10-2017

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Notes/Citation Information
Published in *Endocrine*, v. 58, issue 1, p. 47-58.

Digital Object Identifier (DOI)
https://doi.org/10.1007/s12020-017-1379-1

HHS Public Access

Author manuscript

Endocrine. Author manuscript; available in PMC 2018 October 01.

Published in final edited form as:

Endocrine. 2017 October ; 58(1): 47–58. doi:10.1007/s12020-017-1379-1.

The Dual Role of Group V Secretory Phospholipase A_2 in Pancreatic β-cells

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Abstract

Group X (GX) and group V (GV) secretory phospholipase A_2 ($sPLA_2$) potently release arachidonic acid (AA) from the plasma membrane of intact cells. We previously demonstrated that GX $sPLA_2$ negatively regulates glucose-stimulated insulin secretion (GSIS) by a prostaglandin E2 (PGE2)-dependent mechanism. In this study we investigated whether GV $sPLA_2$ similarly regulates GSIS. GSIS was significantly decreased in islets isolated from GV $sPLA_2$-deficient (GV KO) mice compared to wild-type (WT) mice. Similarly, GSIS was significantly decreased in MIN6 cells, a murine pancreatic beta cell line with siRNA-mediated GV $sPLA_2$ suppression. MIN6 cells overexpressing GV $sPLA_2$ (MIN6-GV) showed a significant increase in GSIS compared to control cells. The amount of AA released into the media by MIN6-GV cells was significantly higher compared to control cells. However, MIN6-GV cells did not exhibit enhanced PGE2 production or decreased cAMP content compared to control MIN6 cells. Surprisingly, GV KO mice exhibited a significant increase in plasma insulin levels following i.p. injection of glucose compared to WT mice. This increase in GSIS in GV KO mice was associated with a significant increase in pancreatic islet size and number of proliferating cells in β-islets compared to WT mice. Thus, deficiency of GV $sPLA_2$ results in diminished GSIS in isolated pancreatic beta-cells. However, the reduced GSIS in islets lacking GV $sPLA_2$ appears to be compensated by increased islet mass in GV KO mice.

Keywords

GV $sPLA_2$; type 2 diabetes; insulin secretion; Arachidonic acid; β-islet mass

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Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in studies involving mice were in accordance with the guidelines of the University of Kentucky Institutional Animal Care and Use Committees. The article does not contain any studies with human participants performed by any of the authors.

Introduction

Type 2 diabetes (T2D) is increasing at an alarming rate worldwide, largely due to an epidemic of obesity and reduced physical activity. T2D results from chronic insulin resistance that ultimately leads to a progressive decline in pancreatic β-cell function. Although insulin resistance precedes the development of hyperglycemia in subjects that eventually develop T2D, the disease manifests itself in insulin-resistant subjects only with the onset of β-cell dysfunction and reduced β-islet mass [1].

Accumulating evidence indicates that arachidonic acid (AA) and its metabolites play key roles in regulating β-cell function. AA constitutes >30% of the glycerolipid fatty acid mass in rodent islets [2], and stimulation of β-cells with glucose is accompanied by release of free AA. Inhibition of AA release impairs glucose-stimulated insulin secretion (GSIS) [3]. The exact mechanisms linking AA and insulin secretion are not clearly understood. While AA is in general considered to be an activator of GSIS, a major metabolite of AA, prostaglandin E2 (PGE2), is a known inhibitor of GSIS [4-9]. It is believed that PGE2 exerts its effect by interacting with its receptor, EP3, thus decreasing adenylyl cyclase activity with a subsequent reduction in cAMP, a known activator of GSIS [10, 11]. Apart from their effects on GSIS, AA and its metabolites have also been implicated in the regulation of β-cell mass. For example, transgenic overexpression of cyclooxygenase-2 (COX-2), a rate-limiting enzyme for prostanoid biosynthesis including PGE2, resulted in significantly decreased pancreatic β-cell number in mice, leading to a significant increase in blood glucose levels. The reduction in β-cell mass in COX-2 transgenic mice was shown to be due to inhibited β-cell proliferation [12].

The phospholipase A_2 (PLA_2) family comprises a group of intracellular and secreted enzymes that hydrolyze phospholipids to yield free fatty acids and lysophosholipids. This reaction is considered to be the initial, rate-limiting step of AA metabolism leading to the production of bioactive lipids including prostaglandins and leukotrienes. Calcium-independent PLA_2 ($iPLA_2$) enzymes have been implicated in glucose-stimulated AA release. Moreover, inhibition of $iPLA_2\beta$ using a pharmacological inhibitor, bromoenol lactone suicide substrate, inhibited AA release and GSIS in vitro [3, 13]. Similarly, knockdown of $iPLA_2\beta$ expression in INS1 cells decreased insulin secretion [14]. Transgenic overexpression of iPLA2β in islet β-cells resulted in enhanced GSIS; consistently, islets from male $iPLA_2\beta$ -null mice exhibited blunted insulin secretion. The role of cytosolic phospholipase A_2 ($cPLA_2$) in insulin secretion has also been investigated. Overexpression of $cPLA_2$ enhances exocytosis of insulin from β-cells [15]. However, sustained expression of $cPLA_2$ has been reported to upregulate uncoupling protein-2 (UCP-2) and consequent mitochondrial uncoupling, which drastically reduces the capacity of β-cells to respond to nutrients [16]. Consistently, another study documented that overexpression of $cPLA_2$ has no effect on insulin content or the basal rate of insulin secretion, yet negatively effects GSIS, probably due to the prolonged exposure of β-cells to AA [17].

Among the secretory PLA_2 ($sPLA_2$) family members, group IB $sPLA_2$ is reportedly expressed in pancreatic islets of rodents and localized within insulin granules. Stimulation with glucose results in the co-secretion of insulin and group IB $sPLA_2$ [18]. However, the

role of the enzyme in GSIS or pancreatic β-cell function is not known. Recently, we reported that another member of the $sPLA_2$ family, group X $sPLA_2$ (GX $sPLA_2$) is expressed in insulin-producing cells of mouse pancreatic islets and negatively regulates GSIS by enhancing the production of PGE2. Moreover, C57BL/6 mice deficient in GX $sPLA_2$ had increased plasma insulin levels following glucose challenge compared to wild-type mice [19].

Thus, based on available evidence, it appears that during glucose-stimulation, the liberation of AA positively modulates insulin secretion, whereas the generation of AA metabolites such as PGE2 negatively modulates insulin secretion. Therefore, the regulatory effect of individual PLA_2's likely depends on the potency and kinetics of AA/PGE2 production. In this study, we investigated the role of yet another member of the $sPLA_2$ family, group V $sPLA_2$ (GV $sPLA_2$) in pancreatic β-cell function. In vitro studies using artificial phospholipid substrates indicate that GV and GX $sPLA_2$s are the most potent in hydrolyzing phosphatidylcholine [20], the major phospholipid on mammalian cell membranes. However, it is not clear whether these two closely related $sPLA_2$ family members perform redundant physiological functions in vivo.

Materials and Methods

Biochemical Reagents and Assays

Assays for Insulin (Crystal Chem Inc), PGE2 metabolites (Cayman) and cAMP (ENZO Life Sciences) were performed according to the manufacturers' instructions. Phospholipase activity in conditioned media was measured using a colorimetric assay as we previously described [21] with 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG; Matreya LLC) as a substrate. Briefly, mixed micelles were prepared by warming 7 mg of POPG to 37°C in a 0.2 mL mixture of 4.0% (wt/vol) Nonidet-40 and 2.0% sodium deoxycholate, and then adding 1.8 mL warm assay buffer (0.12 mol/L Tris-HCl pH 8, 12 mmol/L $CaCl_2$, 0.1 mmol/L EDTA). For enzyme assays, 10 μL of conditioned media was added to 40 μL of substrate solution. After incubating for 20 minutes at 37°C, the amount of free fatty acids (FFA) released was quantified using a NEFA-C kit (Wako Chemicals); phospholipase activity was calculated as the nmol of FFA released in 20 minutes per mg cell protein.

Animals

GV *$sPLA_2$*-deficient (GV KO) mice, backcrossed >10 times with C57BL/6 mice, were originally provided by Dr. Jonathan Arm [22]. Mice were maintained on a 10-h light/14-h dark cycle and received standard mouse chow and water *ad libitum.* Male mice were used throughout the study. All procedures were in accordance with the guidelines of the University of Kentucky Institutional Animal Care and Use Committees.

Islet isolation

Mouse islets were isolated via intraductal collagenase (Sigma) digestion and ficoll gradient centrifugation as described earlier [19]. A detailed method will be provided on request. Following isolation, similar-sized islets were selected and hand-picked and maintained in RPMI containing 5 mM glucose, 10% (v/v) FBS and penicillin and streptomycin.

Immunohistochemistry

Pancreata from WT and GV KO mice were embedded in paraffin and 5 μm-thick sections were mounted on glass slides; processing of the tissues on glass slides was done as described earlier [19]. The sections were immunostained using rabbit anti-mouse GV $sPLA_2$ (gift from Dr. M. Gelb, University of Washington) and goat anti-mouse insulin (Santa Cruz Biotechnology), at a dilution of 1:100 for anti-GV $sPLA_2$ and 1:500 for anti-insulin. For fluorescent images, Alexa Fluor-conjugated secondary antibodies were used (Invitrogen). For Ki67 staining, the sections were immunostained using rabbit anti-Ki67 (Abcam,1:150).

β-cell mass, average islet size, and β-cell proliferation

β-cell mass and average islet size were determined as described earlier [23]. Entire pancreata were removed from 4 GV KO and 4 WT mice (16 weeks old), adhering fat tissues as well as other nonpancreatic tissues were removed, and the organ was then weighed and fixed as described above and cut into 5 μm-thick sections. Every 30^{th} section (a total of 7-8 sections per pancreas) was immunostained for insulin, and then imaged under X10 magnification using NIS elements software (Nikon Instruments, Inc.). β-cell mass was calculated by first obtaining the fraction of the total cross-sectional area of the pancreatic tissue immunopositive for insulin and then multiplying the pancreatic weight by this fraction. Average islet size was calculated by dividing the total islet area by the total number of islets analyzed.

The number of nuclei positive for Ki67 within insulin-positive cells was counted to determine β-cell proliferation. Approximately 40 islets from 2 sections per mouse were analyzed in the proliferation assay (n= 3 mice per group).

In vivo GSIS, glucose tolerance and insulin tolerance tests

In vivo GSIS was performed as described earlier [19]. Mice were fasted for 16 h, and then plasma samples were collected from the retro-orbital sinus before and 15 min after *i.p.* glucose injection (3g/kg). For glucose tolerance tests, mice were fasted for 6 h. Blood glucose concentrations were quantified using a glucometer (Contour; Bayer Laboratories) immediately before and 15, 30, 60, 90, and 120 min following intraperitoneal (i.p) administration of glucose (1.5 g/kg body wt). Insulin tolerance was assessed following a 4-h fast by quantifying blood glucose concentrations at 0, 30, 60, 90 and 120 min after administration of human insulin (0.5 U/kg body wt i.p; Novolin, Novo Nordisk).

Cell culture and transfections

MIN6 cells were cultured in complete media (DMEM supplemented with 15% heat-inactivated fetal bovine serum, 2 mmol/l L-glutamine, 45 mmol/l β-mercaptoethanol, 100 units/ml pencillin and 100 μg/ml streptomycin). C-terminal Flag-tagged cDNA for GV $sPLA_2$ was constructed by PCR using forward (F) and reverse (R) primers containing Hind III and EcoRI restriction sites respectively: 5′-TACCCAAGCTTATGAAGGGTCTCCTCACA-3′(F) and 5′-GCGGAATTCTTACTTGTCATCGTCGTCCTTGTAGTCGCAGAGGAAGTTGGG-3′ (R) and mouse GV $sPLA_2$ cDNA as a template. The PCR product was inserted into the mammalian expression vector pcDNA 3.0 (Invitrogen, Carlsbad, CA) to yield a coding

sequence that expressed GV $sPLA_2$ with a C-terminal FLAG epitope tag. DNA sequencing was performed to confirm the integrity of the expression construct. The plasmids were transfected into MIN6 cells using Nucleofector Kit following the manufacturer's instructions and the program T-016 (Nucleofector Kit V; Lonza).

Arachidonic acid (AA) release assay

MIN6 cells transiently transfected with either the “empty” pcDNA 3.0 plasmid (MIN6-C) or the pcDNA3.0 GV $sPLA_2$ expression construct (MIN6-GV) were assayed 24 h after transfection. The transfected cells (~5×10^5cells/well pf 12-well plate) were incubated in 1 ml of complete medium (DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin) containing 0.1 μCi [^{3}H]arachidonate (200 Ci/mmol; American Radiochemicals Inc, Saint Louis, MO) for 12-16 h. The cells were then washed three times with complete medium followed by 6 h incubation in the same medium. The amount of tritium associated with cells and released into the medium was determined by scintillation counting.

Gene silencing with Small Interfering RNA (siRNA)

A set of pre-designed synthetic oligonucleotides directed to mouse GV $sPLA_2$ (Thermo Scientific, On-TARGETplus SMARTpool siRNA; L-042189-01-0010; mouse PLA2G5 18784) was used. The oligonucleotides were transfected into MIN6 cells using Nucleofector Kit (Nucleofector Kit V; Lonza) and the program T-016. Scrambled siRNA (Thermo Scientific; Non-targeting siRNA#1; D-001810-20) was used as control. The siRNAs were transfected at a final concentration of 2 μg of siRNA pool or non-targeting siRNA per 2×10^6 MIN6 cells. Cells were collected 24 h after transfection for RNA preparation to confirm gene silencing by real-time RT-PCR and used for GSIS assay 48 h after transfection.

Real time RT-PCR

Total RNA was prepared from MIN6 cells using the RNeasy Mini kit (Promega). Quantification was performed in duplicate using the standard curve method and normalized to 18S RNA. The primer sequences used for GV $sPLA_2$ mRNA detection were 5′-AGG GGG CTT GCT AGA ACT CA -3′ (F) and 5′-CAA TCA GTG CCA TCC TTG G -3′ (R).

In vitro insulin secretion assays

In vitro GSIS assays were carried out as described earlier [19]. Briefly, after isolation and handpicking, islets from WT and GV KO mice were cultured overnight in RPMI media. Islets were then selected and transferred to culture inserts (Greiner bio-one) in 12-well plates (25 islets per well), washed, and equilibrated for 1 h in Buffer 1 (DMEM supplemented with 38 mM sodium bicarbonate, 4 mM L-glutamine, 1 mM sodium pyruvate, 4.65 mM HEPES and 1 g/l BSA) containing 5 mM glucose, and then incubated successively for 40 min in Buffer 1 containing 5 mM glucose (low glucose) followed by 40 min in Buffer 1 containing 20 mM glucose (high glucose). At the end of the incubations, insulin content in the low and high glucose-containing buffers was assayed and normalized to total cellular insulin content, which was determined after lysing islets in acid-ethanol (75% ethanol, 0.2 mol/l HCl). To ensure that islets from the two strains of mice were challenged sufficiently to detect

differences in response to glucose, we performed additional *in vitro* GSIS assays in which islets were equilibrated for 1 h in Buffer 1 containing low glucose (1 mM) and then incubated successively for 40 min in Buffer 1 containing physiological glucose (5 mM) followed by 40 min in Buffer 1 containing glucose (20 mM). The impact of GV $sPLA_2$ on GSIS was not different under the two pre-equilibration conditions (data not shown). For GSIS in MIN6-C and MIN6-GV cells, cells (70-80% confluent) in 24-well plates were washed once with Kreb's Ringer buffer containing 0.2% BSA (KRB-BSA) and 5 mM glucose and then equilibrated in the same buffer for 1 h. The media was replaced with fresh KRB-BSA supplemented with either 5 mM (low) glucose or 20 mM (high) glucose for 40 min. Insulin concentrations in conditioned media were normalized to total cell protein.

Statistics

Data are expressed as mean ± SEM. Results were analyzed by *t* test or by 1-way ANOVA followed by a Bonferroni post-test. $P<0.05$ was considered statistically significant. All statistical analyses were carried out using GraphPad Prism 4.

Results

GV $sPLA_2$ is expressed in mouse pancreatic islets

Recently, we reported that group X secretory phospholipase A_2 (GX $sPLA_2$) is expressed in pancreatic β-islet cells and negatively regulates insulin secretion through a mechanism mediated by cyclooxygenase-2-dependent prostaglandin E2 production [19]. In this study, we investigated whether a closely related member of the $sPLA_2$ family, group V $sPLA_2$, is expressed and functional in mouse β-islet cells. Double immunofluorescent staining of pancreatic sections from C57BL/6 mice showed positive immunoreactivity for GV $sPLA_2$ (Fig. 1; green staining) that was most intense in regions immunopositive for insulin (red staining), indicating that GV $sPLA_2$ is expressed in insulin-producing β-cells in mouse pancreas (Fig. 1; yellow on merged image). As expected, GV $sPLA_2$ immunoreactivity was not detected in pancreatic sections from GV KO mice (Fig. 1).

GV $sPLA_2$ augments glucose-stimulated insulin secretion (GSIS) in mouse pancreatic β-cell line

GV $sPLA_2$ is known to potentiate the release of arachidonic acid (AA) leading to eicosanoid generation, including PGE2, in several cell types [22, 24]. PGE2 is a negative regulator of glucose-stimulated insulin secretion (GSIS), whereas non-esterified AA is an activator of GSIS [6, 8, 25-28]. Therefore, to assess whether GV $sPLA_2$ regulates GSIS, we investigated the impact of forced overexpression in MIN6 cells, a mouse pancreatic β-cell line that expresses endogenous GV $sPLA_2$ (data not shown). Transient transfection of GV $sPLA_2$ cDNA resulted in a 1.8-fold increase in phospholipase activity secreted into the culture media of MIN6 cells (MIN6-GV) compared to media from cells transfected with control plasmid (MIN6-C; Fig. 2a). Insulin secretion was similar in MIN6-C and MIN6-GV cells incubated in low glucose (5 mM), indicating that overexpression of GV $sPLA_2$ does not impact insulin secretion under basal conditions (Fig. 2b). In contrast, insulin secretion in cells incubated in high glucose (20 mM) was significantly increased in MIN6-GV (2.8±0.19 μg/mg cell protein) compared to MIN6-C (1.95±0.06 μg/mg cell protein; p=0.0015).

Moreover, when expressed as fold-increase over basal, the magnitude of GSIS was significantly greater in cells overexpressing GV $sPLA_2$ (6.4±0.43) compared to control cells (4.8±0.16; p=0.0052; data not shown). The effect of GV $sPLA_2$ overexpression to enhance GSIS occurred in the absence of alterations in total cellular insulin content (Fig. 2c).

GV $sPLA_2$ enhances AA release but not PGE2 production in β-cells

Given the recognized importance of AA and its metabolite, PGE2, in regulating GSIS [6, 8, 27, 28], we next investigated the extent to which GV $sPLA_2$ overexpression enhances AA and PGE2 production in β-cells. MIN6-GV cells demonstrated a modest but significant increase in AA release compared to MIN6-C cells, consistent with GV $sPLA_2$'s known ability to hydrolyze glycerophospholipids on mammalian cell membranes (Fig. 2d) [29]. However, increased AA release was not associated with a significant increase in PGE2 secretion by MIN6-GV cells (Fig. 2e). Moreover, cellular cAMP was not significantly different in MIN6-GV compared to MIN6-C cells (Fig. 2f), as would be expected if enhanced GV $sPLA_2$ activity resulted in increased PGE2 generation. Thus, in contrast to what we observed with GX $sPLA_2$ [19], *it appears that AA released by MIN6 cells through the action of GV $sPLA_2$ is of insufficient magnitude, or is not effectively coupled to the prostaglandin synthetic pathway, to provide a detectable increase in PGE_2 production.*

Deficiency of endogenous GV $sPLA_2$ attenuates GSIS in β-cells

To understand the role of endogenous GV $sPLA_2$ in β -cell function, we next investigated the impact of siRNA-mediated silencing on GSIS in MIN6 cells. GV $sPLA_2$ expression was suppressed ~90% in MIN6 cells 24 h after transient transfection with siRNA targeting GV $sPLA_2$ (GV-siRNA; Fig. 3a). While the magnitude of GSIS in GV-siRNA cells (7.03±0.18 μg/mg cell protein) was significantly reduced compared to Scr-siRNA cells (8.8±0.35 μg/mg cell protein; p=0.004), basal insulin secretion was also significantly lower in GV-siRNA cells compared to Scr-siRNA cells (Fig. 3b). As a consequence, the fold-increase in insulin secretion over basal conditions was not significantly different for the two cell lines (6.5±0.16 for GV-siRNA versus 6.1±0.24 for Scr-siRNA).

The lack of a pronounced effect of siRNA-mediated GV suppression on GSIS in MIN6 cells may be the result of residual GV $sPLA_2$ activity in the silenced cells. It is also possible that GV $sPLA_2$ plays a relatively small role in regulating GSIS in this immortalized cell line. Thus, to more definitively assess the impact of endogenous GV $sPLA_2$ on β -cell function, we performed experiments with islets isolated from WT and GV KO mice. To rule out any potential differences in GSIS due to variations in islet size [30], similar-sized islets from WT and GV KO mice were used for the assay. We also confirmed that GX $sPLA_2$ expression was not significantly different in pancreatic islets isolated from GV KO mice compared to WT mice (data not shown). There was no significant difference in basal insulin secretion (5 mM glucose) between islets from WT and GV KO mice (Fig. 3c). As expected, insulin secretion by WT islets increased significantly in response to high glucose (20 mM). Conversely, GSIS was almost totally abolished in islets lacking GV $sPLA_2$ (Fig. 3c). Deficiency of GV $sPLA_2$ in primary islets did not significantly change total insulin content (Fig. 3d), consistent with findings in MIN6 cells (Fig. 2c). Taken together, our results demonstrate that GV $sPLA_2$

enhances GSIS in pancreatic β-cells, in marked contrast to our previous findings with GX $sPLA_2$ [19].

GV KO mice demonstrate enhanced GSIS in vivo

Our *in vitro* findings that GV $sPLA_2$ augments GSIS in cultured MIN6 cells and isolated islets prompted us to investigate the *in vivo* effect of GV $sPLA_2$ deficiency on glucose homeostasis in mice. 6-month-old GV KO mice showed a trend for decreased fasting glucose levels compared to age-matched WT mice (Fig. 4a), which was associated with a non-significant increase in fasting insulin levels in GV KO mice compared to WT (0 min, Fig. 4b). As expected, plasma insulin levels were significantly increased in both WT and GV KO mice 15 minutes after i.p glucose injection (3 mg/kg body weight). Surprisingly, in contrast to *in vitro* and *ex vivo* results, plasma insulin levels 15 min following glucose injection were significantly higher in GV KO mice compared to WT mice (Fig. 4b). Glucose disposal and insulin tolerance were not significantly different between the two strains of mice (Fig. 4c, d), nor was there a significant difference in body weights (data not shown).

GV KO mice have increased average islet size, β-cell mass and proliferation

Despite an apparent defect in GSIS in pancreatic islets that are deficient in GV $sPLA_2$ (Fig. 3c), GV KO mice demonstrated increased GSIS *in vivo* (Fig. 4b). This discrepancy motivated us to investigate potential differences in pancreatic β islets between GV KO and WT mice. Extensive morphometric analysis of pancreatic tissue identified a significant 1.5-fold increase in β-islet mass (estimated by determining the portion of total pancreatic area in tissue sections corresponding to insulin-positive β-islets and the respective pancreatic weights) in GV KO compared to WT pancreas (Fig. 5a). There was no significant difference in pancreatic weights between the two strains (Fig. 5b). Therefore, an increase in β-islet mass must reflect an increase in islet number or an increase in islet size, or both. There was no difference in average number of islets in the pancreas of GV KO mice compared to WT mice (data not shown). However, average islet size was significantly larger for GV KO mice compared to WT mice (Fig. 5c). β-cell proliferation was assessed in pancreatic sections from 12-weeks old WT and GV KO (Fig. 5d shows representative images of GV KO pancreatic section) mice by determining the total number of insulin-positive cells (red staining) that were also positive for Ki67 (green staining) and DAPI nuclear stain. This analysis showed a significant increase in the number of proliferating cells in β-islets in GV KO mice compared to WT (Fig. 5e).

Discussion

There is a large body of evidence pointing to arachidonic acid (AA) and its biologically active metabolites as key regulators of insulin secretion by β-cells [6, 25, 26, 28, 31, 32]. The important role of AA in β-cells is underscored by its relatively high abundance in islets, representing ~30% of the total mass of fatty acyl glycerophospholipids [33]. The liberation of AA from cellular membranes is dependent on the action of PLA_2 enzymes, which also represents the initial, rate-limiting step for the production of a myriad of AA-derived bioactive lipid mediators, including prostaglandins and leukotrienes. Despite the central role of PLA_2's in AA metabolism, the relative contributions of the various intracellular and

secreted forms of these enzymes in β-cells and their respective impact on the regulation of insulin secretion has not been fully defined. Thus, we have set out to investigate whether GV or GX $sPLA_2$ play a role in regulating insulin secretion by β-cells. These two related enzymes have been shown to hydrolyze cell membrane phospholipids with much higher efficiency compared to other $sPLA_2$ family members due to their high binding affinity to phosphatidylcholine (PC) [34, 35]. We previously reported that GX $sPLA_2$ suppresses GSIS through a cyclooxygenase-2-dependent mechanism [19]. In this report, we provide evidence that GV $sPLA_2$ regulates β-cell function in a manner that is distinct from GX $sPLA_2$.

Like GX $sPLA_2$, GV $sPLA_2$ is expressed by insulin-producing cells in mouse pancreas and by MIN6 cells, a mouse pancreatic β-cell line. However, in contrast to GX $sPLA_2$, gain and loss of function studies showed that GV $sPLA_2$ enhances GSIS in MIN6 cells. Moreover, primary mouse islet cells deficient in GV $sPLA_2$ exhibit significantly decreased GSIS ex vivo compared to pancreatic islets from wild-type mice. Taken together, our studies demonstrate the unexpected finding that whereas GX $sPLA_2$ expressed by β-cells suppresses GSIS, the related GV $sPLA_2$ enzyme serves to enhance GSIS. It is interesting to note we were unable to detect a significant increase in PGE_2 production in MIN6 cells with forced overexpression of GV $sPLA_2$, despite a significant 1.8-fold increase in PLA_2 activity secreted by these cells. In comparable studies of GX $sPLA_2$, a 2.2-fold increase in $sPLA_2$ activity in transfected MIN6 cells was associated with a robust 2.5-fold increase in PGE_2 generation [19]. The ability of GX $sPLA_2$ to reduce GSIS was blocked in cells treated either with a cyclooxygenase-2 inhibitor or an EP3 receptor antagonist, demonstrating that the suppressive effect of GX $sPLA_2$ is dependent on PGE2 synthesis and signaling through the EP3 receptor. The PGE2-EP3 receptor axis is thought to impact GSIS through decreased adenylyl cyclase activity and consequent reductions in cAMP, an established activator of insulin secretion [10, 11]. Our findings that overexpression of GX $sPLA_2$ [19] but not GV $sPLA_2$ (Fig. 2f), enhances intracellular cAMP concentrations in MIN6 cells is consistent with the conclusion that AA is coupled to increased PGE_2 production only in the case of GX $sPLA_2$. We speculate that increased GSIS in GV $sPLA_2$-overexpressing MIN6 cells is a consequence of increased non-esterified AA in these cells, in line with previous studies showing that treatments that increase endogenous AA in β-cells leads to augmented insulin secretion [36]. The mechanisms for AA-mediated activation of GSIS are not totally understood. After glucose-induced depolarization of the plasma membrane and subsequent release of insulin, Kv2.1 channels present on the β-cell membrane serve to repolarize the membrane and consequently limit the duration of insulin secretion [37]. By attenuating Kv2.1 activity, AA prolongs the action potential, resulting in the amplification of GSIS for longer periods [38].

As noted above, our data indicate that AA liberated by GV $sPLA_2$ versus GX $sPLA_2$ has distinct metabolic fates in MIN6 cells, such that GX $sPLA_2$ appears to be uniquely coupled to the prostaglandin synthesis pathway. Several in vitro studies point to differences in subcellular localization and actions for the two enzymes, which might account for our observations in β-cells. Though it is generally believed that GX $sPLA_2$ hydrolyzes phospholipids in the outer leaflet of the plasma membrane, there is evidence that this enzyme may release AA prior to secretion [20]. It has also been suggested that GX $sPLA_2$ is taken up by cells through its high affinity binding to the M-type receptor [39-41], whereas

GV $sPLA_2$ may be internalized and trafficked to intracellular compartments by heparin sulphate proteoglycans [42, 43]. Unlike GV $sPLA_2$, which is expressed as the mature enzyme, GX $sPLA_2$ is originally produced as a proenzyme that must be proteolytically cleaved by furin-like proprotein convertases in order to exert full catalytic activity [44, 45]. Thus, compartmentalization of GX $sPLA_2$ activity may be dictated by the subcellular location of the activating convertase. It is also important to note that local Ca^{2+} concentrations may influence the relative hydrolytic activity of the two enzymes, since in vitro studies indicate the requirement for Ca^{2+} is 10-fold higher for GV $sPLA_2$ compared to GX $sPLA_2$ [46]. Clearly, additional studies are required to determine whether spatial segregation of the catalytic activities of Group V and Group X $sPLA_2$ is responsible for the observed functional differences of these two enzymes in β-cells.

Intriguingly, our in vivo results show that whole-body ablation of GV $sPLA_2$ results in enhanced GSIS in mice (Fig. 4b), contrary to the suppressive effect of GV $sPLA_2$ deficiency observed in isolated islets ex vivo (Fig. 3c). Enhanced GSIS in GV $sPLA_2$-deficient mice was associated with a significant increase in islet mass as well as markers of β -cell proliferation, *which are likely to at least partially overcome* the reduction in GSIS observed for pancreatic islets isolated from GV $sPLA_2$-deficient mice. Recently, Sato et al. reported that diet-induced obesity induces a robust increase in adipocyte expression of GV $sPLA_2$. Additionally, GV $sPLA_2$-deficient mice had significantly increased body weight and greater insulin resistance, mainly in white adipose tissue, compared to wild-type littermates after high fat diet feeding [47]. Interestingly, high fat diet-induced hyperinsulinemia was greater in GV $sPLA_2$-deficient mice compared to wild-type mice, whereas glucose disposal and fasting blood glucose levels were similar for the two strains. The authors also showed that GSIS was significantly more robust in obese GV $sPLA_2$-deficient mice compared to control, although the mechanism for increased GSIS was not investigated. While it is difficult to tease out the systemic versus local effects of GV $sPLA_2$ in β-cell function, our studies in mice fed a normal rodent diet indicate that whole-body GV $sPLA_2$ deficiency is associated with increased β-cell proliferation, an increase in islet mass, and overall capacity to secrete insulin, which may ultimately serve a protective effect in the setting of profound insulin resistance. *The observed effects on β-cell proliferation and islet mass in the GV KO mice may be the consequence of GV $sPLA_2$ deficiency in tissues other than the pancreas. Additional studies using tissue-specific knock-out mice are necessary to fully understand the biology of these intriguing PLA_2's.*

Conclusions

Our results demonstrate that GV $sPLA_2$ plays multiple roles in regulating β-cell function in mice. Based on in vitro studies in MIN6 cells and isolated pancreatic islets, it is clear that GV $sPLA_2$ acts in an autocrine and/or paracrine manner to enhance GSIS, possibly by mediating the release of AA from membrane glycerophospholipids. This activity is in marked contrast to GX $sPLA_2$, which we previously showed suppresses GSIS by providing AA substrate for PGE2 generation in β-cells [19]. The opposing roles of GV and GX $sPLA_2$ in regulating insulin secretion underscore the fact that these two related enzymes are not functionally redundant. Additional in depth studies are necessary to fully understand the biology of these intriguing PLA_2's. Despite the capacity of GV $sPLA_2$ to enhance insulin

secretion by β-cells, the aggregate effect of whole-body GV $sPLA_2$ deficiency was to augment GSIS in mice, an outcome that was associated with increased β-cell proliferation and pancreatic islet mass. These data are consistent with a previous report that GV $sPLA_2$-deficient mice are partially protected from impaired GSIS associated with severe obesity-induced insulin resistance [47]. Thus, GV $sPLA_2$ may provide a potential target for improving β-cell compensation in the setting of insulin resistance.

Acknowledgments

We thank Dr. Sabire Ozcan, University of Kentucky for providing MIN6 cells and Dr. M. Gelb, University of Washington for supply of anti-mouse GV $sPLA_2$ antibody. We also thank Dr. Wendy Katz for helping us with paraffin embedding and tissue sectioning.

Funding: This work was supported in whole or in part by National Institutes of Health Grant R01 DK082419 (to N. R. W.) and the NIGMS Grant P20 GM103527 (to P. S.).

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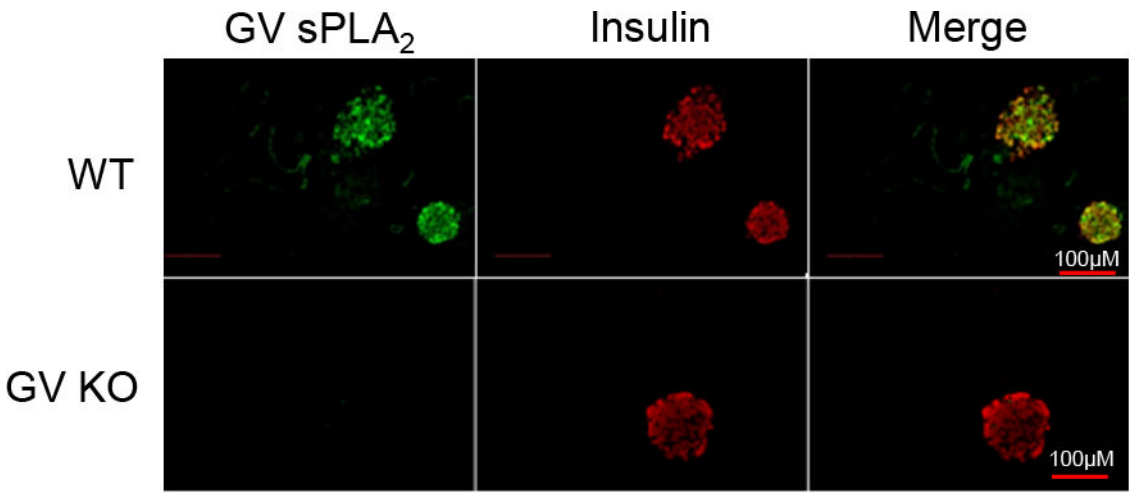

Fig 1.

GV $sPLA_2$ is expressed in mouse pancreatic islet cells. Pancreatic sections from WT and GV KO mice were co-stained by indirect immunofluorescence for GV $sPLA_2$ (green) and insulin (red) and visualized (20 × magnification) by fluorescence microscopy. A merged image shows expression of GV $sPLA_2$ in insulin-producing cells of WT pancreas (yellow).

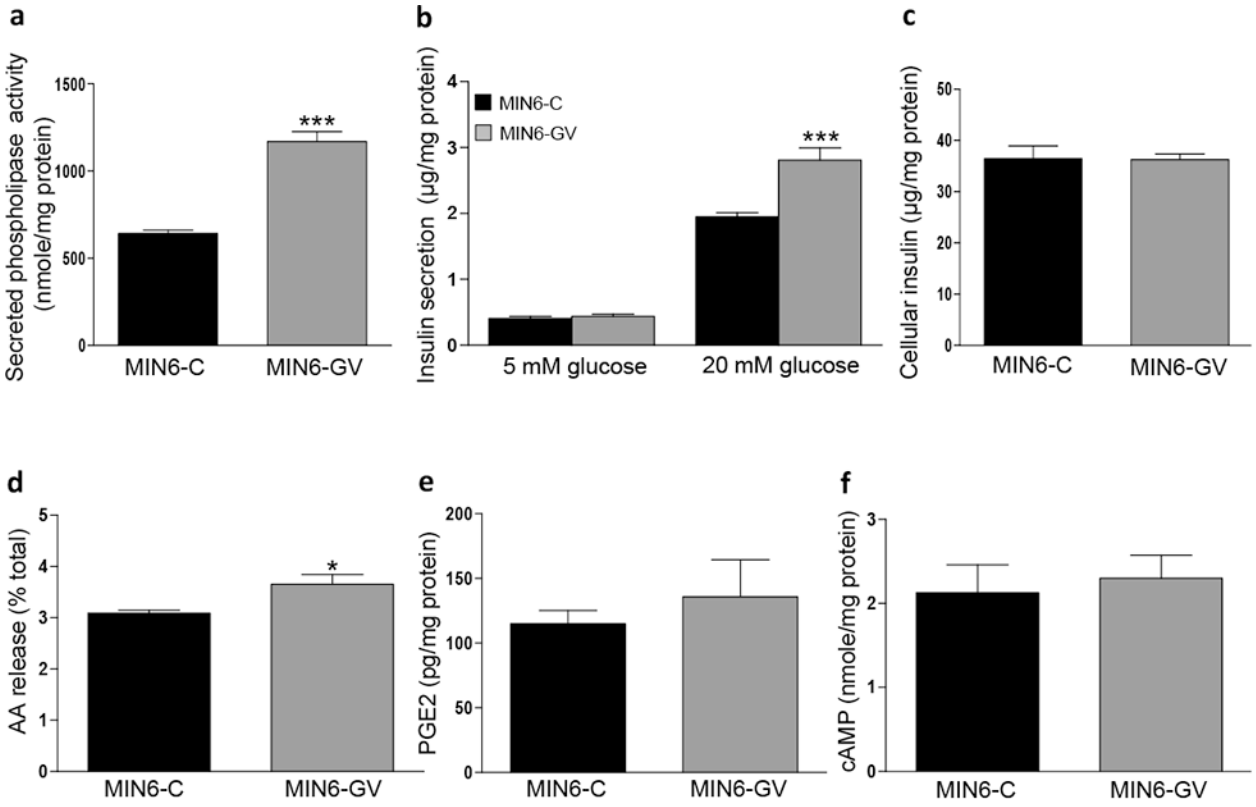

Fig 2.

GV $sPLA_2$ activates glucose-stimulated insulin secretion (GSIS) and enhances AA release in MIN6 cells. MIN6 cells were transfected with a control expression plasmid (MIN6-C) or an expression plasmid encoding GV $sPLA_2$ (MIN6-GV) as described under "Materials and Methods." a, Phospholipase activity in 48 h conditioned media from MIN6-C or MIN6-GV cell cultures was determined and normalized to cell protein. b, GSIS was performed in MIN6-C and MIN6-GV cells 48 h after transfection as described in "Materials and Methods." Insulin levels in the media were determined and normalized to total cell protein. c, Total cellular insulin content of MIN6-C and MIN6-GV cells was assessed as described in "Materials and Methods." d, [^{3}H]-AA release by MIN6-C and MIN6-GV cells was quantified and expressed as the percent of total cellular [^{3}H]-AA as described in "Materials and Methods." e, PGE2 levels in culture media from MIN6-C and MIN6-GV cells 48 h after transfection, normalized to total cell protein. f, cellular cAMP content in MIN6-C and MIN6-GV cells was determined 48 h after transfection and normalized to total cell protein. Data are from 4 independent transfections per construct and are presented as mean ± S.E; *$p<0.05$. ***, $p < 0.001$.

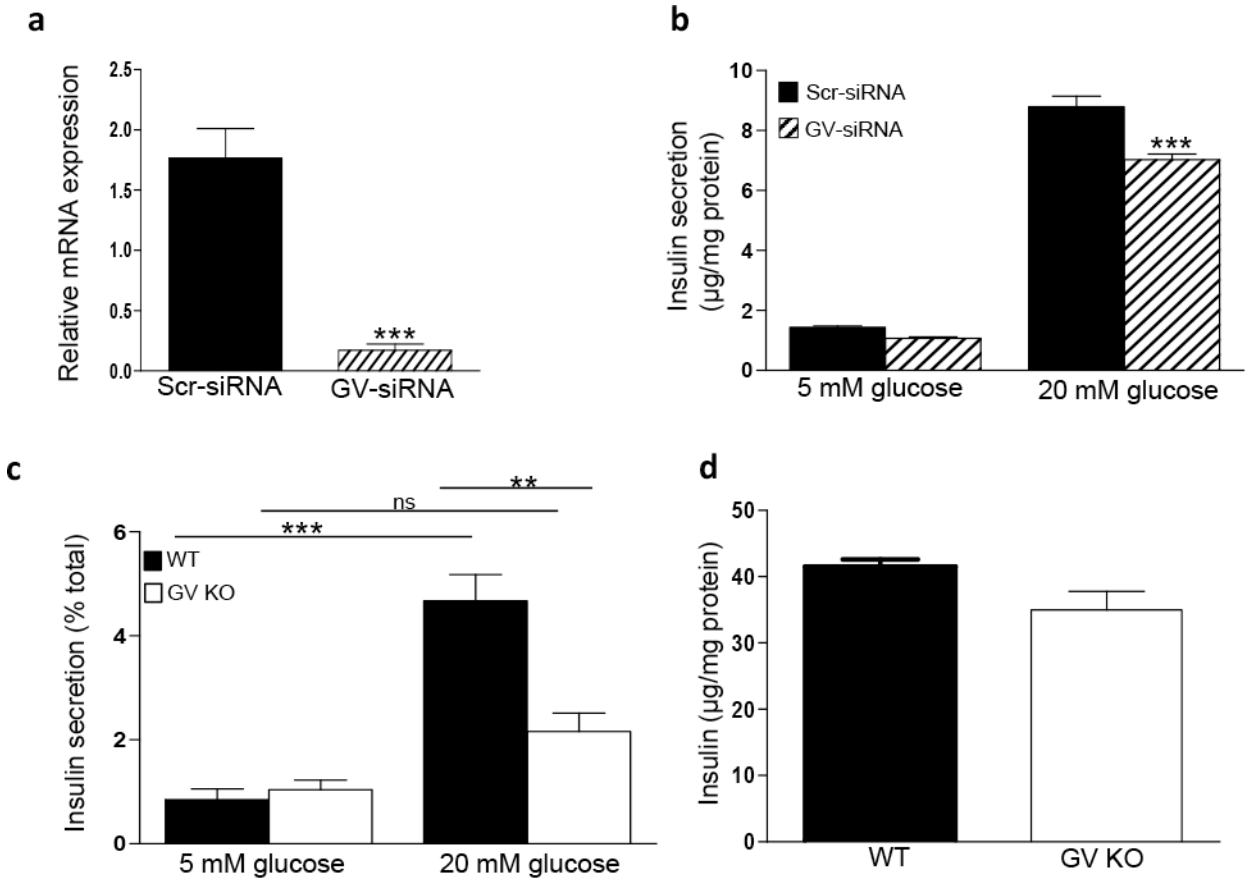

Fig 3.

GV $sPLA_2$ deficiency suppresses GSIS in MIN6 cells and primary mouse pancreatic islet cells. MIN6 cells were transfected with a control siRNA (Scr-siRNA) or siRNA directed to GV $sPLA_2$ (GV-siRNA), as described under "Materials and Methods." a, RNAs were prepared from Scr-siRNA and GV-siRNA cells 24 h after transfection for quantification of GV $sPLA_2$ mRNA abundance. b, GSIS was performed in Scr-siRNA cells and GV-siRNA cells as described in "Materials and Methods." Insulin secreted into the media was assayed and normalized to total cell protein. c, GSIS assay was performed using islets (25 similar-sized islets per mouse) isolated from 4-month-old WT and GV KO mice (n=4). Islets were incubated successively for 40 min in buffer containing 5 mM glucose (low glucose) and for 40 min in buffer containing 20 mM glucose (high glucose). Insulin in the assay media was determined and normalized to total cellular insulin in the corresponding islets. d, Total islet insulin content in 25 similar-sized islets from WT and GV KO mice were estimated and normalized to total protein. Data are presented as mean ± S.E; ns-not significant; **p<0.01;***p<0.001.

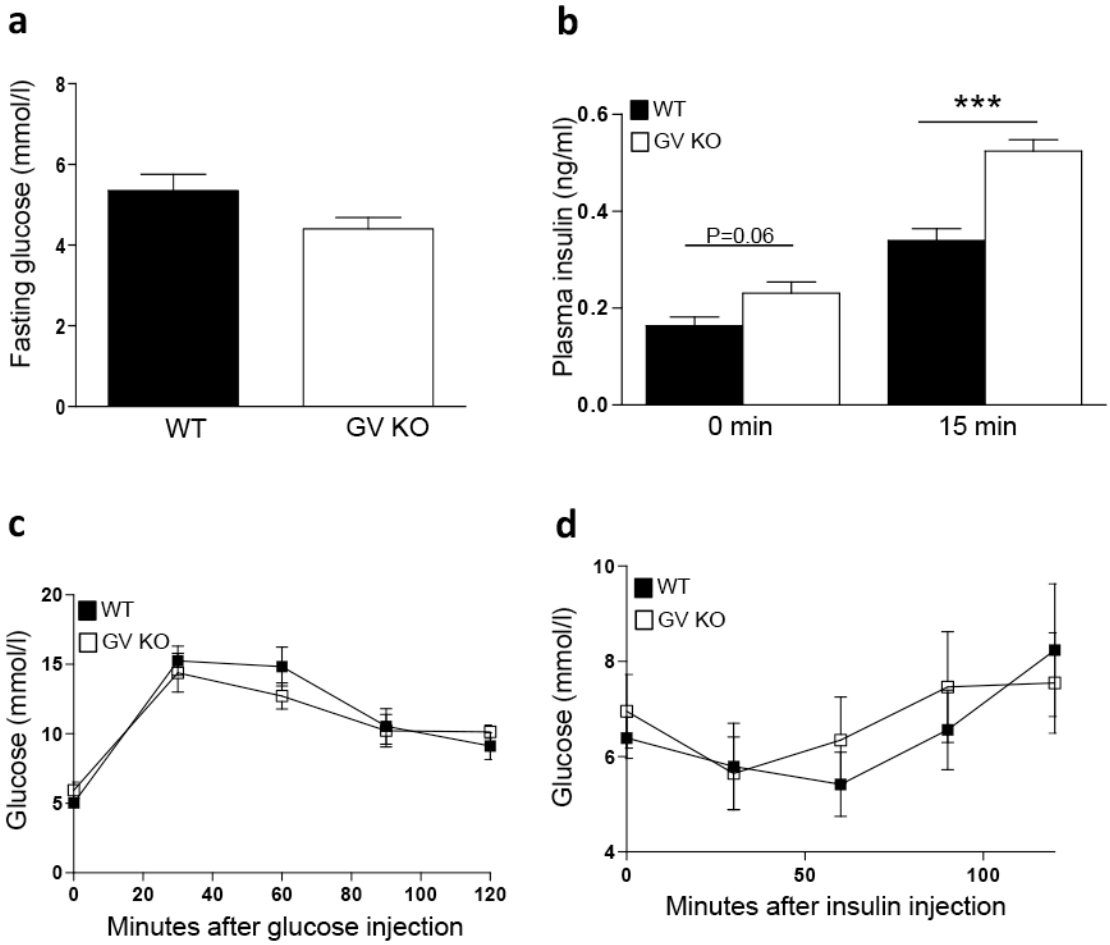

Fig 4.

Enhanced in vivo GSIS in GV KO mice. a, Blood glucose levels after 16 h fast in WT and GV KO mice. b, Plasma insulin levels were determined before and 15 min after i.p. glucose injection (3 g/kg; n=4-5) in 6-month old WT and GV KO mice. c, 6- month old WT and GV KO mice were fasted for 6 h prior to i.p. injection of 1.5 mg glucose/g body weight, and glucose concentrations in the blood were determined at the indicated time after injection (n=5). d, 6- month old WT and GV KO mice were fasted for 4 h prior to i.p. glucose injection of human insulin (0.5 U/kg body weight), and glucose concentrations in the blood were determined at the indicated time after injection (n=4-5). e, body weight of WT and GV KO mice (n=4/strain). Data are presented as mean ± S.E; ***p<0.001.

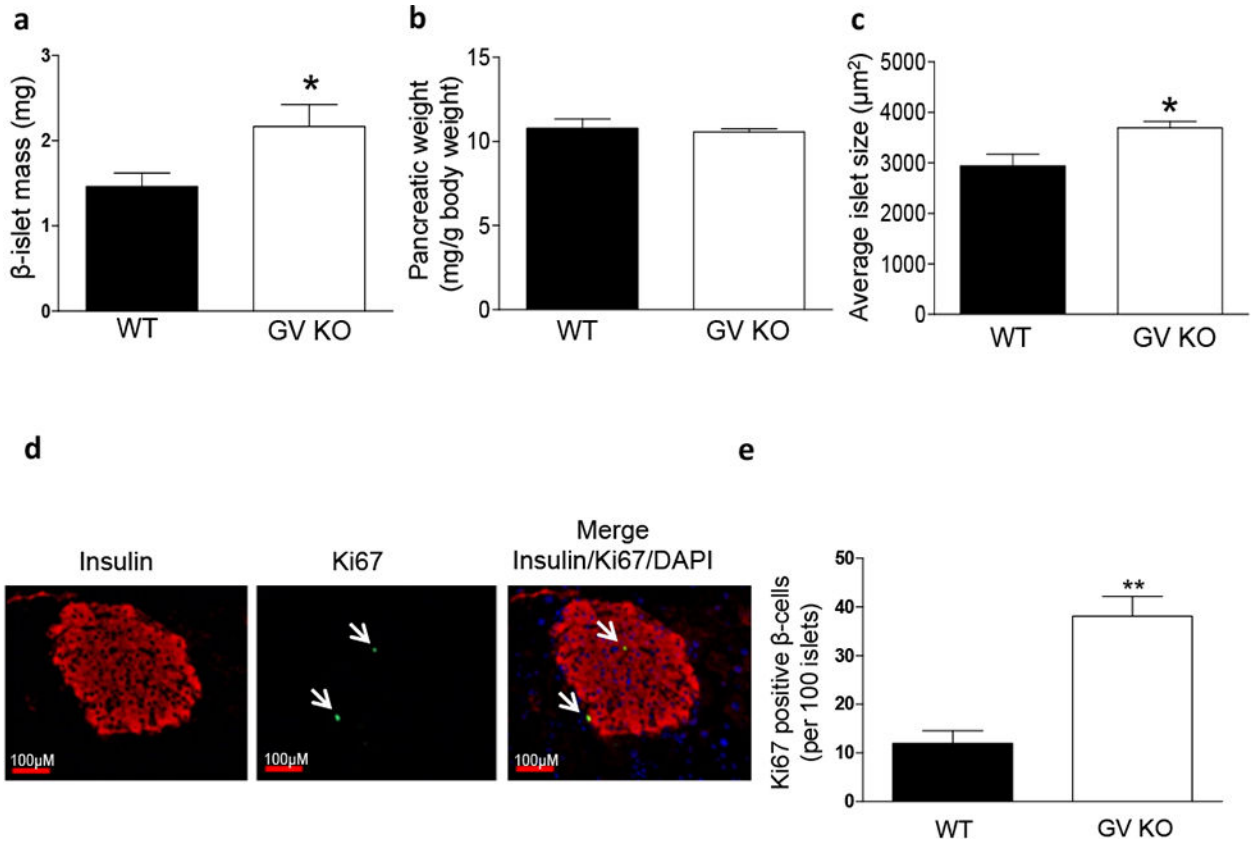

Fig 5.

Increased β-islet mass, average islet size and β-cell proliferation in the pancreas of GV KO mice. a, Pancreatic β-islet mass of WT and GV KO mice (n=4/strain; 5-8 sections were analyzed from each mouse) were calculated by obtaining the fraction of the area of pancreatic tissue positive for insulin staining and multiplying this by the pancreatic weight as described in detail in the “Materials and Methods.” b, Pancreatic weight of WT and GV KO mice (n=4/strain). c, Average islet size of WT and GV KO pancreata (n=4/strain; 5-8 sections/mouse) was calculated by dividing the total area of pancreatic tissue positive for insulin staining by the total number of islets. d, Representative immunofluorescence images of pancreatic islets from GV KO mice at 20X magnification showing insulin (red) and Ki67 (indicated by arrows pointing to green staining). A merged image shows expression of Ki67 in insulin-producing cells of GV KO pancreas, co-localized with DAPI staining. e, Quantification of number of β-cells with Ki67-positive nuclei per 100 islets in WT and GV KO mice (n=3/group; 30-50 islets from 2 sections per mouse). Data are presented as mean ± S.E; *p<0.05; ***p<0.01.