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GABA Promotes Human β -Cell Proliferation and Modulates Glucose Homeostasis

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γ -Aminobutyric acid (GABA) exerts protective and regenerative effects on mouse islet β -cells. However, in humans it is unknown whether it can increase β -cell mass and improve glucose homeostasis. To address this question, we transplanted a suboptimal mass of human islets into immunodeficient NOD-scid- γ mice with streptozotocin-induced diabetes. GABA treatment increased grafted β -cell proliferation, while decreasing apoptosis, leading to enhanced β -cell mass. This was associated with increased circulating human insulin and reduced glucagon levels. Importantly, GABA administration lowered blood glucose levels and improved glucose excursion rates. We investigated GABA receptor expression and signaling mechanisms. In human islets, GABA activated a calcium-dependent signaling pathway through both GABA A receptor and GABA B receptor. This activated the phosphatidylinositol 3-kinase–Akt and CREB–IRS-2 signaling pathways that convey GABA signals responsible for β -cell proliferation and survival. Our findings suggest that GABA regulates human β -cell mass and may be beneficial for the treatment of diabetes or improvement of islet transplantation.

Expanding β -cell mass by promoting β -cell regeneration is a major goal of diabetes therapy. β -Cell proliferation has

been shown to be the major source of β -cell renewal in adult rodents (1) and perhaps in humans as well (2). In vitro, human β -cells have generally responded poorly to mediators that stimulate mouse β -cells. In vivo, the proliferation of adult human β -cells is very low, but an increase has been detected in a patient with recent-onset type 1 diabetes (T1D) (3), suggesting a regenerative capacity. This is supported by recent studies showing that mild hyperglycemia can increase human β -cell proliferation in vivo (4,5). These observations suggest that it may be possible to use stimuli to induce the β -cell regeneration and promote β -cell mass in the diabetic condition.

γ -Aminobutyric acid (GABA) is produced by pancreatic β -cells in large quantities (6). It is an inhibitory neurotransmitter in the adult brain (7), but in the developing brain it exerts trophic effects including cell proliferation and dendritic maturation via a depolarization effect (8). In the β -cells, GABA induces membrane depolarization and increases insulin secretion (9), while in the α -cells it induces membrane hyperpolarization and suppresses glucagon secretion (10). In mice, we previously observed that it enhanced β -cell proliferation and reduced β -cell death, which reversed T1D (9). Indeed, in various disease models, GABA exerts trophic effects on β -cells and

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protects against diabetes (9,11). More recent studies reveal that GABA also protects human β -cells against apoptosis and increases their replication rate (12,13). However, it is unknown whether it can increase functional human β -cell mass and improve glucose homeostasis under in vivo conditions.

In this study, we investigated stimulatory effects of GABA on human β -cells in vivo and in vitro. In order to evaluate the effect of GABA on expanding functional human β -cell mass in vivo, we transplanted a marginal (sub-optimal) dose of human islets under the kidney capsule of diabetic NOD-scid- γ (NSG) mice. We report here that GABA stimulates functional human β -cell mass expansion and improves glucose homeostasis in the diabetic condition.

RESEARCH DESIGN AND METHODS

Human Islet Isolation

Human islets were isolated as previously described (14). Pancreata from deceased nondiabetic adult human donors were retrieved after consent was obtained by Transplant Québec (Montreal, Canada). The pancreas was intraductally loaded with cold Clzyme Collagenase HA (VitaCyte LLP) and neutral protease (Neutral Protease NB; SERVA Electrophoresis GmbH) enzymes, cut into pieces, and transferred to a sterile chamber for warm digestion at 37°C in a closed-loop circuit. Dissociation was stopped using ice-cold dilution buffer containing 10% normal human AB serum, once 50% of the islets were seen to be free of surrounding acinar tissue under dithizone staining. Islets were purified on a continuous iodixanol-based density gradient (OptiPrep; Axis-Shield), using a COBE 2991 Cell Processor (Terumo BCT). Yield, purity, and viability were determined, and glucose-stimulated insulin secretion assays were performed. The clinical data of donors used for islet transplantation experiments are shown (Supplementary Table 1).

Human Islet Transplantation and In Vivo Assays

All animal handlings were in accordance with approved Institutional Animal Care and Use Committee protocols at St. Michael's Hospital. Male NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NOD-scid IL2r γ ^{null}, NSG) (denoted NOD-scid- γ or NSG) mice (five animals per group) were rendered diabetic with streptozotocin (STZ) injections (125 mg/kg for two consecutive days; Sigma). Mice with blood glucose (BG) ≥ 18 mmol/L for >2 days were selected for experiments and transplanted with a suboptimal dose of human islets (1,500 islet equivalents [IEQ]) under the kidney capsule as previously described (4,5). This leaves a margin to observe whether GABA treatment improves hyperglycemia in these diabetic mice. Mice were then treated with or without GABA (6 mg/mL; Sigma) in drinking water. One week after transplantation, subtherapeutic dose of subcutaneous insulin (0.2 units/mouse; Novolin ge Toronto, Novo Nordisk) was administered daily as treatment for dehydration owing to high BG to all animals. The injection of insulin was omitted 24 h prior to the bioassays or

48 h prior to the blood sampling to avoid the drug effect. BG was measured using a glucose meter. Five weeks post-transplantation, mice were killed after receiving BrdU injection (100 mg/kg, 6 h prior); the blood was collected for the measurement of human insulin (human insulin ELISA kit, Mercodia, Uppsala, Sweden) and glucagon (glucagon ELISA kit, Millipore); the graft-containing kidneys and pancreases were paraffin embedded and prepared for histological analysis as previously described (9). For glucose-stimulated insulin secretion, human islets were placed into 48-well plates in serum-free Hank's buffer containing 2 mmol/L or 11 mmol/L glucose treated with or without GABA at indicated concentrations in the presence or absence of relevant agonists or antagonists for 30 min at 37°C. Insulin levels were measured by a human insulin RIA kit (Millipore, Billerica, MA).

Human Islet Culture and In Vitro Assays

Isolated human islets were maintained in CMRL-1066 medium (Gibco) supplemented with 10% FBS, penicillin, streptomycin, and L-glutamine. For mRNA and apoptosis assays, CMRL-1066 medium containing 2% heat-inactivated FBS was used. Cytokines (interleukin-1 β , tumor necrosis factor- α , and interferon- γ) were from Sigma. For phosphorylation assay, the islets were pretreated with picROTOXIN (Tocris Bioscience), saclofen (Sigma), or nifedipine (Sigma).

Immunohistochemistry, β -Cell Count, and Islet Mass Analysis

Tissue harvesting and processing were performed as we described previously (9,15). For rodent pancreas, we cut the rodent pancreatic issues from a single pancreas into 8–10 segments; they were randomly arranged and embedded into one block, thus permitting analysis of the entire pancreas in a single section to avoid any orientation issues (9,15). For human islet mass analysis, kidney grafts were cut in longitudinal orientation and random sections were analyzed. More than 4,000–5,500 β -cells were examined from at least two to four different random sections from individual grafts in each group. Proliferative β -cells were identified by insulin-BrdU or insulin-Ki67 dual staining. Islets were dual-stained for insulin and glucagon for β -cell and α -cell mass analysis. The primary antibodies used were as follows: guinea pig anti-insulin IgG (1:800; Dako), rabbit anti-glucagon IgG (1:500; Dako), anti-BrdU (1:100; Sigma), or anti-Ki67 (1:200; Abcam). The staining was detected with fluorescent (1:1,000; Cy3- and FITC-conjugated IgG, The Jackson Laboratory) or biotinylated secondary antibodies, and in the latter case, samples were incubated with avidin-biotin-peroxidase complex (Vector Laboratories) before chromogen staining with DAB (Vector Laboratories) and subsequent hematoxylin counterstaining. Insulin⁺ BrdU⁺ (or Ki67⁺) β -cells were counted within the human grafts. β -Cell and α -cell mass were evaluated by quantification of positive pixels in the selected graft area using the Aperio count algorithm (Aperio ImageScope), which is preconfigured for

quantification of insulin and/or glucagon versus total graft area—similar to that previously described (16).

Immunoblotting

Immunoblotting was performed as previously described (16). Primary antibodies (p-Akt [Ser⁴³⁷] 1:500, total Akt 1:3,000, p-CREB [Ser¹³³] 1:1,000, total CREB 1:1,000, cleaved caspase-3 1:1,000, and total caspase-3 1:1,000) were from Cell Signaling, and GAPDH antibody (1:10,000) was from Boster Immunoleader. Horseradish peroxidase-conjugated secondary antibodies (1:5,000–20,000) were from Jackson ImmunoResearch. Protein band densities were quantified using the ImageJ program.

mRNA Extraction, Reverse Transcription, and Quantitative PCR

RNA isolation and reverse transcription from 1 µg RNA was performed using Qiazol (Qiagen) and reverse transcriptase (Fermentas) according to the manufacturers' instructions. Real-time quantitative PCR reaction was conducted on ABI ViiA 7 (Applied Biosystems) in 8 µL volume of SYBR Green PCR reagents (Thermo Scientific) under conditions recommended by the supplier. For insulin receptor substrate (IRS)-2 mRNA detection, the following primers were used: 5'TCTCTCAGGAAAAGCAGCGA3' (forward) and 5'TGGCGATGTAGTTGAGACCA3' (reverse). Results were normalized to β-actin, and relative quantification analysis was performed using the $2^{-\Delta\Delta C_t}$ method.

Reactive Oxygen Species Assay

The levels of reactive oxygen species (ROS) were measured using a 2',7'-dichlorofluoresceindiacetate fluorogenic dye-based cellular ROS detection assay kit (Abcam) according to manufacturer's protocol.

Intracellular Ca²⁺ Measurement

Intracellular Ca²⁺ was measured using Fura-2, AM (Molecular Probes). Isolated human islet β-cells were preloaded with Fura-2, AM (2 µmol/L), washed, and transferred to a thermal-controlled chamber and perfused with GABA or muscimol with or without inhibitors focally in the recording solution (3) while the recordings were made with an intensified charge-coupled device camera. The fluorescent signal was recorded with a time-lapse protocol, and the fluorescence intensity (i.e., Poenie-Tsien) ratios of images were calculated with Image-Pro 5.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel and GraphPad Prism 6 (GraphPad Software). Student *t* test or one-way ANOVA with Dunnett post hoc test were used as appropriate. Data are means ± SE. *P* values <0.05 are considered significant.

RESULTS

GABA Enhanced Human β-Cell Mass and Elevated Human Insulin Levels

Direct in vivo studies of human β-cell proliferation have been limited by the lack of biopsy material or quantitative noninvasive methods to assess β-cell mass (17). Here, we

used an in vivo model by transplanting a marginal (sub-optimal) mass of human islets. The islets were inserted under the kidney capsule of NOD-scid-γ (NSG) mice with STZ-induced diabetes. We then treated the recipient mice with or without oral GABA, administered through the drinking water (6 mg/mL) for 5 weeks. Immunohistochemical analysis showed that GABA induced β-cell replication in the islet grafts, as demonstrated by an increased number of BrdU⁺ β-cells (Fig. 1A and B) and the ratio of β-cells to grafted islet cells (Fig. 1A and C). However, the ratio of α-cells per graft was not significantly changed. In untreated mice, the rate of human β-cell proliferation was $0.4 \pm 0.07\%$, consistent with previous findings under similar conditions (4,5). Administration of GABA significantly increased the rate of β-cell proliferation by approximately fivefold, revealing a potent stimulatory effect.

We examined circulating insulin levels in the recipient mice using a human insulin-specific ELISA kit and found that the administration of GABA significantly increased plasma insulin levels compared with untreated mice (Fig. 1D). The specific detection of human insulin was further confirmed by pancreatic histochemistry, which showed that STZ injection destroyed nearly all the mouse pancreatic β-cells, with the residual islet containing mostly α-cells (Supplementary Fig. 1). Notably, the treatment of GABA increased circulating human insulin levels while reducing glucagon levels as determined by the ELISA (Fig. 1E). Serial BG testing showed that marginal islet transplantation in the diabetic mice reduced BG levels in both groups. However, BG levels were significantly lower in the GABA-treated group (Fig. 1F) and associated with improved glucose excursion rates, as demonstrated by the intraperitoneal glucose tolerance testing (Fig. 1G). The transplantation experiments were performed three times with three different donors, and similar results were obtained. Of note, higher doses of GABA (up to 30 mg/mL in the drinking water) yielded similar results (Supplementary Fig. 2).

In vitro, in similarity to the in vivo results, we observed that GABA increases human β-cell replication as determined by the increased numbers of Ki67⁺ and BrdU⁺ β-cells (Fig. 2A and B). Furthermore, GABA-increased proliferative human islet β-cells were blocked by type A receptor (GABA_AR) antagonist picrotoxin or partially by the type B receptor (GABA_BR) antagonist saclofen (Supplementary Fig. 3A). This is consistent with observations in clonal β-cells that GABA increased β-cell thymidine incorporation, which was blocked by GABA_AR and GABA_BR antagonists (Supplementary Fig. 3B), suggesting the involvement of both types of GABA receptors and in accord with a recent study in human islets under in vivo and in vivo settings (13).

GABA Induced GABA Receptor Activation and Evoked Calcium Influx

Pancreatic β-cells express two GABA receptors, i.e., GABA_AR and GABA_BR (18). We recently reported that GABA stimulation activates a Ca²⁺-dependent intracellular

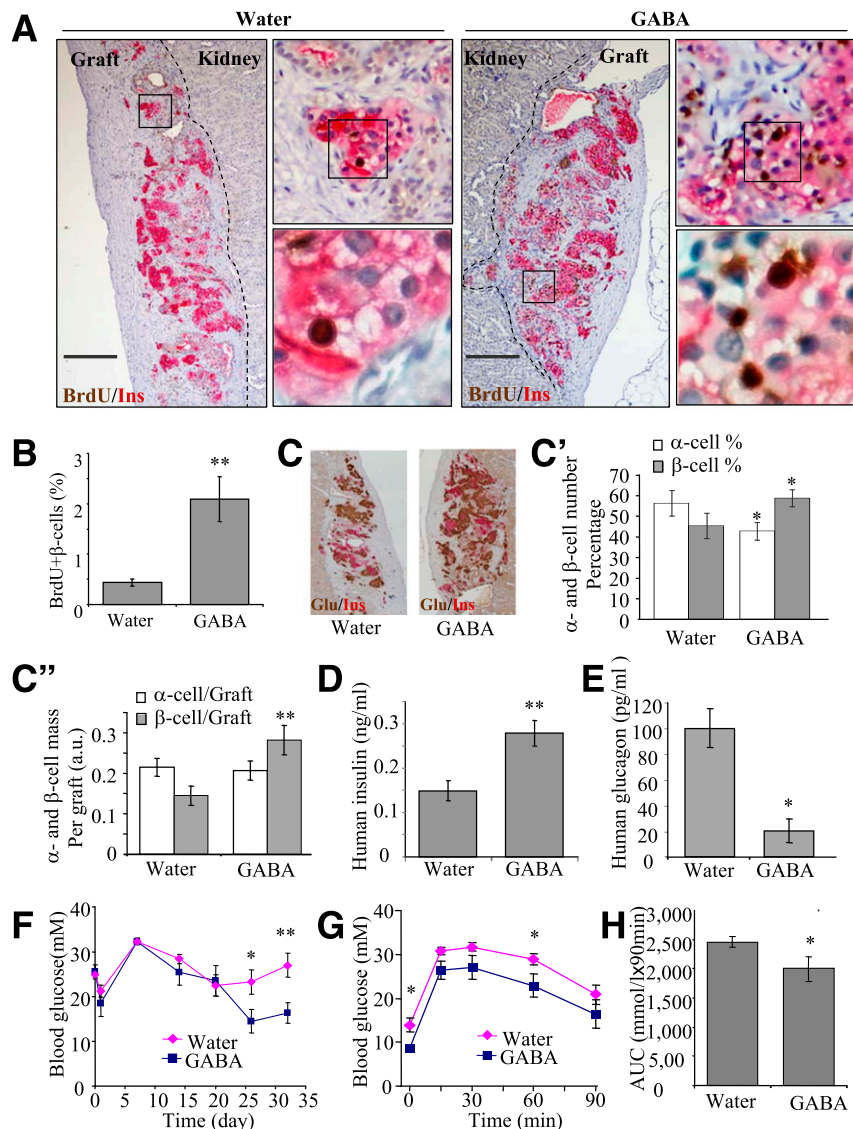


Figure 1—GABA increased β -cell proliferation of transplanted human islets and improved glucose homeostasis. STZ NOD-SCID mice ($n = 5$ for each group) were transplanted with human islets of suboptimal number (1,500 IEQ) under the kidney capsule and treated with or without GABA (6 mg/mL in drinking water). **A**: Representative images of islet grafts double stained with insulin (red) and BrdU (brown). Scale bars are 200 μ m. **B**: GABA treatment increased β -cell proliferation determined by counting the number of BrdU $^{+}$ β -cells over the total β -cell number. From 4,000 to 5,500 β -cells were counted from at least two to four different sections from individual grafts in each group. **C**: GABA increased β -cell mass but not α -cell mass, which were evaluated by quantifying the positive pixel in the entire graft and quantified as percentage of islet cells (**C'**) and islet cell mass per graft (**C''**). Circulating human insulin (**D**) and glucagon (**E**) levels were measured by specific ELISA kits. **F**: BG levels were measured in the course of GABA administration. **G**: Intraperitoneal glucose tolerance testing was performed at the end of the feeding course, and area under curve (AUC) is shown (**H**). Data are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs. control, $n = 5$. a.u., arbitrary units; Ins, insulin.

signaling pathway involving phosphatidylinositol 3-kinase (PI3K)-Akt, which appears to be important in conveying trophic effects to rodent β -cells (9). To investigate whether this pathway is active in human β -cells, we conducted intracellular Ca^{2+} -imaging assays. We observed that both GABA and the GABA $_A$ R agonist muscimol evoked Ca^{2+} influx that was diminished by GABA $_A$ R antagonism or Ca^{2+} channel blockage (Fig. 3A), suggesting GABA $_A$ R-mediated Ca^{2+} channel activation. Furthermore, GABA-stimulated elevation of intracellular Ca^{2+} was also attenuated by

GABA $_B$ R antagonist treatment (Fig. 3A). This is consistent with previous findings that activation of GABA $_B$ R results in a rise in Ca^{2+} concentration that, however, is released from intracellular Ca^{2+} stores (19). We conclude that GABA increases intracellular Ca^{2+} in human β -cells through the activation of both GABA $_A$ R and GABA $_B$ R. To determine whether GABA-induced intracellular Ca^{2+} is linked to a secretory event, we performed glucose-stimulated insulin secretion in isolated human islets. We found that GABA increased insulin secretion in human islets in a GABA receptor

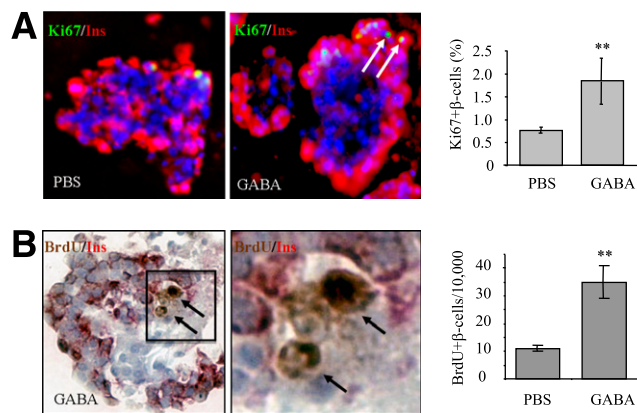


Figure 2—GABA induces β -cell proliferation in vitro. Human islets were incubated with BrdU (50 μ M/L) and treated with or without GABA (100 μ M/L) for 24 h. Islets were double stained for insulin (red) and Ki67 (green) and visualized with a fluorescent microscope. A portion of treated islets was paraffin embedded and double stained for insulin (red) and BrdU (brown). GABA induced Ki67⁺ (A) and BrdU⁺ (B) β -cells. Arrows indicate Ki67⁺ and BrdU⁺ β -cells. The bar graph shows quantitative results of three assays of islets from three donors. ** $P < 0.01$ vs. control. Ins, insulin.

antagonism-sensitive fashion (Supplementary Fig. 3C), consistent with previous findings by us (12) and others (18).

GABA Activated Akt and CREB Pathways Independently

The Akt signaling pathway is pivotal in regulating β -cell mass in rodents (20). We hypothesized that in human β -cells, the GABA-induced Ca^{2+} initiates signaling events that lead to the activation of the Akt pathway. Here, we show by Western blotting that GABA promoted Akt activation in human β -cells, which was sensitive to either GABA receptor or Ca^{2+} channel blockade (Fig. 3B), consistent with our findings in rodent islet cells (9) and insulinoma cells (Supplementary Fig. 4).

In an effort to identify other key target molecules that mediate GABA trophic signals in human β -cells, we found that CREB, a transcription factor that is crucial in β -cell gene expression and function, was remarkably phosphorylated upon GABA receptor activation. In particular, our data show, in an in vitro setting of human islets, that GABA stimulation increases CREB phosphorylation, which can be inhibited by GABA_AR or GABA_BR antagonists, as well as calcium channel blockade with nifedipine (Fig. 3B). Interestingly, this was simultaneously associated with increased mRNA expression level of IRS-2 (Fig. 3C). Furthermore, we found that pharmacological inhibition of PI3K-Akt did not reduce GABA-induced CREB phosphorylation, and blockade of protein kinase A (PKA)-dependent CREB activation did not attenuate GABA-induced Akt phosphorylation in human islets (Fig. 4A) and INS-1 cells (Supplementary Fig. 5). This suggests that GABA-stimulated β -cell signaling involves the activation of Akt and CREB pathways independently. These findings lead us to postulate that GABA acts on human β -cells through

a mechanism involving Ca^{2+} influx, PI3K-Akt activation, and CREB-IRS-2 signaling (Fig. 4B).

GABA Protected β -Cells Against Apoptosis Under In Vitro and In Vivo Conditions

Under in vitro conditions, we found that GABA significantly attenuated cytokine-induced human islet apoptosis (Supplementary Fig. 6A and B), which was associated with suppressed cytokine-induced ROS production in human islets (Supplementary Fig. 6C). This is consistent with anti-inflammatory and immunosuppressive GABA-mediated effects reported by us (9,12) and others (13). Indeed, under in vivo conditions, treatment of GABA significantly reduced β -cell apoptosis in the human islet grafts in the recipient mice, as determined by insulin-TUNEL dual staining (Supplementary Fig. 6D), suggesting protective effects of GABA in human islet β -cells under in vivo conditions.

DISCUSSION

In this study, we demonstrated proliferative and protective effects of GABA on human islets. Our findings indicate that GABA enhances grafted human islet β -cell mass and increases circulating human insulin levels, while decreasing glucagon levels. Importantly, GABA treatment decreased BG levels and improved glucose tolerance in these diabetic NSG mice. The elimination of their endogenous β -cells by injection of high-dose STZ rendered the grafted human islets the sole resource of insulin production. Therefore, the improved glucose homeostasis observed in these diabetic recipient mice is attributed to the enhanced functional human islet β -cell mass. These findings suggest that the trophic effects of GABA on human islet β -cells are physiologically relevant and that it may contribute to the improved glucose homeostasis in diabetic conditions.

It is very likely that the improved hyperglycemia in the diabetic GABA-treated NSG mice is due to enhanced β -cell mass, elevated insulin secretion, and suppressed glucagon release. Of note, despite the fact that the α -cell mass per graft was not significantly changed in the GABA-treated mice, they displayed significantly reduced serum glucagon levels. This presumably resulted, at least in part, from increased inraislet insulin action. Indeed, insulin is a physiological suppressor of glucagon secretion (21). Furthermore, our previous work showed that the paracrine insulin, in cooperation with GABA, exerts suppressive effects on glucagon secretion in the α -cells (10).

It is interesting to note that the glucose-induced suppression of glucagon release is significantly blunted in the islets of T2D patients. This has been attributed in part to declined inraislet insulin (21), decreased inraislet GABA levels (22), and/or reduced GABA receptor signaling (23). These reports are consistent with clinical observations that T2D patients have exaggerated glucagon responses under glucagon stimulatory conditions (24).

The molecular mechanisms by which GABA exerts trophic effects on β -cells are not well understood. GABA_AR is a pentameric ligand-activated chloride channel that

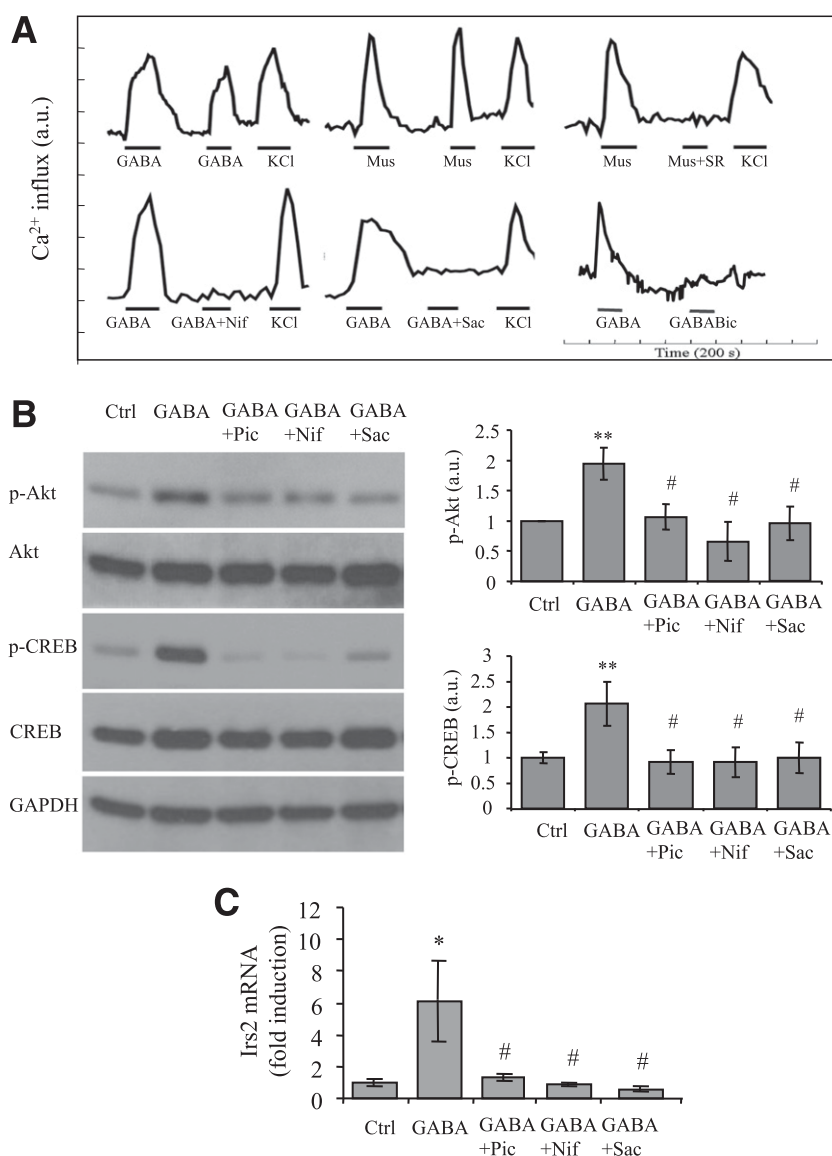


Figure 3—GABA induced Akt and CREB phosphorylation and increased IRS-2 mRNA expression in human islets. **A:** GABA evokes Ca²⁺ influx in isolated adult human islets. Islets were perfused with GABA (200 μ M) or the GABA_A agonist muscimol (Mus) in the presence or absence of antagonists as indicated. Intracellular Ca²⁺ was measured using Fura-2, AM. Shown is a representation of $n = 11$ –26 from two different donors. **B:** GABA induced Akt and CREB phosphorylation in human islets, which was inhibited by picrotoxin, nifedipine, and saclofen. Representative blots are shown. The bar graphs represent quantitative results of three to nine assays from five islet donors. **C:** GABA increased IRS-2 mRNA expression in isolated human islets. Islets were treated with GABA (100 μ M) in the presence of indicated inhibitors for 16 h, followed by mRNA extraction and quantitative PCR using specific primers for human IRS-2. The data shown are representative results of three assays using islets from two donors. GABA_A agonist: muscimol (20 μ M). GABA_A antagonists: picrotoxin (Pic) (100 μ M), bicuculline (Bic) (100 μ M), or gabazine (SR-95531, SR) (1,000 μ M). GABA_B antagonist: saclofen (Sac) (100 μ M). Ca²⁺ channel blocker: nifedipine (Nif) (1 μ M). * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, # $P < 0.05$ vs. GABA-treated group. a.u., arbitrary units; Ctrl, control.

consists of various combinations of several subunits (i.e., 2 α , 2 β , and a third subunit) such that multiple GABA_ARs can be assembled. Activation of GABA_AR allows movement of Cl[−] in or out of the cells, thus modulating membrane potentials (25). In developing neurons, GABA induces depolarizing effects that result in Ca²⁺ influx and activation of Ca²⁺-dependent signaling events involving PI3K (26) in modulating a variety of cellular processes such as proliferation and differentiation (8). Here, we report that GABA

induces membrane depolarization and promotes calcium influx into β -cells. In rodents, the PI3K-Akt signaling pathway is known to be pivotal for the regulation of β -cell mass and function in response to GLP-1 and glucose (27). We hypothesized that the GABA-induced membrane depolarization and elevation of intracellular Ca²⁺ initiate Ca²⁺-dependent signaling events that result in the activation of the PI3K-Akt pathway in human β -cells. In accord with this hypothesis, we found that GABA triggered

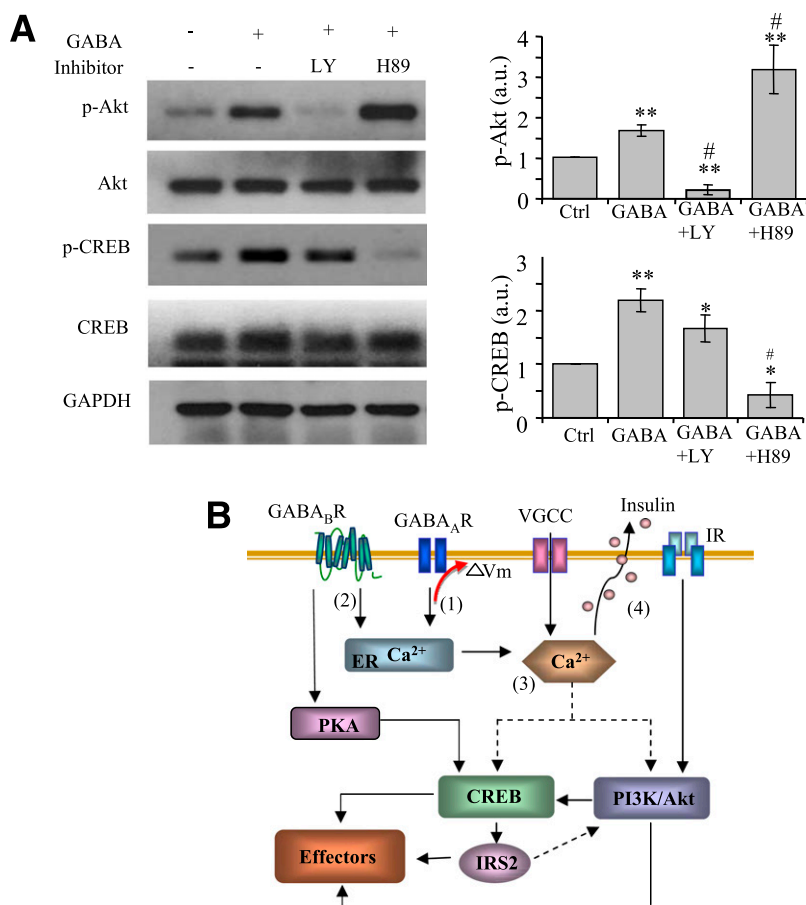


Figure 4—GABA induced Akt and CREB phosphorylation independently. **A:** GABA-induced Akt and CREB phosphorylation are independent of each other. Human islets were serum starved for 2 h and treated with GABA for 1 h in the presence or absence of PI3K inhibitor LY294002 (LY) (1 μ mol/L) or protein kinase A (PKA) inhibitor H89 (1 μ mol/L) as indicated. Inhibitors were added 30 min prior to GABA treatment. **B:** Model of GABA signaling in the β -cells. 1) GABA_AR activation causes membrane depolarization, which leads to Ca²⁺ influx. 2) Activation of GABA_BR causes release of Ca²⁺ from intracellular storage and PKA activation (19). 3) Ca²⁺-dependent activation of PI3K-Akt (37) and CREB (38), which regulates IRS-2 expression (39). 4) Increased intracellular Ca²⁺ promotes insulin secretion, and autocrine insulin action activates PI3K-Akt signaling pathway. Data are means $\pm$ SEM. * P < 0.05, ** P < 0.01 vs. control, # P < 0.05 vs. GABA-treated group, n = 3. a.u., arbitrary units; Ctrl, control; ER, endoplasmic reticulum; IR, insulin receptor; VGCC, voltage-gated calcium channel.

the Ca²⁺-dependent signaling pathway involving activation of PI3K-Akt signaling, and this is likely an important mechanism underlying its action in the human β -cells.

Our observations also show that GABA stimulated CREB activation. This CREB protein is a key transcription factor for the maintenance of appropriate glucose sensing, insulin exocytosis, insulin gene transcription, and β -cell growth and survival (28–30). Activation of CREB, in response to a variety of pharmacological stimuli including GLP-1, initiates the transcription of target genes in the β -cells (30). The role of CREB in regulating β -cell mass homeostasis is supported by the finding that mice lacking CREB in their β -cells have diminished expression of IRS-2 (28) and display excessive β -cell apoptosis (31). Previous findings suggested that the activation of CREB is one of the key targets of the Akt signaling pathway (32). Interestingly, our findings showed that in human islets, GABA-induced CREB activation was not suppressed upon inhibiting the

PI3K-Akt signaling pathway, whereas blockade of PKA-dependent CREB activation did not affect GABA-stimulated Akt activation. This suggests that the two signaling pathways downstream of GABA_AR and GABA_BR are both actively involved in conveying GABA actions in the human β -cells. GABA_BR is a G-protein-coupled receptor consisting of two nonvariable subunits, and upon activation it initiates cAMP signaling and Ca²⁺-dependent signaling. Of note, previous studies demonstrated that under membrane depolarizing conditions, activation of GABA_AR triggered voltage-gated calcium channel-dependent Ca²⁺ influx and Ca²⁺ release from intracellular stores, whereas GABA_BR activation evoked intracellular Ca²⁺ only (19). Our observations that the GABA_BR-mediated activation of the cAMP-PKA signaling is independent of the PI3K-Akt pathway appear to be of physiological relevance. This provides a plausible mechanism by which GABA may be bypassing Akt activation in subjects with insulin resistance.

Pancreatic β -cells are susceptible to injury under glucolipotoxicity or inflammatory cytokine-producing conditions. This appears to be due at least in part to the production of ROS (33) causing β -cell apoptosis and a loss of functional β -cell mass (34). The NSG mice used in this study have impaired innate and adaptive immunity and likely produce much reduced inflammatory cytokines (35). However, the transplanted organs in these mice are also exposed to other nonspecific inflammatory stimuli that generate nitric oxide and ROS. Notably, the newly transplanted islets are essentially avascular. This ischemic microenvironment followed by reperfusion as a result of revascularization produces conditions known to induce detrimental ROS in transplanted organs (36). In this study, we show that GABA protects human β -cells from apoptosis induced by inflammatory cytokines in vitro and from the spontaneous apoptosis observed in transplanted islets in vivo. This protective effect undoubtedly contributes to the increase in β -cell mass observed.

The trophic effects of GABA in human islets appear to be physiologically relevant and might contribute to the recovery or preservation of β -cell mass in diabetic patients. In T1D, a major limitation of β -cell replacement therapy by islet transplantation or other methods is the persistent autoimmune destruction of these cells, primarily by apoptosis. Thus, there is only a limited chance of therapeutic success unless this immune component is suppressed. GABA has the rare combination of properties of stimulating β -cell growth, suppressing inflammation (insulinitis), and inhibiting apoptosis. These features point to a possible application in the treatment of T1D. Moreover, our findings suggest that GABA might improve the outcome of clinical islet transplantation.

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Duality of Interest. Q.W. is an inventor of GABA-related patents and serves on the Scientific Advisory Board of Diamyd Medical. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. I.P. and J.Z. performed the experiments, analyzed data, and wrote the manuscript. X.L. performed islet transplantation. M.D. and Z.-P.F. performed the calcium measurement. D.O.S. contributed to in vitro apoptosis studies and data analysis. Z.Z., Y.L., D.L.G., and M.A. provided intellectual input. C.L. and A.T. contributed to cell line Western blot assays. E.S. contributed to islet image capture and data analysis. C.H. and S.P. isolated the human islets. R.B. provided technique assistance in islet transplantation. G.J.P. contributed to experimental design and preparation of the manuscript. Q.W. conceived and designed the study, analyzed data, wrote the manuscript, and was responsible for the overall study. Q.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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