been spent on understanding insulin's signaling mechanisms. Multiple signaling pathways have been identified that account for insulin's effect on metabolism.

More than 30 years ago, dissociations of insulin actions suggested the presence of an insulin second messenger [3]. Later, a hypothesis was put forward that glycosylphosphatidylinositols (GPIs) served as precursors for an insulin mediator [4]. In this model, insulin's interaction with its receptor stimulated a phospholipase to cleave GPIs to release inositol glycans [5,6]. These inositol glycans then served as second messengers to mediate some of the signaling events of insulin including stimulation of glucose uptake and oxidation, increasing pyruvate dehydrogenase activity [7] and glycogen synthase phosphatase [8], and inhibiting cyclic adenosine monophosphate (AMP) phosphodiesterase [9,10] and protein kinase A activity [8]. The involvement of GPIs was based upon the observations that insulin stimulated cleavage of a phospholipid that could be metabolically labeled with glucosamine, galactosamine, and inositol. Mass spectrometer analysis confirmed the presence of these constituents of partially purified preparations of these "precursors." Larner et al [11] later identified one class of glycans containing *myo*-inositol and glucosamine [12] and another containing D-*chiro*-inositol and galactosamine. Its novel β1-4 linkage was established by 2-dimensional nuclear magnetic resonance, and it was chemically synthesized. It regulates subcellular glucose disposal by activating protein phosphatase 2C that dephosphorylates and activates glycogen synthase and pyruvate

* Corresponding author. Eli Lilly & Company, Indianapolis, IN 46285. Tel.: +1 317 277 9933; fax: +1 317 276 4198.
E-mail address: deegma@lilly.com (M.A. Deeg).

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dehydrogenase phosphatase that dephosphorylates and activates mitochondrial pyruvate dehydrogenase. Glycogen synthase and pyruvate dehydrogenase are the rate-limiting enzymes of nonoxidative and oxidative glucose disposal. Additional evidence for a role of inositol glycans in insulin action include the following: (1) antibodies to the *core* GPI *myo*-inositol glycan inhibited insulin action in BC3H1 cells [13], (2) purified and structurally defined inositol glycans [11,14-17] and a *pseudo*-disaccharide containing D-*chiro*-inositol as pinitol 3-*O*-methyl ether and galactosamine mimicked insulin action in vivo and in cells [18], and (3) insulin did not stimulate glycogen synthesis in K562 cells defective in GPI biosynthesis [19].

As an alternative approach to examining this hypothesis, we focused on GPI phospholipases. The only GPI-specific phospholipase identified to date in mammals is GPI-specific phospholipase D (GPI-PLD). The highest level of GPI-PLD expression occurs in the liver, but GPI-PLD can be found in nearly every tissue and cell type [20,21]. Serum contains a high level of GPI-PLD where it associates with a high-density lipoprotein–like particle [22,23]. The function of serum GPI-PLD is as yet unclear, as GPI-PLD appears to catalytically inactive in serum [24]; but elevations in serum GPI-PLD are associated with alterations in serum triglyceride-rich lipoprotein metabolism [25]. Glycosylphosphatidylinositol-specific phospholipase D is also associated with various cellular membrane fractions including the plasma membrane were GPI-PLD associates with lipid rafts [26] including caveolae. To test the hypothesis that GPIs may be involved in insulin's action, we overexpressed GPI-PLD using adenovirus-mediated gene transfer and examined its affect on glucose tolerance.

2. Materials and methods

2.1. Materials

βTC6f7 cells [27] were obtained from Dr Shimon Efrat (Albert Einstein College of Medicine, New York, NY). βTC6f7 cells were maintained in Dulbecco modified Eagle medium (DMEM) containing 25 mmol/L glucose supplemented with 15% horse serum and 2.5% fetal bovine serum (growth medium).

2.2. Animals

C57BL/6 male mice (8 weeks of age) were purchased from Harlan (Indianapolis, IN). Mice were maintained in a temperature-controlled (25°C) atmosphere with a 12:12-hour light-dark cycle with free access to food and water. Animals were fed a chow diet (protein 20%, fat 9%, and carbohydrates 71% of total kilocalories; Purina Mills, St Louis, MO; Mouse Chow 20). All animal studies were approved by the Institutional Animal Care and Use Committee of Indiana University and the Roudebush VA Medical Center.

2.3. Intraperitoneal glucose tolerance test/insulin tolerance test

To determine if GPI-PLD plays a direct role in glucose metabolism, C57BL/6 mice were injected with saline, a control adenovirus expressing β-galactosidase (AdLacZ, 10^9 plaque-forming units [pfu]), or GPI-PLD (AdGPI-PLD, 10^9 pfu) as previously described [25]. Serum alanine transferase activity was determined to confirm hepatic infection of adenovirus (Table 1). Approximately 95% to 99% of the adenovirus was expressed in the liver [25]. Seven days post–virus administration, an intraperitoneal glucose tolerance test or an insulin tolerance test was performed. For the intraperitoneal glucose tolerance test, mice were fasted for 24 hours and then given glucose (2 g/kg intraperitoneally); and serum glucose was measured at 0, 30, 60, 90, and 120 minutes and serum insulin at 0, 30, and 60 minutes. For the insulin tolerance test, mice were fasted for 4 hours and then treated with insulin (0.75 U/kg body weight intraperitoneally); and serum glucose was determined at 0, 15, 30, 45, 60, 90, and 120 minutes. Blood was obtained via tail vein.

2.4. Insulin secretion studies

For insulin secretion studies, cells were plated in 35-mm dishes in 2 mL of growth medium. When cells reached 60% confluence, cells were transduced with various amounts of AdGPI-PLD or AdLacZ in DMEM containing 25 mmol/L glucose. Five hours after the addition of the virus-containing medium, the medium was switched to DMEM containing 25 mmol/L glucose and 10% fetal bovine serum and incubated for an additional 19 hours. The medium was then switched to DMEM containing 5 mmol/L glucose supplemented with fatty-acid–free bovine serum albumin (1 mg/mL). After 24 hours, the cells were rinsed twice with 1 mL of Krebs-Ringer bicarbonate buffer with 0.1% bovine serum albumin containing no glucose and preincubated for

Table 1
Effect of GPI-PLD overexpression on serum chemistries

	Control	AdLacZ	AdGPI-PLD
Cholesterol (mmol/L)	2.90 ± 0.28 (7)	2.85 ± 0.56 (8)	3.45 ± 0.89 (9)
Triglycerides (mmol/L)	0.85 ± 0.12 (10)	0.96 ± 0.16 (17)	1.24 ± 0.26 (19)*
Alanine transferase (U/L)	72 ± 30 (8)	285 ± 190 (13)†	468 ± 320 (17)†
β-Hydroxybutyrate (mmol/L)	0.13 ± 0.05 (6)	0.08 ± 0.04 (9)†	0.09 ± 0.03 (11)
Glucose (mg/dL)	174 ± 10 (6)	180 ± 20 (9)	153 ± 18 (11)‡
Serum GPI-PLD (U/mL)	3.42 ± 0.57 (10)	3.02 ± 0.64 (17)	17.20 ± 2.25 (19)*

C57BL/6 mice were treated with saline or infected with AdLacZ or AdGPI-PLD as described in "Materials and methods." Seven days after infection, serum chemistries were determined after a 4-hour fast.

* $P < .001$ vs control or AdLacZ, by 1-way ANOVA.

† $P < .05$ vs control, by 1-way ANOVA.

‡ $P < .01$ vs AdLacz, by 1-way ANOVA.

an additional 1 hour in the same medium. The secretion study was initiated by removing the medium and adding 1 mL of Krebs-Ringer bicarbonate buffer with 0.1% bovine serum albumin containing either 0 or 20 mmol/L glucose. After 30 minutes, the medium were removed, centrifuged, and aliquoted for measuring insulin and GPI-PLD activity. The cells were washed twice with phosphate-buffered saline, and cells were extracted with 95% ethanol containing 225 mmol/L HCl at the end of the incubation for determining total cellular insulin.

For experiments measuring insulin messenger RNA (mRNA), βTC6f7 cells were grown as above; and total RNA was extracted using TriPure (Roche Diagnostics, Indianapolis, IN) followed by further purification with Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA) and was stored at −80°C until used for Northern blotting. Preproinsulin mRNA was detected and normalized to β-actin mRNA as previously described [28].

2.5. Analytical assays

Cholesterol, triglycerides, β-hydroxybutyrate, glucose, and alanine transferase assays were performed by the Indiana University Endocrinology Analyte Core using commercially available kits (Sigma-Aldrich, St Louis, MO). Blood for serum chemistry in Table 1 was obtained via cardiac puncture. Serum insulin was determined using a radioimmunoassay (Linco Research, St Charles, MO). The GPI-PLD activity was determined as previously described [25].

2.6. Statistical analysis

Incremental area under the curve (AUC) for glucose in the glucose tolerance test or insulin tolerance test was calculated using the trapezoid rule. Statistical analyses were performed using SigmaStat software (version 3.1; Systat, San Jose, CA). Comparison between 3 groups was done using 1- or 2-way analysis of variance (ANOVA) as appropriate. Data are presented as mean ± SD with the number in parentheses unless otherwise indicated. A $P < .05$ was considered statistically significant.

3. Results

3.1. Overexpressing GPI-PLD improves glucose tolerance

To determine if GPI-PLD is involved in glucose metabolism, we overexpressed GPI-PLD using adenovirus-gene–mediated transfer. We reasoned that if GPI-PLD was involved in insulin signaling, altering its expression in liver would alter hepatic and whole-body glucose metabolism. Mice were treated with vehicle, AdLacZ, or AdGPI-PLD; and serum GPI-PLD and glucose were determined. After 7 days, serum GPI-PLD levels were increased approximately 5-fold (Table 1), whereas the fasting glucose was lower, in the AdGPI-PLD–infected animals compared with the AdLacZ and control mice (Table 1). In contrast, serum triglycerides were increased as previously described [20].

To further characterize this observation, we examined the effect of overexpressing GPI-PLD on glucose and insulin levels during a glucose tolerance test. After 24 hours of fasting, the blood glucoses did not differ between groups (9.3 ± 0.8 mmol/L [6], 10.4 ± 1.4 [9], and 9.4 ± 1.2 [9] for the control, AdLacZ-treated, and AdGPI-PLD–treated animals, respectively). Although the fasting glucoses did not differ between groups, the peak glucose concentration and the incremental AUC area for glucose during the glucose tolerance test was 40% and 30% lower, respectively, in the AdGPI-PLD–treated animals compared with the control or AdLacZ-treated animals (Fig. 1). This change was associated with an increase in serum insulin at 30 minutes in the AdGPI-PLD–treated group (Table 2). To determine if whole-body insulin sensitivity was altered by GPI-PLD, we conducted an insulin tolerance test.

For the insulin tolerance test, the mice were fasted for 4 hours. The fasting glucose levels were 9.9 ± 0.9 (6), 9.8 ± 0.7 (9), and 8.5 ± 1.2 (9) mg/dL for the control, AdLacZ-treated, and AdGPI-PLD–treated animals. Although the fasting glucose levels were significantly lower in the AdGPI-PLD–treated animals ($P < .01$ vs control or AdLacZ), the incremental AUC for glucose in response to insulin was not different between groups (Fig. 2). In addition, the rate of fall of glucose during the first 15 minutes of the insulin tolerance test did not differ between groups (1.9 ± 1.7 [6], 1.5 ± 1.1 [9], and 1.2 ± 1.7 [9] mmol/L per 15 minutes for the control, AdLacZ-treated, and AdGPI-PLD–treated animals, respectively).

Together, these results demonstrate that overexpressing GPI-PLD improves glucose tolerance with an increase in insulin levels without a change in insulin sensitivity. Although skeletal muscle glucose uptake accounts for the majority of insulin-stimulated glucose uptake in vivo [29], these experiments do not preclude an effect of GPI-PLD on hepatic insulin sensitivity or glucose uptake or an effect on islet β-cell activity. Because systemic administration of adenovirus can infect the islet β-cell [30], we examined the effect of overexpressing GPI-PLD on insulin secretion in vitro.

3.2. Overexpressing GPI-PLD in an insulinoma cell line enhances glucose-stimulated insulin secretion

To determine if overexpressing GPI-PLD alters insulin secretion, βTC6f7 cells were treated with buffer (control) or transduced with increasing amounts of either AdLacZ or AdGPI-PLD; and the effects on insulin secretion and content were examined. The control virus had no effect on GPI-PLD content or secretion, whereas overexpressing GPI-PLD increased GPI-PLD secretion in a concentration-dependent manner (Fig. 3A). In contrast, glucose-stimulated insulin release was enhanced in the GPI-PLD–overexpressing cells compared with AdLacZ-transduced cells (Fig. 3B). Glucose

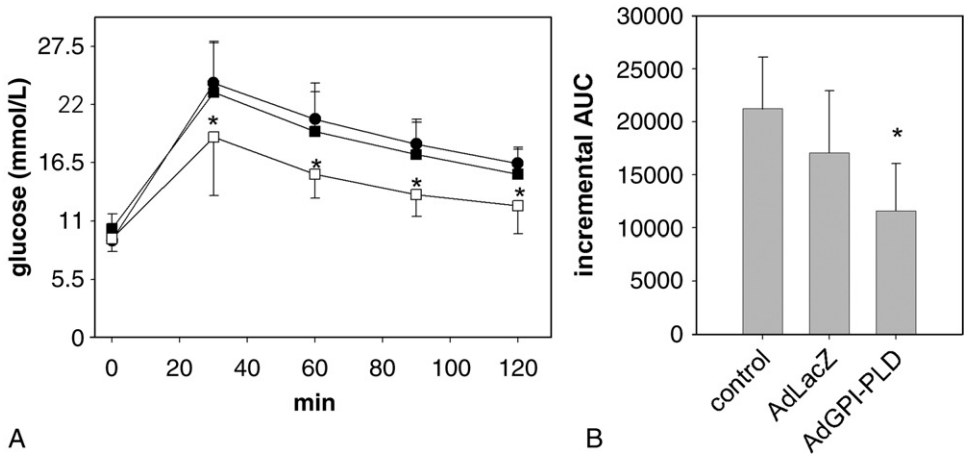

Fig. 1. Overexpressing GPI-PLD improves glucose tolerance. C57BL/6 mice were transduced with AdLacZ (■) or AdGPI-PLD (□) or treated with saline (●) as described in "Materials and methods." A, After 7 days, mice were fasted for 24 hours and then given glucose (2 g/kg intraperitoneally); and serum glucose was measured at 0, 30, 60, 90, and 12 minutes. n = 5 to 9 per time point. B, Area under the glucose curve was calculated by the trapezoid rule. *$P < .05$ by 1-way ANOVA.

stimulated insulin secretion 2-fold in control cells, whereas in GPI-PLD–overexpressing cells, glucose-stimulated insulin secretion was increased 4-fold.

To determine if this effect of GPI-PLD could be accounted for by changes in insulin content, we determined the effect of GPI-PLD on insulin content. Total insulin content was similar in control and AdLacZ-treated cells, but overexpressing GPI-PLD did result in a concentration-dependent increase in total insulin content (Fig. 3C). At the highest concentration of AdGPI-PLD, total insulin content increased approximately 35%. In contrast, overexpressing GPI-PLD did not affect the preproinsulin mRNA levels (Fig. 3D).

These results demonstrate that overexpressing GPI-PLD enhances glucose-stimulated insulin secretion that may in part be due to an increase in insulin content in β-cells.

4. Discussion

Glycosylphosphatidylinositols have been implicated in mediating the action of numerous hormones, including insulin; and defective inositol glycan signaling has been implicated in type 2 diabetes mellitus [31], polycystic ovarian syndrome [32], and preeclampsia [33]. Taking an alternative approach, we increased GPI-PLD expression, resulting in a decrease in fasting glucose and an improvement in glucose tolerance. With our experimental approach, liver, skeletal muscle, and islet β-cells could all potentially be involved.

Table 2
Serum insulin levels during the intraperitoneal glucose tolerance test

Treatment	0 min	30 min	60 min
Control	78.3 ± 8.3 (5)	83.3 ± 16.6 (6)	86.6 ± 10.0 (5)
AdLacZ	73.3 ± 8.3 (7)	74.9 ± 8.3 (9)	69.9 ± 6.7 (7)
AdGPI-PLD	79.9 ± 8.3 (9)	109.9 ± 35.0 (9)*	79.9 ± 13.3 (8)

Serum insulin levels during the intraperitoneal glucose tolerance test from Fig. 1. Values are in picomoles per liter.

* $P < .001$ vs 30-minute AdLacZ and 0- and 60-minute AdGPI-PLD, by 2-way ANOVA.

The GPI-PLD–induced changes in liver are most likely to be involved given that the majority of adenovirus given systemically is cleared and expressed in the liver. It is possible that the hepatic infection per se could have resulted in a change in glucose metabolism secondary to the inflammatory response in the liver. However, our observation that the vehicle- and AdLacZ-treated animals have identical glucose responses suggests that if this effect occurs, it is negligible. Definitive glucose clamp studies are needed to fully characterize the effect of increasing GPI-PLD on skeletal muscle and hepatic glucose uptake and insulin sensitivity.

How exactly overexpressing GPI-PLD might affect glucose metabolism is not clear. Preliminary microarray experiments in our laboratory showed that overexpressing GPI-PLD in HepG2 cells results in an increase in gene expression leading to increased glycolysis, and cholesterol and fatty acid synthesis. Among the genes affected was phosphofructokinase (1.7-fold), which may be sufficient to explain an effect on hepatic glucose metabolism because the level of phosphofructokinase mRNA expression correlates to the glucose AUC during a glucose tolerance test in mice [34]. Our observation that overexpressing GPI-PLD is associated with a lower fasting glucose after 4 hours but not 24 hours may reflect the fact that beyond 4 hours of fasting, small rodents are in a semistarvation state with increases in gluconeogenesis [35], fatty acid, and amino acid metabolism [36,37].

In addition to an effect on liver, it is possible that nonhepatic tissues, including skeletal muscle and islet β-cells, were transduced with GPI-PLD. This cannot be

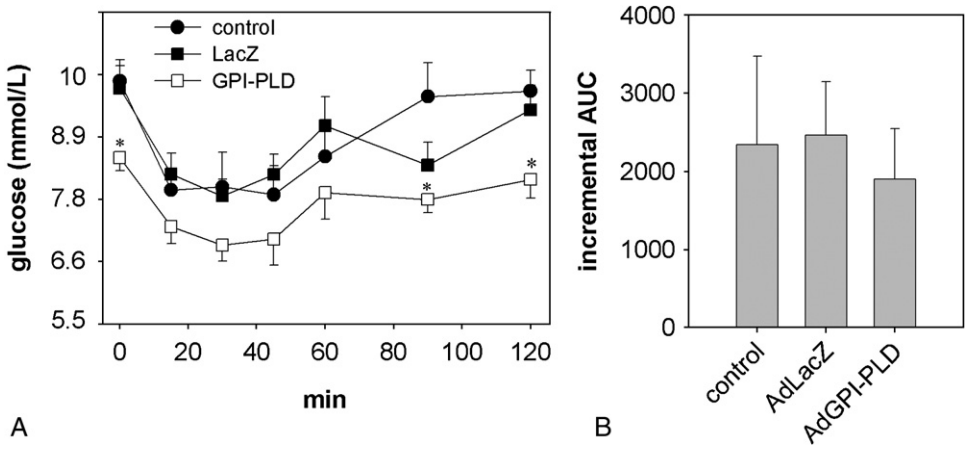

Fig. 2. Overexpressing GPI-PLD does not alter the response to insulin. C57BL/6 mice were infected with AdLacZ (■) (n = 9) or AdGPI-PLD (□) (n = 9) or treated with saline (●) (n = 6) as described in "Materials and methods." A, After 7 days, mice were fasted for 4 hours and then treated with insulin (0.75 U/kg body weight intraperitoneally); and serum glucose was determined at 0, 15, 30, 45, 60, 90, and 120 minutes. B, Area under the glucose curve was calculated by the trapezoid rule. *$P < .05$ by 1-way ANOVA.

completely discounted because very small amounts of GPI-PLD are expected to be overexpressed in nonhepatic tissues [25,30]. Glycosylphosphatidylinositol-specific phospholipase D has been suggested to play a role in glucose metabolism in skeletal muscle, as a GPI-PLD has been implicated in translocation of the glucose transporter 4 transporter in a cell-free rat skeletal muscle system [38], and an mRNA related to GPI-PLD (corresponding only to the C-terminal β propeller and not the N-terminal catalytic domain) is increased in skeletal muscle of *ob*/*ob* mice relative to wild-type mice [39]. The vast majority of the glucose uptake during an insulin tolerance test occurs in skeletal muscle.

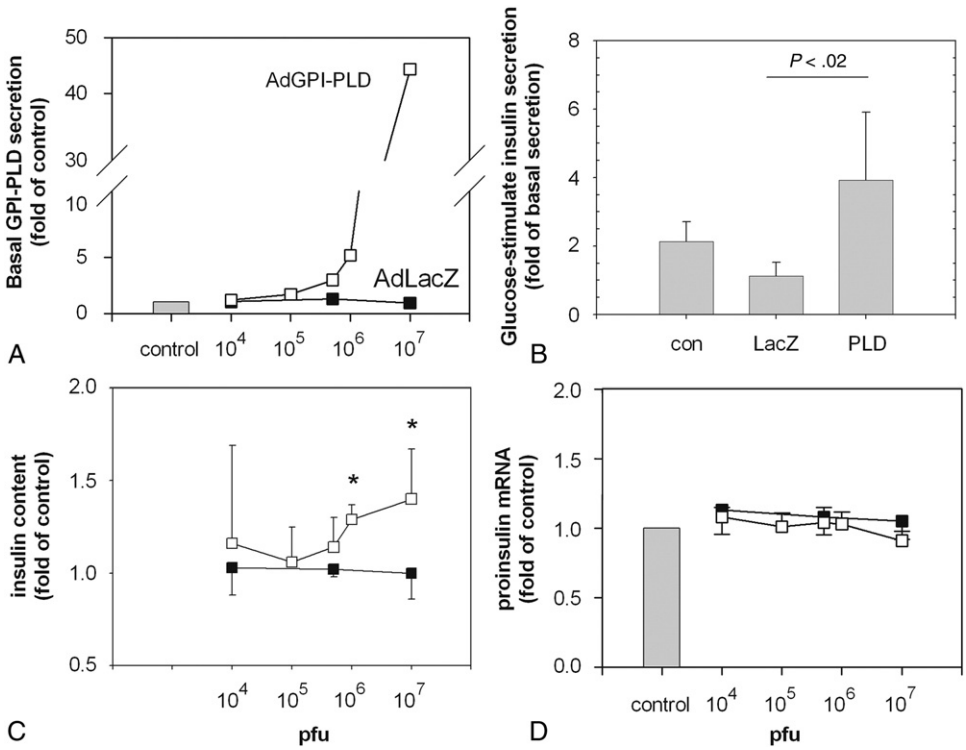

Fig. 3. Effect of GPI-PLD overexpression on insulin secretion in βTC6f7 cells. βTC6f cells were treated with vehicle or transduced with AdLacZ or AdGPI-PLD with the amount of virus as indicated as described in "Materials and methods"; and basal GPI-PLD secretion (A), insulin content (C), and proinsulin mRNA (D) were measured. B, βTC6f7 cells were treated with vehicle or transduced with 10^7 pfu of AdLacZ (LacZ) or AdGPI-PLD (PLD) and insulin secretion in the absence (0 mmol/L glucose) or presence of glucose (20 mmol/L) as described in "Materials and methods." All experiments are the results of 4 independent experiments done in triplicate. *$P < .05$ by 1-way ANOVA.

Because overexpressing GPI-PLD did not affect the glucose fall or incremental AUC in response to insulin, it is unlikely that the insulin sensitivity of skeletal muscle was affected by GPI-PLD overexpression.

Our observation that systemic administration of AdGPI-PLD resulted in an increase in serum insulin response during a glucose tolerance test suggests an effect on islet β-cells. Systemic administration of adenovirus has been shown to transduce a small percentage of islet β-cells [30]. Although it is possible that there is an indirect effect to improve islet β-cell function, it is clear from our in vitro experiments that overexpressing GPI-PLD in a β-cell line improves glucose-stimulated insulin secretion; therefore, enhanced insulin secretion may also contribute to the improvement in glucose tolerance we observed in vivo. However, this effect on β-cells probably does not account for our observation that overexpressing GPI-PLD reduces fasting glucose, as fasting insulin was not different between control and AdGPI-PLD–treated mice. It is interesting to note that in mouse models of insulin resistance, islet β-cell expression of GPI-PLD is increased [40], suggesting that GPI-PLD may be part of the compensatory response to increased insulin demand. The GPI-PLD enhancement of insulin secretion most likely occurs via activation of protein kinase Cα [41].

If GPI-PLD plays a role in insulin or hormone signaling, then its activity must be regulated. We and others have shown that the expression of GPI-PLD mRNA is altered by various stimuli under different pathologic conditions [42-44]. At a minimum, this increase in GPI-PLD expression results in a constitutively active GPI-PLD involved in the continuous release of GPI-anchored proteins [45-48]. This alters the cell surface expression of GPI-anchored proteins that in turn can affect cell physiology [49]. This cleavage occurs early in the endoplasmic reticulum and Golgi [26]. Although GPI-PLD activity can be found in these compartments, the specific activity is low, suggesting that GPI-PLD–mediated cleavage is slow likely because of the microenvironment of the GPI anchor [50]. Acute regulation of GPI-PLD activity to release of GPI-anchored proteins and/or inositol glycans has not been demonstrated, although insulin has been shown to stimulate release of inositol glycans on a rapid time frame (seconds to minutes) [51].

Finally, the effects of GPI-PLD may be independent of insulin as suggested by the increase in PFK that would lead to an increase in glycolysis. This would be consistent with the increase in liver fatty acid synthesis with the concomitant increase in serum triglycerides we observe with over-expressing GPI-PLD [25]. It is also possible that over-expressing GPI-PLD may affect AMP kinase, as insulin has been shown to acutely inactivate AMP kinase that would lead to increased adenosine triphosphate utilization for lipid synthesis [52,53].

There are numerous limitations to our study. The primary limitation is that at least 2 organs, the liver and islet β-cells, were affected using adenovirus-mediated gene transfer to overexpress GPI-PLD, thereby complicating the interpretation. Numerous experiments are needed to determine the biochemical details in the liver and β-cells to clarify the effect on hepatic insulin signaling, GPI-PLD promoter activity, and insulin secretion. Although C57BL/6 mice were chosen because they have low levels of serum GPI-PLD [54], they do not display abnormal glucose metabolism. Additional experiments are needed to determine if GPI-PLD affects glucose metabolism and insulin secretion in models of insulin resistance and diabetes.

In summary, increased expression of GPI-PLD is associated with improvements in fasting glucose and glucose tolerance. Additional experiments are required to examine the mechanism by which this occurs, but a role for inositol glycans is not precluded.

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