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Insulin secretion stimulated by L-arginine and its metabolite L-ornithine depends on $G\alpha_{i2}$

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Leiss V, Flockerzie K, Novakovic A, Rath M, Schönsiegel A, Birnbaumer L, Schürmann A, Harteneck C, Nürnberg B. Insulin secretion stimulated by L-arginine and its metabolite L-ornithine depends on $G\alpha_{i2}$. *Am J Physiol Endocrinol Metab* 307: E800–E812, 2014. First published September 9, 2014; doi:10.1152/ajpendo.00337.2014.—*Bordetella pertussis* toxin (PTx), also known as islet-activating protein, induces insulin secretion by ADP-ribosylation of inhibitory G proteins. PTx-induced insulin secretion may result either from inactivation of $G\alpha_o$ proteins or from combined inactivation of $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ isoforms. However, the specific role of $G\alpha_{i2}$ in pancreatic β-cells still remains unknown. In global ($G\alpha_{i2}^{-/-}$) and β-cell-specific ($G\alpha_{i2}^{\beta cko}$) gene-targeted $G\alpha_{i2}$ mouse models, we studied glucose homeostasis and islet functions. Insulin secretion experiments and intracellular Ca^{2+} measurements were used to characterize $G\alpha_{i2}$ function in vitro. $G\alpha_{i2}^{-/-}$ and $G\alpha_{i2}^{\beta cko}$ mice showed an unexpected metabolic phenotype, i.e., significantly lower plasma insulin levels upon intraperitoneal glucose challenge in $G\alpha_{i2}^{-/-}$ and $G\alpha_{i2}^{\beta cko}$ mice, whereas plasma glucose concentrations were unchanged in $G\alpha_{i2}^{-/-}$ but significantly increased in $G\alpha_{i2}^{\beta cko}$ mice. These findings indicate a novel albeit unexpected role for $G\alpha_{i2}$ in the expression, turnover, and/or release of insulin from islets. Detection of insulin secretion in isolated islets did not show differences in response to high (16 mM) glucose concentrations between control and β-cell-specific $G\alpha_{i2}$-deficient mice. In contrast, the two- to threefold increase in insulin secretion evoked by L-arginine or L-ornithine (in the presence of 16 mM glucose) was significantly reduced in islets lacking $G\alpha_{i2}$. In accord with a reduced level of insulin secretion, intracellular calcium concentrations induced by the agonistic amino acid L-arginine did not reach control levels in β-cells. The presented analysis of gene-targeted mice provides novel insights in the role of β-cell $G\alpha_{i2}$ showing that amino acid-induced insulin-release depends on $G\alpha_{i2}$.

$G\alpha_{i2}$; insulin secretion; β-cell; L-arginine; GPCR

GLUCOSE IS THE PRINCIPAL STIMULATOR of insulin secretion from pancreatic β-cells. Together with regulators such as other nutrients or hormones, it adjusts insulin secretion according to physiological demands. A disruption of this tightly controlled process can lead to diabetes mellitus and its comorbidities. Physiological regulators of insulin release include not only glucose but also other nutrients such as free fatty acids or amino acids on the one hand, and hormones including glucagon and norepinephrine on the other hand. They all have in common that they signal via seven-transmembrane G protein-coupled receptors (GPCR) present on the surface of pancreatic β-cells (1, 28, 33). Upon ligand binding, GPCRs activate heterotrimeric G proteins, thereby modulating the activity of cellular effectors. It is widely accepted that insulin release from pancreatic β-cells can be triggered via $G\alpha_s$- and/or G_q/G_{11}-dependent mechanisms (2, 41). Studies in isolated systems and in animals, using pertussis toxin (PTx), also known as islet-activating protein, suggest $G\alpha_i/G\alpha_o$-dependent signaling as being important for inhibition of insulin secretion (11, 14, 15). PTx specifically catalyzes the ADP-ribosylation of a cysteine residue located four residues from the carboxyl terminus of the α-subunits of $G\alpha_i/G\alpha_o$ proteins. Thus, PTx treatment disrupts GPCR stimulation of the G protein and thereby disconnects it from signal transduction. The α-subunits of the PTx-sensitive G proteins include three $G\alpha_i$ ($G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$) proteins, which are the product of three different genes, and two $G\alpha_o$ splice variants i.e., $G\alpha_{o1}$ and $G\alpha_{o2}$ (3). $G\alpha_i/G\alpha_o$ proteins show a sequence identity of up to 95% with overall sequence homology being more pronounced among the three $G\alpha_i$ isoforms (37). Due to this high homology, it has been suggested that all $G\alpha_i$ and $G\alpha_o$ proteins serve the same functions; namely, they are activated by the same or a similar set of GPCRs and appear to signal to a fully overlapping set of effectors. However, combined analysis of global and islet cell-specific $G\alpha_{o1}$- and/or $G\alpha_{o2}$-deficient mouse lines and PTx treatment identified $G\alpha_{o2}$ as the main inhibitory G protein related to insulin release (32, 34, 42). Glucose-induced insulin secretion (34) and insulin containing vesicle docking (42) were increased in the absence of $G\alpha_{o2}$. Moreover, PTx treatment of $G\alpha_o$-deficient mice had a negligible effect on the already disarranged insulin secretion (42). These findings indicate that $G\alpha_o$ proteins are the major isoform responsible for PTx-sensitive inhibition of insulin secretion. Additional evidence suggests that $G\alpha_{o2}$ mediates the somatostatin (SST)- and galanin-induced receptor-to-effector signal transduction in β-cells (32). Together, these findings support a prominent role for $G\alpha_{o2}$ as the major regulator in GPCR-induced inhibition of insulin secretion. It is presently largely unclear whether the PTx-sensitive $G\alpha_{i2}$ isoform expressed in islets or elsewhere, such as intestine, skeletal muscle, or adipose tissue is of any significance for insulin secretion in vitro and/or glucose homeostasis in vivo. To elucidate the biological role and gain insight into the cellular mechanisms of

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$G\alpha_{i2}$ protein function in pancreatic β-cells, we examined global and β-cell-specific $G\alpha_{i2}$-deficient mice. We show that $G\alpha_{i2}$ mediates L-arginine-induced insulin secretion by modulation of corresponding changes in $[Ca^{2+}]_i$ that trigger exocytosis. Decreased plasma insulin levels in $G\alpha_{i2}$-targeted animals leading to an impaired glucose tolerance supported these in vitro findings. These data strongly promote and unexpectedly stress a stimulatory role for $G\alpha_{i2}$ in insulin secretion, showing that although PTx inhibits $G\alpha_o$ and $G\alpha_{i2}$ proteins these two isoforms have distinct and opposite roles in the regulation of insulin secretion. Whereas $G\alpha_o$ inhibits, $G\alpha_{i2}$ stimulates insulin secretion from β-cells.

MATERIALS AND METHODS

Experimental animals. The generation of the global $G\alpha_{i2}$-deficient mice ($G\alpha_{i2}^{-/-}$) has been described previously (29). To prolong life expectancy of the $G\alpha_{i2}^{-/-}$ animals, mice were bred and kept in individually ventilated cages (IVC) under specific-pathogen-free conditions (39) and had free access to water and standard chow. β-Cell-specific deletion of $G\alpha_{i2}$ ($G\alpha_{i2}^{\beta cko}$) was achieved by crossing the floxed $G\alpha_{i2}$ mouse line (27) and the Rip-$Cre^{+/tg}$ mouse line (12), both on a C57BL/6N background. The Rip-$Cre^{+/tg}$ mouse line was a kind gift from Pedro Luis Herrera, Geneva. $G\alpha_{i2}^{\beta cko}$ and their littermate controls (ctrl) were kept under specific-pathogen-free conditions with free access to water and standard chow. For all experiments, gene-targeted $G\alpha_{i2}$ animals were compared with their littermate wild types (WT) or controls (genotype: $G\alpha_{i2}^{+/+}$;Rip-$Cre^{+/tg}$ or $G\alpha_{i2}^{+/fl}$;Rip-$Cre^{+/tg}$) on a C57BL/6N background (age 6–24 wk). All animals analyzed within this study were maintained and bred in the animal facility of the Institute of Experimental and Clinical Pharmacology and Toxicology, Eberhard Karls University, Tübingen. All experimental procedures were approved by the local government's Committee on Animal Care and Welfare Tübingen.

Isolation of pancreatic islets. Islets were isolated according to procedure in Ref. 20. Upon a retrograde injection of 2 ml of collagenase solution (1.9 U/ml collagenase; Sigma Aldrich, Munich, Germany) in 1× HBSS (Life Technologies, Darmstadt, Germany) via the bile duct, fully perfused pancreata were incubated for 10 min at 37°C. Islets were picked using a 200-μl pipette during several washing steps.

Whole pancreatic insulin content. Pancreata were removed and weighed. After a homogenization step in 7 ml of ice-cold 1.5% HCl in 70% ethanol, pancreata were incubated in acid ethanol for 24 h at 4°C. Insulin content was determined in the supernatant with the commercially available ultrasensitive insulin ELISA method (Mercodia, Uppsala, Sweden). Pancreatic insulin content was normalized to the weight of the pancreas.

Islet morphology. Whole pancreatic tissue was fixed in 4% formaldehyde for 24 h and embedded in paraffin at a stretch according to standard procedures, and longitudinal 6-μm serial sections were performed. Every 250 μm, pancreata were stained with hematoxylin and eosin, and every single islet within the stained pancreatic slice was quantified. Average islet and cell size was determined by hand, whereas the number of nuclei were calculated with the AxioVision software (Zeiss, Jena, Germany). Mean values derived from each individual mouse were used to calculate means ± SE of three mice in each group.

In vitro experiments with isolated islets. The effects of treatments on insulin secretion were assessed using static incubation experiments (20). Overnight-cultured islets [RPMI 1640 (GE Healthcare Europe, Freiburg, Germany), 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin] were grouped as batches of 10 islets and incubated in Krebs-buffered HEPES saline (120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM HEPES, 20 mM $NaHCO_3$, 0.5 mg/ml BSA, pH 7.4) containing 3 mM glucose in humidified 5% CO_2 at 37°C for 30 min. Subsequently, the supernatant was discarded, and fresh Krebs-buffered HEPES saline was added supplemented with either 3 or 16 mM glucose with or without L-arginine (10 mM), L-ornithine (10 mM), L-leucine (100 μM), palmitate (0.2 mM), clonidine (1 μM), SST (0.1 μM), or KCl (30 mM). The islets were incubated in humidified 5% CO_2 air at 37°C for 1 h. Afterward, islets were treated with ice-cold acid ethanol (1.5% HCl, 70% ethanol) for 1 h. For each condition, aforementioned above 3 and 16 mM glucose was used to test the quality of islet preparation. The amount of secreted insulin in the supernatant as well as the whole insulin content was determined with a commercially available ultrasensitive insulin ELISA method (DRG Diagnostics, Marburg, Germany). Islet DNA was isolated with the Isolate II Genomic DNA kit (Bioline, Luckenwalde, Germany), and the islet DNA content was measured (30). Based on the identical DNA and whole insulin contents of $G\alpha_{i2}^{\beta cko}$ and ctrl islets, the amounts of secreted insulin were indicated % of insulin content.

Immunoblot analysis. Freshly isolated islets were homogenized in 0.8 μl of protein lysis buffer per islet. The protein extractions were performed on pooled islets isolated from a single animal. To achieve electrophoretical separation of $G\alpha_i$ isoforms, separation was performed in gels containing 6 M urea. The proteins were visualized by immunodetection using the following primary antibodies described elsewhere (7, 10, 21, 39): rabbit anti-$G\alpha_{i2}$ (1:8,000 islets, 1:20,000 hypothalamus), rabbit anti-$G\alpha_{i3}$ (1:5,000), rabbit anti-$G\alpha_{o1/o2}$ (1:2,000), and rabbit anti-$G\beta_{common}$ (1:9,000). Antibodies against $G\alpha_{s(short)/s(long)}$ (1:1,000; SC-383) and $G\alpha_q$ (1:1,000; SC-393) were purchased from Cell signaling. Equal loading was verified with antibodies against mouse anti-α-tubulin (1:4,000, Sigma-Aldrich) or mouse anti-β-actin (1:4,000 islets, 1:40,000 hypothalamus; Sigma-Aldrich). The protein levels of $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_q$, $G\alpha_s$, $G\beta_1$, and $G\beta_2$ were quantified using densitometric analysis software (Image Lab; Bio-Rad, Gräfelfing, Germany) and were normalized to the α-tubulin or β-actin levels of the same samples.

Immunofluorescence. Immunodetection was performed on serial cryosections (8 μm) of pancreata fixed in 4% paraformaldehyde. For antigen retrieval, slices were treated with 0.3% Triton X-100-PBS for 30 min. To avoid unspecific antibody binding, slices were incubated in 5% normal goat serum (NGS)-PBS prior to incubations in primary antibody dilutions. Primary antibodies used were specific for rabbit anti-$G\alpha_{i2}$ (1:1,500), mouse anti-glucagon (1:5,000; Sigma-Aldrich, Hamburg, Germany), and guinea pig anti-insulin (1:5,000; DAKO, Hamburg, Germany) and diluted in 1.5% NGS-PBS. Binding of primary antibodies was performed at 4°C overnight. The complexes were detected with secondary antibodies conjugated to fluorescent dyes (1:200 dilution in PBS, Invitrogen). Slices were embedded in Roti-Mount-Fluoro-Dapi (Carl Roth, Karlsruhe, Germany). Fluorescence was visualized using either the Zeiss laser scanning microscope LSM 510 or the Zeiss Axio Image.M2 microscope (Zeiss, Jena, Germany).

Glucose tolerance tests. Mice were fasted overnight, followed by an intraperitoneal (ip) injection of glucose (2 mg/g body wt). Blood samples were collected via the tail vein, and blood glucose levels were measured using a Contour glucometer (Bayer, Leverkusen, Germany).

L-Ornithine test. Mice were fasted overnight but supplemented with 1 mM L-ornithine in their drinking water to ensure sufficient high basal L-ornithine levels in the plasma. Mice received 100 μl of a 1.32 mg/ml L-ornithine solution by ip injection, and blood samples were collected via tail vein before administration and after 1 min to determine plasma insulin levels with the commercially available ultrasensitive insulin ELISA method (see above).

Insulin tolerance test. Mice were fasted for 4 h, followed by an ip injection of insulin (1 mU/g body wt). Blood glucose levels were measured using a Contour glucometer (Bayer).

Plasma analysis. Plasma levels of insulin were measured using a commercially available insulin ELISA method (see above), whereas plasma C-peptide levels were determined with the 96-well fluorescent Milliplex immunoassay (Millipore, Darmstadt, Germany).

Ca^{2+} imaging. Intracellular calcium measurements were performed as previously described (16). Isolated islets were dispersed by trypsin-EDTA digestion, plated on glass coverslides, and allowed to recover for 4 h in humidified 5% CO_2 air at in 37°C. Cells were loaded with Fura 2-AM (5 μM) 30 min prior to calcium measurements. Changes of intracellular calcium concentrations $[Ca^{2+}]_i$ were recorded with a Till Photonics Oligochrome V (FEI, Gräfeling, Germany). After 4 min of baseline recordings in the presence of 5 mM glucose, dispersed islet cells were treated with 16 mM glucose, 10 mM L-arginine, 10 mM D-arginine, or a combination of 16 mM glucose and L-arginine for 10 min. Maximum depolarization of β-cells was evaluated by the use of 30 mM KCl. Emitted fluorescence was excited at 340 and 380 nm and measured at 520 nm, and the ratio 340/380 was calculated. Cells were identified as β-cells, when being inactive at 5 mM glucose, with stable baseline recordings, and an obvious response to the applied stimuli (e.g., 16 mM glucose, 10 mM L-arginine). Cells without Ca^{2+} responses to the respective stimuli were excluded from the measurements and analysis.

Statistical analysis. Data are presented as means ± SE of individual data points. Data were analyzed using Student's *t*-test for unpaired groups. $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of β-cell-specific $G\alpha_{i2}$-deficient mice. The generation and characterization of global $G\alpha_{i2}$-deficient ($G\alpha_{i2}^{-/-}$) mice has been described (27, 29, 39). The β-cell-specific $G\alpha_{i2}$-deficient mice, termed $G\alpha_{i2}^{\beta cko}$ (genotype: $G\alpha_{i2}^{fl/fl}$;Rip-$Cre^{+/tg}$), were generated by crossing Rip-Cre mice (12) with mice carrying floxed $G\alpha_{i2}$ alleles (27). The resulting progeny, i.e., the different genotypes, were born at expected Mendelian ratios and were viable and fertile. Since the expression of the Rip-Cre transgene has been reported to have metabolic effects per se (19), we used Rip-Cre-positive littermates throughout this study as controls (ctrl genotype: $G\alpha_{i2}^{+/+}$; Rip-$Cre^{+/tg}$ or $G\alpha_{i2}^{+/fl}$;Rip-$Cre^{+/tg}$) for experiments including $G\alpha_{i2}^{\beta cko}$ mice after having confirmed that heterozygous mice, e.g., $G\alpha_{i2}^{+/fl}$;Rip-$Cre^{+/tg}$ mice were indistinguishable from $G\alpha_{i2}^{+/+}$;Rip-$Cre^{+/tg}$ animals. Recombination was validated by PCR analysis of genomic DNA (Fig. 1, *A* and *B*). Whereas DNA isolated from hearts and islets from ctrl animals as well as cardiac DNA of the $G\alpha_{i2}^{\beta cko}$ mice remained in the premutant state, islet DNA of $G\alpha_{i2}^{\beta cko}$ mice showed clear recombination by appearance of an additional 390-bp band (Fig. 1, *A* and *B*). Since strong hypothalamic recombination has been reported in different Rip-Cre mouse lines (38), we analyzed DNA isolated from ctrl and $G\alpha_{i2}^{\beta cko}$ hypothalami. The absence of the 390-bp band indicative for recombination argues against significant recombination events in the hypothalamic brain region of our mouse line (Fig. 1*B*). The expression pattern of $G\alpha_{i2}$ protein in pancreatic islets was studied by immunoblot analysis. Using specific antibodies against the COOH-terminal sequence of $G\alpha_{i2}$, we detected the $G\alpha_{i2}$ protein in islet homogenates of WT and ctrl mice, whereas $G\alpha_{i2}$ was strongly reduced by ~90% in $G\alpha_{i2}^{\beta cko}$ animals (Fig. 1, *C* and *D*), and absent in $G\alpha_{i2}^{-/-}$ islet lysates (Fig. 1*C*). Remaining $G\alpha_{i2}$ immunoreactivity in $G\alpha_{i2}^{\beta cko}$ likely results from the expression of $G\alpha_{i2}$ in other endocrine cell types i.e., α-, δ-, ε-, or PP-cells (see below). To further verify the cell-specific ablation of $G\alpha_{i2}$ in $G\alpha_{i2}^{\beta cko}$ tissues, immunoblot analysis of hypothalamic lysates from ctrl and $G\alpha_{i2}^{\beta cko}$ animals were performed. $G\alpha_{i2}$ protein was absent in hypothalamic protein extracts from $G\alpha_{i2}^{-/-}$ mice (Fig. 1*E*), whereas the $G\alpha_{i2}$ protein levels of $G\alpha_{i2}^{\beta cko}$ were unaffected, i.e., at control levels (Fig. 1, *E* and *F*). The combined analysis of $G\alpha_{i2}^{\beta cko}$ tissues by genomic PCRs and immunoblot make it very unlikely that the studied physiological effects resulted from cells other than the pancreatic β-cells.

Examination of Rip-Cre-mediated recombination was performed by staining of $G\alpha_{i2}$ protein in pancreatic cryosections on the cellular level. β-Cells were visualized by insulin-specific antibodies (Fig. 1*G*). A diffuse yellow background staining was detectable; however, the specific $G\alpha_{i2}$ staining was restricted in proximity to the plasma membrane. Insulin (blue) was found mainly in the cytoplasm in ctrl, $G\alpha_{i2}^{\beta cko}$, and $G\alpha_{i2}^{-/-}$ islets (left). In ctrl islets, $G\alpha_{i2}$ staining mainly localized to the plasma membrane (*top middle*) and was detectable in insulin-positive and insulin-negative cells (*top right*). In contrast, only a few primarily outer cells of the $G\alpha_{i2}^{\beta cko}$ islets showed the specific membrane-close $G\alpha_{i2}$ staining (*middle*, arrows). These $G\alpha_{i2}$-positive cells were all identified as insulin negative and therefore non-β-cells (*middle right*). In $G\alpha_{i2}^{-/-}$ pancreata, the membrane-close $G\alpha_{i2}$ staining was absent in all islet cells, insulin-negative and insulin-positive ones (*bottom middle* and *bottom right*). These results validated that $G\alpha_{i2}$ and insulin could be found only in ctrl β-cells, whereas $G\alpha_{i2}^{\beta cko}$ β-cells lacked the $G\alpha_{i2}$ protein. Therefore, a successful recombination in $G\alpha_{i2}^{\beta cko}$ islets was restricted to β-cells and demonstrated on a cellular level.

Analysis of G protein subunit levels in $G\alpha_{i2}^{-/-}$ and $G\alpha_{i2}^{\beta cko}$ islets of Langerhans. Compensatory upregulation of closely related proteins is a threat of many studies using gene-deficient animals. The expression of almost all $G\alpha_{i/o}$ isoforms in pancreatic islets as well as $G\alpha_q$, and $G\alpha_s$ has been described. All of them perform major functions in the insulin secretion machinery. To get insight into possible upregulation and compensation in $G\alpha_{i2}$ deficiency by other G protein family members, $G\alpha_{i2}^{-/-}$ (Fig. 2) and $G\alpha_{i2}^{\beta cko}$ (Fig. 3) islet homogenates were immunoblotted for putative changes in the expression of $G\alpha_{i3}$, $G\alpha_{o1}$, $G\alpha_{o2}$, $G\alpha_q$, $G\alpha_{s(short)}$, and $G\alpha_{s(long)}$. The expression levels of the $G\alpha_{o1}$, $G\alpha_{o2}$, $G\alpha_q$, and $G\alpha_s$ isoforms remained at the control values in $G\alpha_{i2}^{-/-}$ (Fig. 2, *A–C*) and $G\alpha_{i2}^{\beta cko}$ islets (Fig. 3, *A–C*). In contrast, the highly homologous isoform $G\alpha_{i3}$ was significantly upregulated in $G\alpha_{i2}^{-/-}$ islets (Fig. 2*D*) (146.3 ± 14.3% of wt, $P < 0.001$). Importantly, in $G\alpha_{i2}^{\beta cko}$ islets, $G\alpha_{i3}$ protein levels remained unchanged (119.7 ± 12.2% of control, $P = 0.22$; Fig. 3*D*).

G proteins are heterotrimeric proteins consisting of an α-subunit and a βγ-complex. Upon ligand activation of a GPCR, the α-subunit dissociates from the receptor and from the βγ-complex. Both the α-subunit and the βγ-complex modulate the activity of a variety of effectors, including ion channels and enzymes. Due to high $G\alpha_i$ expression levels, the receptor-dependent activation of $G\alpha_i$ results in the release of significant amounts of βγ-complexes (4). Moreover, it has been shown that stabilization of GDP-bound Gα by Gβγ is likely a major mechanism for the maintenance of stoichiometry between Gα and Gβγ. (9, 18). Therefore, an important point was to investigate whether expression levels of the β-isoforms were altered in $G\alpha_{i2}^{-/-}$ and $G\alpha_{i2}^{\beta cko}$ islets. Unaltered Gβ-protein levels, but simultaneously reduced Gα protein levels, might result in permanently free βγ-complexes and increased βγ-mediated effects. This could mask the physiological consequences of $G\alpha_{i2}$ deletion. At least five different Gβ subunits ($G\beta_1$–$G\beta_5$)

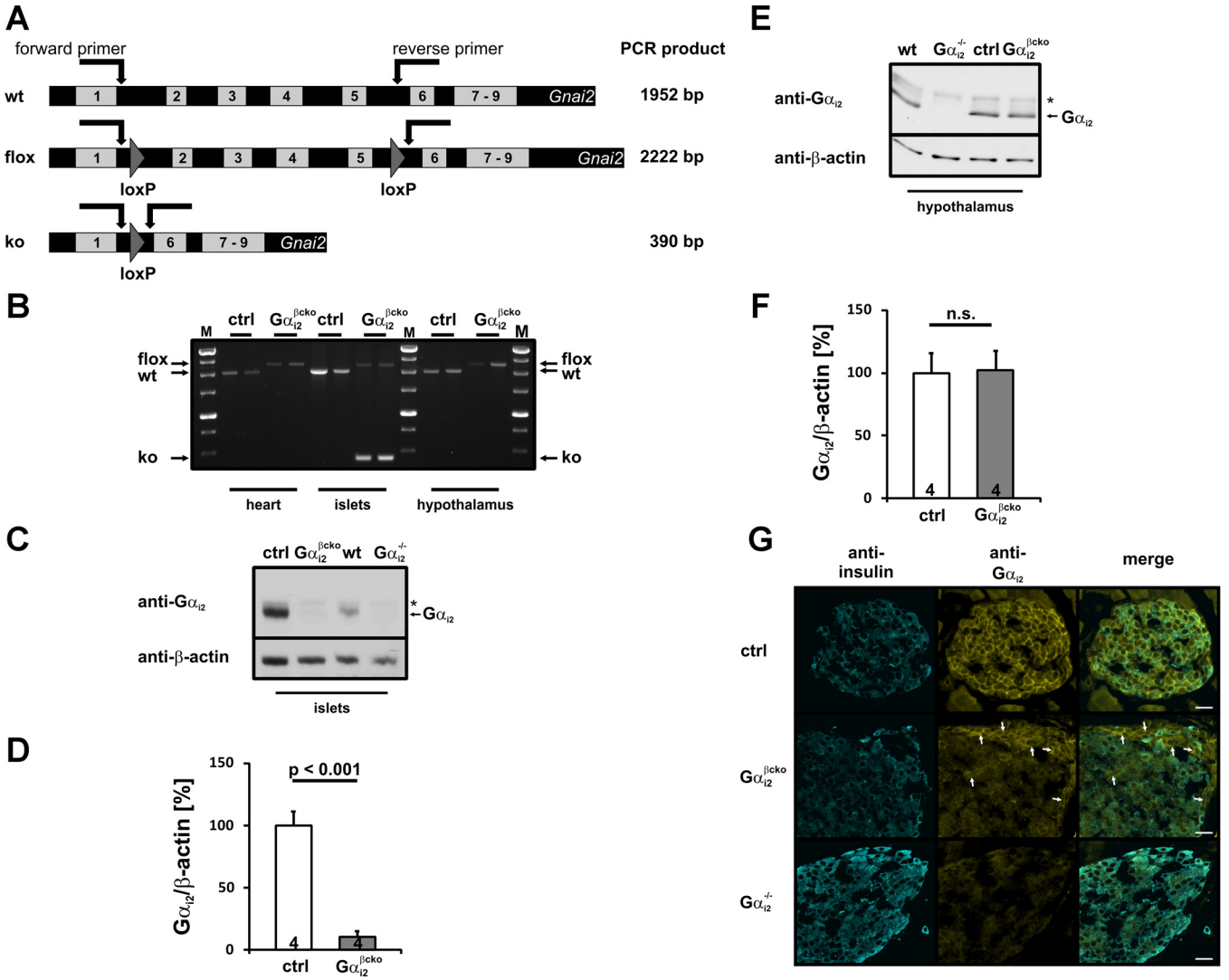

Fig. 1. $G\alpha_{i2}$ expression analysis in β-cell-specific ($G\alpha_{i2}^{\beta cko}$) and globally $G\alpha_{i2}$-deficient ($G\alpha_{i2}^{-/-}$) mice *A*: schematic overview of the *Gnai2* gene and PCR strategy for recombination analyses. *B*: genomic DNA analysis of heart, islets, and hypothalamus isolated from control (ctrl) and $G\alpha_{i2}^{\beta cko}$ animals. Recombination is restricted to $G\alpha_{i2}^{\beta cko}$ islets. *C*: representative immunoblots of islet homogenates isolated from ctrl, $G\alpha_{i2}^{\beta cko}$, wild-type (wt), and $G\alpha_{i2}^{-/-}$ mice. $G\alpha_{i2}$ protein is almost absent in $G\alpha_{i2}^{\beta cko}$ islets and completely absent in $G\alpha_{i2}^{-/-}$ islets. The weak *top* band (*) may have resulted from $G\alpha_{i1}$ expression in islets. Equal loading was confirmed by β-actin detection. *D*: statistical analysis of $G\alpha_{i2}$ expression levels in ctrl and $G\alpha_{i2}^{\beta cko}$ islets. Islets from 4 animals were analyzed in ≥3 independent experiments. *E*: representative immunoblot analysis of hypothalamic homogenates isolated from ctrl and $G\alpha_{i2}^{\beta cko}$ mice. As a negative control, hypothalamic homogenates from $G\alpha_{i2}^{-/-}$ mice were loaded. The *top* band (*) might result from $G\alpha_{i1}$ expression in the brain. Equal loading was confirmed by β-actin detection. *F*: statistical analysis of $G\alpha_{i2}$ expression levels in hypothalami of ctrl and $G\alpha_{i2}^{\beta cko}$ mice. Hypothalamic tissue from 4 animals was analyzed in ≥3 independent experiments. *G*: representative immunostainings of pancreatic cryosections stained against $G\alpha_{i2}$ (yellow) and insulin (blue). $G\alpha_{i2}$ shows proximity to the plasma membrane, whereas insulin is mainly found in the cytoplasm. In ctrl islets, $G\alpha_{i2}$ staining is detectable in both insulin-positive β-cells and insulin-negative cells. $G\alpha_{i2}$ protein is absent in $G\alpha_{i2}^{\beta cko}$ β-cells but can be observed in insulin-negative cells. $G\alpha_{i2}$ staining is completely absent in all $G\alpha_{i2}^{-/-}$ islet cells. Diffuse yellow staining is background staining. Scale bars, 20 μm.

have been described (13), with β_1 and β_2 being predominantly and ubiquitously expressed. In both mutant mouse lines, lower expression of $G\beta_1$ ($G\alpha_{i2}^{-/-}$ 64.3 ± 9.6% of wt, $P = 0.06$; $G\alpha_{i2}^{\beta cko}$ 61.1 ± 19.5% of ctrl, $P = 0.1$) and $G\beta_2$ ($G\alpha_{i2}^{-/-}$ 80.3 ± 4.3% of wt, $P < 0.01$; $G\alpha_{i2}^{\beta cko}$ 58.2 ± 8.2% of ctrl, $P < 0.01$) were noted in islet homogenates compared with the respective controls (Figs. 2*E* and 3*E*). However, the reduction of $G\beta_2$ levels was more prominent in $G\alpha_{i2}^{\beta cko}$ islets than in $G\alpha_{i2}^{-/-}$ islets, most likely due to increased $G\alpha_{i3}$ protein levels (145%) in $G\alpha_{i2}^{-/-}$ islets. The reduced Gβ protein levels let it seem rather unlikely that effects observed in the gene-targeted mice resulted from increased Gβγ complex activity.

Metabolic evaluation of $G\alpha_{i2}^{-/-}$ and $G\alpha_{i2}^{\beta cko}$ mice. To study the consequences of $G\alpha_{i2}$ deletion on the metabolism, we performed metabolic analysis of WT vs. $G\alpha_{i2}^{-/-}$ (Fig. 4) and ctrl vs. $G\alpha_{i2}^{\beta cko}$ (Fig. 5) mice. Global $G\alpha_{i2}$ deficiency is associated with a reduced body weight gain (22, 29), which was still observed when animals were kept under specific pathogen-free conditions (SPF) in IVCs (Fig. 4*A*). Differences in ad libitum-fed blood glucose levels were not obvious (Fig. 4*B*). Next an ip glucose tolerance test after overnight fasting was performed to analyze the impact of $G\alpha_{i2}$ deletion on the glucose homeostasis of these animals. Global deletion of $G\alpha_{i2}$ did not affect blood glucose levels during the glucose challenge

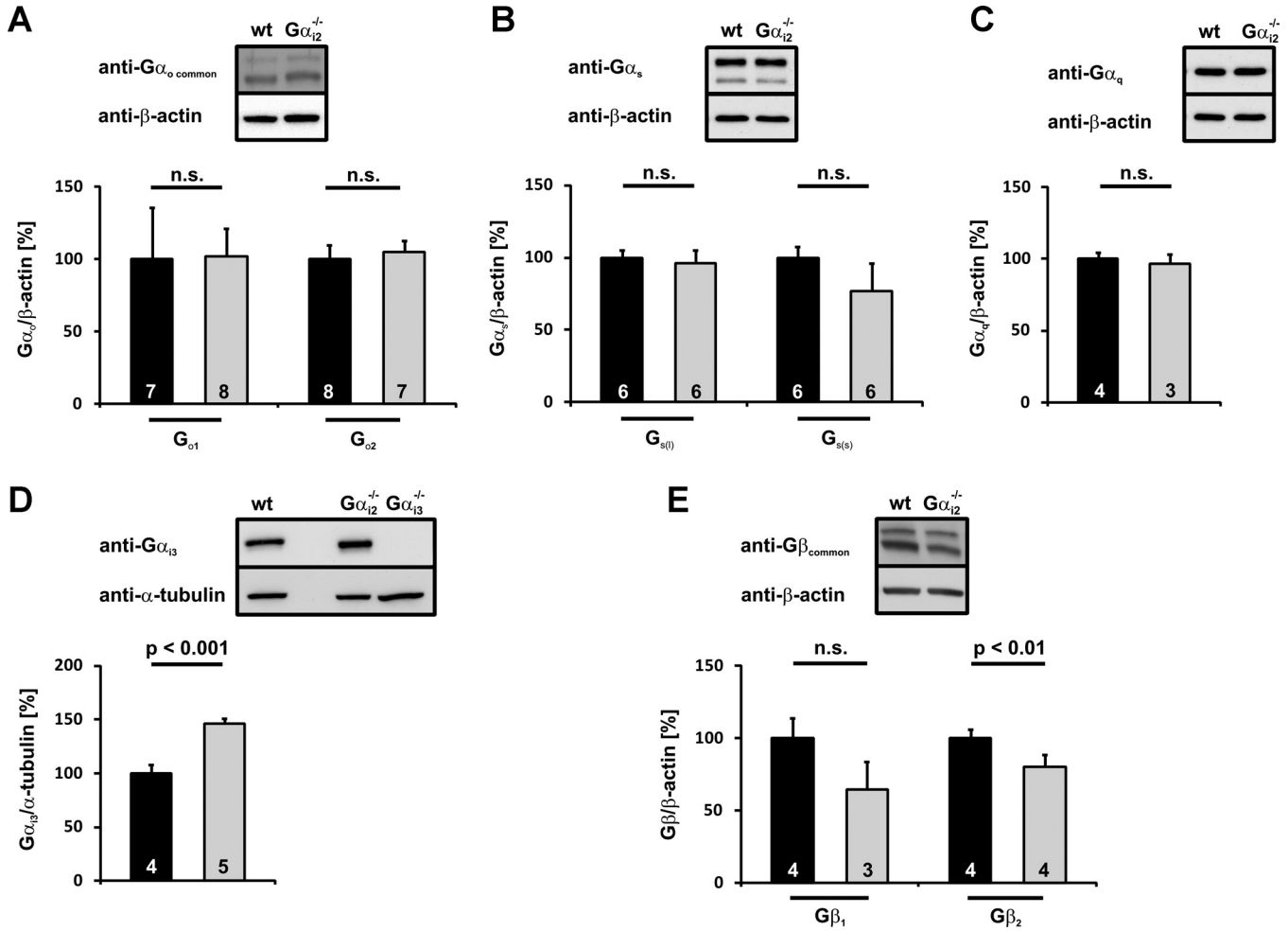

Fig. 2. Statistical analysis of different Gα- and Gβ-subunit expression patterns in $G\alpha_{i2}^{-/-}$ islets. $G\alpha_{o1}$, $G\alpha_{o2}$ (*A*), $G\alpha_{s(long)}$, $G\alpha_{s(short)}$ (*B*), and $G\alpha_q$ (*C*) expression levels are not affected by $G\alpha_{i2}$ deletion in $G\alpha_{i2}^{-/-}$ islets. *D*: $G\alpha_{i3}$ protein upregulation is detectable in $G\alpha_{i2}^{-/-}$ islets. To verify $G\alpha_{i3}$ antibody specificity, $G\alpha_{i3}^{-/-}$ islets were loaded. *E*: $G\beta_1$ and $G\beta_2$ protein levels are downregulated in $G\alpha_{i2}^{-/-}$ islets. $G\beta_2$ downregulation gains statistical significance. *Insets*: representative immunoblots of islet homogenates from wt and $G\alpha_{i2}^{-/-}$. Equal loading was confirmed by β-actin or α-tubulin detection.

(Fig. 4*C*). Interestingly, significantly lower insulin levels were measured in $G\alpha_{i2}^{-/-}$ mice at 15, 30, and 60 min after glucose injection compared with WT mice (Fig. 4*D*). In addition, $G\alpha_{i2}^{-/-}$ animals displayed an improvement in insulin tolerance (Fig. 4*E*). To assess whether β-cell-specific deletion of $G\alpha_{i2}$ influences the body weight of $G\alpha_{i2}^{\beta cko}$ in a similar way as observed in $G\alpha_{i2}^{-/-}$ animals, we recorded body weight and fasted blood glucose levels over time in $G\alpha_{i2}^{\beta cko}$ and ctrl animals (Fig. 5, *A* and *B*). Continuous measurements did not reveal any differences between these two groups. Again, we monitored blood glucose levels following an ip injection of glucose after an overnight fasting period in the $G\alpha_{i2}^{\beta cko}$ and ctrl mice (Fig. 5*C*). β-Cell-specific $G\alpha_{i2}$ deletion caused significantly increased blood glucose levels in the $G\alpha_{i2}^{\beta cko}$ mice 30, 60, and 120 min after the glucose challenge, pointing to an impaired glucose tolerance. The parallel analysis of plasma insulin levels during the glucose tolerance test revealed significantly lower plasma insulin levels at 15, 30, and 60 min in $G\alpha_{i2}^{\beta cko}$ mice vs. the littermate controls (Fig. 5*D*). Consistent with the reduced plasma insulin, plasma C-peptide levels were significantly decreased at 15 and 30 min after the glucose bolus in $G\alpha_{i2}^{\beta cko}$ mice (Fig. 5*E*). Insulin sensitivity, tested by the injection of insulin to 4-h-fasted $G\alpha_{i2}^{\beta cko}$ and ctrl mice, revealed no differences between the two groups (Fig. 5*F*). These findings indicate that $G\alpha_{i2}$ is involved in the expression, turnover, or release of insulin from pancreatic islets. Higher plasma insulin levels would have been expected from previous PTx-based studies. Surprisingly, $G\alpha_{i2}$ deletion restricted to β-cells lowered plasma insulin levels. Collectively, these results indicate that deletion of $G\alpha_{i2}$ in β-cells impairs glucose tolerance by decreasing insulin secretion, suggesting that $G\alpha_{i2}$ is a positive regulator of insulin secretion.

Islet function in vitro. In $G\alpha_{i2}^{\beta cko}$ mice, $G\alpha_{i2}$ deletion is restricted to β-cells, $G\alpha_{i3}$ is not upregulated in this conditional gene-targeted mouse line, and body weight is not affected by β-cell $G\alpha_{i2}$ deletion. Thus, these mice are an appropriate model for studying the molecular mechanism of $G\alpha_{i2}$ signaling in isolated islets independently of systemic effects. No abnormalities were detected in morphometric analysis of $G\alpha_{i2}^{\beta cko}$ islets. Islet area, number of nuclei and islet size (Fig. 6, *A–E*), pancreas and islet insulin content, and whole islet DNA content were indistinguishable between ctrl and $G\alpha_{i2}^{\beta cko}$ islets (Fig. 6, *F–H*). We performed insulin secretion studies on islets isolated from $G\alpha_{i2}^{\beta cko}$ and ctrl mice. Insulin release was studied either

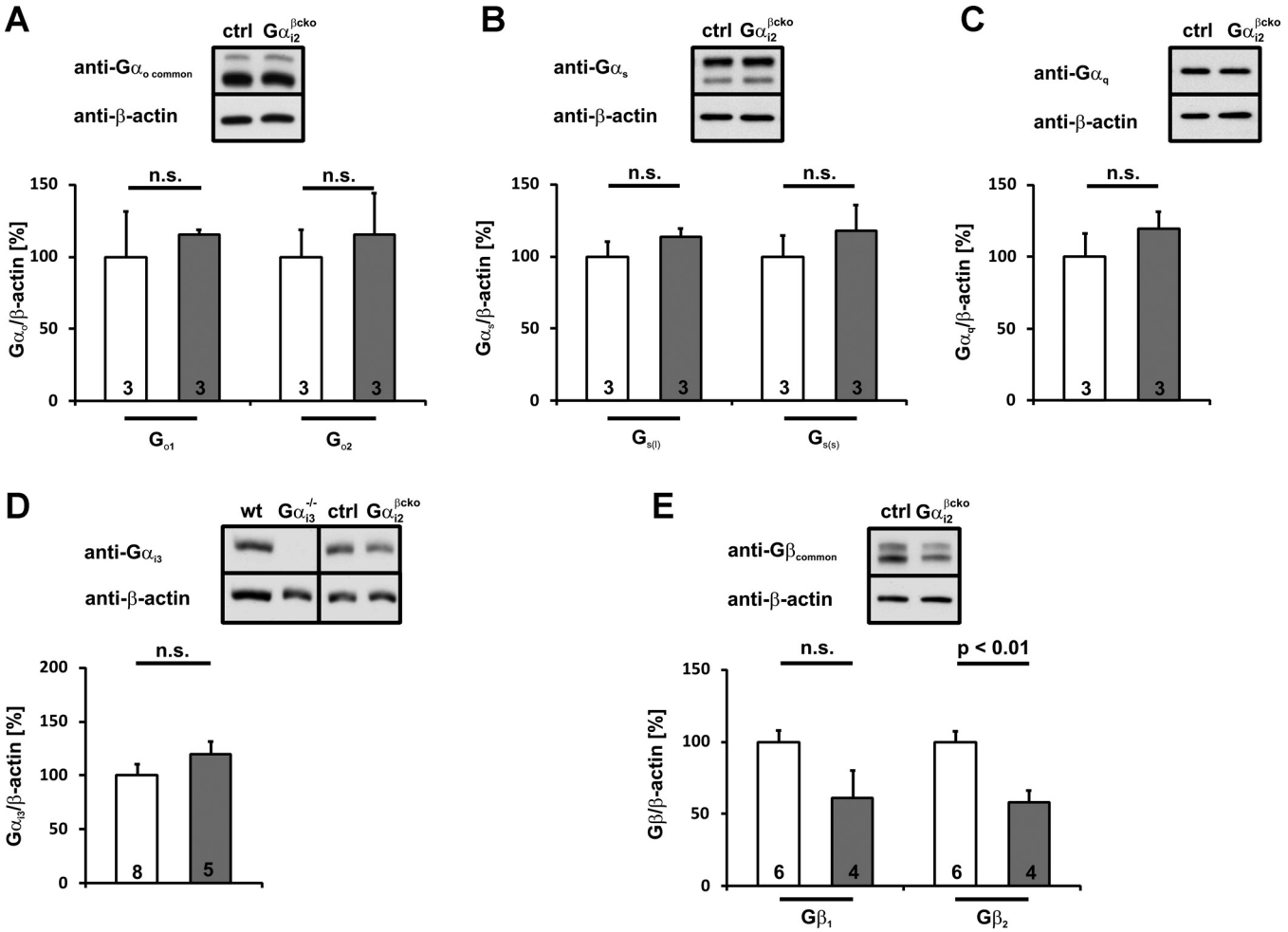

Fig. 3. Statistical analysis of different Gα- and Gβ-subunit expression patterns in $G\alpha_{i2}^{\beta cko}$ islets. $G\alpha_{o1}$, $G\alpha_{o2}$ (*A*), $G\alpha_{s(long)}$, $G\alpha_{s(short)}$ (*B*), $G\alpha_q$ (C), and $G\alpha_{i3}$ (*D*) protein expression levels are not influenced by $G\alpha_{i2}$-deletion in $G\alpha_{i2}^{\beta cko}$ islets. $G\alpha_{i3}$ antibody specificity was shown by loading of $G\alpha_{i3}^{-/-}$ islets. *E*: downregulation of $G\beta_1$ and $G\beta_2$ expression levels in $G\alpha_{i2}^{\beta cko}$ islets. $G\beta_2$ downregulation gains statistical significance. *Insets*: representative immunoblots of islet homogenates from wt and $G\alpha_{i3}^{-/-}$ or ctrl and $G\alpha_{i2}^{\beta cko}$ animals. Equal loading was confirmed by β-actin detection.

under low (3 mM) or high (16 mM) glucose concentrations. All $G\alpha_{i2}^{\beta cko}$ and ctrl islets produced a robust insulin-secretory response to high glucose concentrations, whereas insulin release was almost absent at low glucose concentrations (Fig. 7*A* and Table 1). Insulin secretion evoked by high K^+ concentration, which bypasses glucose metabolism, was also not affected by the deletion of $G\alpha_{i2}$ in $G\alpha_{i2}^{\beta cko}$ islets (Fig. 7*A*). Since a large body of PTx-based studies suggested that $G\alpha_{i2}$ proteins inhibit insulin secretion (11, 15, 28), we tested clonidine- and somatostatin (SST)-induced inhibition of insulin secretion. The inhibiting effects of clonidine (1 μM) and SST (100 nM) were unaffected by the absence of $G\alpha_{i2}$ in $G\alpha_{i2}^{\beta cko}$ islets (Figs. 7, *B* and *C*). This argues against an inhibitory role of $G\alpha_{i2}$ in the regulation of insulin secretion from β-cells. In contrast, significantly reduced in vivo plasma insulin levels in $G\alpha_{i2}^{\beta cko}$ mice suggest that $G\alpha_{i2}$ modulates glucose-induced insulin release by an unknown parameter, for instance, a factor present in the serum, i.e., fatty acids and/or amino acids, which signals via islet GPCRs (37). To identify the stimulus, we analyzed the effect of the free fatty acid palmitate on insulin secretion in $G\alpha_{i2}^{\beta cko}$ islets. Simultaneous stimulation of insulin with high glucose and palmitate (200 μM) resulted in increased insulin secretion in ctrl and $G\alpha_{i2}^{\beta cko}$ islets (Fig. 8*A*). Next, we studied amino acids. Application of L-leucine (100 μM) did not alter the insulin response either in ctrl or $G\alpha_{i2}^{\beta cko}$ islets (Fig. 8*B*). However, application of L-arginine and L-ornithine surprisingly produced significantly differing insulin secretion responses in ctrl and $G\alpha_{i2}^{\beta cko}$ islets. Costimulation of L-arginine (10 mM) together with glucose (16 mM) showed decreased insulin release from $G\alpha_{i2}^{\beta cko}$ islets compared with ctrl islets (Fig. 8*C*). To test whether L-arginine signals via nitric oxide (NO), we used L-ornithine. In combination with glucose, L-ornithine (10 mM) was able to mimic the effect observed with L-arginine (Fig. 8*D*). None of the tested inhibitors or stimulators used within this setup was able to induce significant insulin secretion from isolated islets in the presence of 3 mM glucose (Table 1). To test whether our findings of significantly reduced L-arginine- and L-ornithine-induced insulin secretion from $G\alpha_{i2}^{\beta cko}$ islets are of any physiological relevance, we performed an in vivo L-ornithine challenge. L-Ornithine was used instead of the more potent stimulus L-arginine to exclude interferences by NO-driven pathways. We studied whether a single dose of L-ornithine (1.32 mg/ml L-ornithine ip) affects plasma insulin levels in vivo in male mice. Whereas plasma

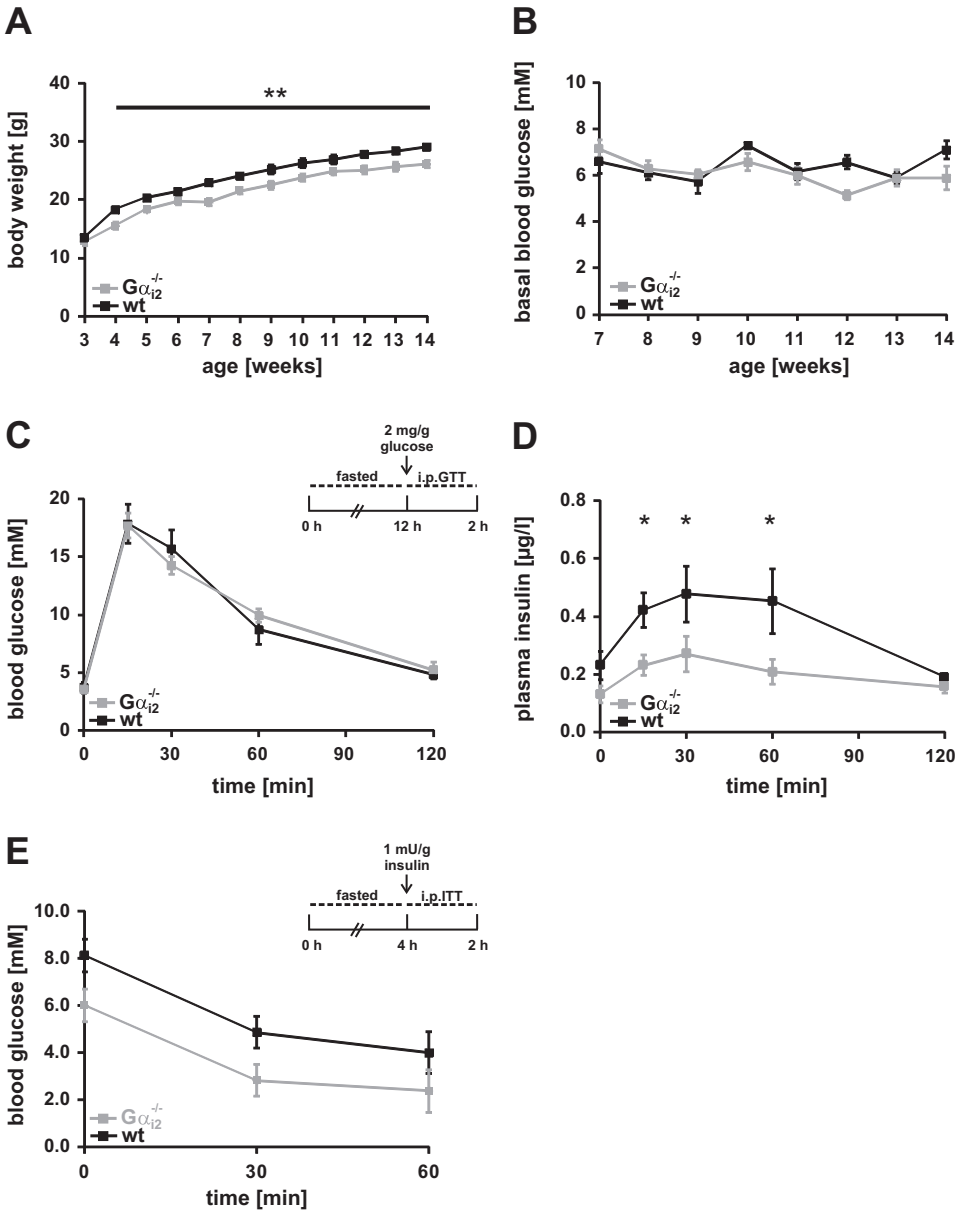

Fig. 4. Metabolic phenotyping of $G\alpha_{i2}^{-/-}$ animals. *A*: body weight gain of wt and $G\alpha_{i2}^{-/-}$ mice. Body weight of $G\alpha_{i2}^{-/-}$ mice is significantly reduced. *B*: blood glucose levels determined in wt and $G\alpha_{i2}^{-/-}$ mice over time, which are comparable between the 2 genotypes ($n = 16$ animals per genotype). *C*: blood glucose levels in 8- to 10-wk-old wt (■) and $G\alpha_{i2}^{-/-}$ (■) mice following ip application of glucose after overnight fasting are equal ($n = 8$ animals per group). *D*: plasma insulin levels during ip glucose tolerance testing in wt and $G\alpha_{i2}^{-/-}$ animals ($n = 8$ animals per group). Plasma insulin levels are significantly reduced at 15, 30, and 60 min. *E*: insulin tolerance testing in 4-h-fasted wt and $G\alpha_{i2}^{-/-}$ mice ($n = 6$ per genotype). $^*P < 0.05$.

insulin levels of control mice immediately increased after the L-ornithine application, plasma insulin levels of $G\alpha_{i2}^{\beta cko}$ animals remained at basal levels (Fig. 8*E*). Taken together, the reduced L-ornithine-induced insulin secretion in vitro and in vivo strengthen the assumption that the PTx-sensitive $G\alpha_{i2}$ isoform is not an inhibitor but a stimulator of insulin secretion.

$[Ca^{2+}]_i$ measurements in islets. To gain detailed insight into the signaling pathway, we aimed to link our in vitro findings to an intracellular correlate. It is commonly accepted that insulin secretion is a result of an increase in $[Ca^{2+}]_i$. This can be determined in single islet cells and is therefore a cell-based correlate for β-cell insulin secretion. Measurements of intracellular calcium changes link the $G\alpha_i$-dependent signaling to insulin secretion at a cellular level. Changes in $[Ca^{2+}]_i$ signals for individual β-cells were recorded in Fura 2-loaded single islet cells. For basal conditions, β-cells were kept at 5 mM glucose before treatment with 16 mM glucose. Control and $G\alpha_{i2}^{\beta cko}$ single islet cells showed a similar increase in $[Ca^{2+}]_i$ in response to the application of 16 mM glucose (Fig. 9*A*). The Ca^{2+} response was significantly reduced in $G\alpha_{i2}^{\beta cko}$ islet cells when stimulated with L-arginine (10 mM) in the presence of basal glucose levels (5 mM) (Fig. 9*B*). Being aware that a simple depolarization event could be caused by the positive charge of the basic amino acid, we tested D-arginine (10 mM), the enantiomer of L-arginine. In contrast to L-arginine, D-arginine did not induce a calcium response either in $G\alpha_{i2}^{\beta cko}$ or in ctrl single islet cells (Fig. 9*C*). In contrast, the L-arginine-induced response was reduced by 25% (Fig. 9*E, left*). The reduced response to L-arginine of $G\alpha_{i2}^{\beta cko}$ cells was even more evident when β-cells were coexposed to high glucose concentrations and L-arginine (Fig. 9*D*). The L-arginine-induced increase in $[Ca^{2+}]_i$ was calculated as the difference between the maxima obtained with glucose plus L-arginine (Fig. 9*D*) minus the maxima obtained with glucose alone (Fig. 9*A*). The cotreatment resulted in a robust increase in $[Ca^{2+}]_i$ in control islets, whereas the response of $G\alpha_{i2}^{\beta cko}$ islet cells was signif-

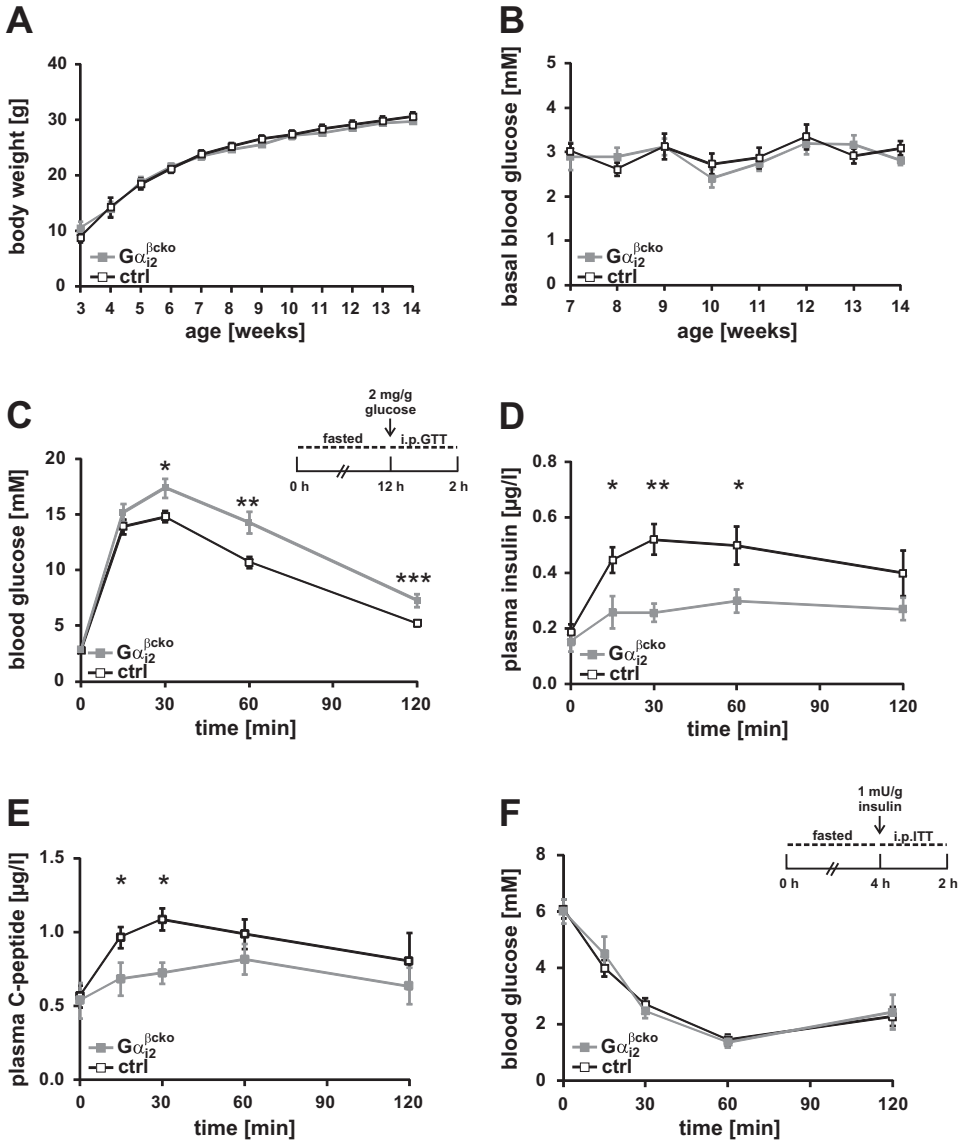

Fig. 5. Metabolic phenotyping of $G\alpha_{i2}^{\beta cko}$ mice. *A*: body weight determined in ctrl and $G\alpha_{i2}^{\beta cko}$ mice over time. Body weight gain was similar in ctrl and $G\alpha_{i2}^{\beta cko}$ mice ($n = 8$ animals per genotype). *B*: fasted blood glucose levels monitored in ctrl (□) and $G\alpha_{i2}^{\beta cko}$ (■) mice over time. *C*: blood glucose levels of 8- to 10-wk-old ctrl and $G\alpha_{i2}^{\beta cko}$ mice following ip application of glucose after overnight fasting ($n = 16$ animals per group). $G\alpha_{i2}^{\beta cko}$ mice have impaired glucose tolerance. Plasma insulin (*D*) and plasma C-peptide (*E*) levels during ip glucose tolerance testing were significantly reduced in $G\alpha_{i2}^{\beta cko}$ mice ($n = 5$–8 animals per group). *F*: insulin tolerance test in 4-h-fasted ctrl and $G\alpha_{i2}^{\beta cko}$ mice ($n = 8$ animals per group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

icantly reduced by 44% (Fig. 9*E, right*). The Ca^{2+} responses evoked by high K^+ were similar in ctrl and $G\alpha_{i2}^{\beta cko}$ β-cells (Fig. 9, *D* and *F*). These analyses identify β-cell $G\alpha_{i2}$ as necessary for the stimulation of insulin secretion by L-arginine in a calcium-dependent manner but independent of a depolarization that may be caused by the positive charge of the amino acid.

DISCUSSION

The present study analyzed the physiological role of $G\alpha_{i2}$ in insulin-secreting β-cells by using global and β-cell-specific $G\alpha_{i2}$ mutants. Significantly lower plasma insulin levels were observed upon glucose challenge in both the $G\alpha_{i2}^{\beta cko}$ and the $G\alpha_{i2}^{-/-}$ animal models. A normal glucose-induced insulin secretion in vitro per se accompanied these in vivo findings. Importantly, the selective and specific stimulatory effects of amino acids such as L-arginine and L-ornithine on insulin secretion were significantly diminished in $G\alpha_{i2}$-deficient β-cells. This was not due to alterations in islet morphology, since islet size, the number of nuclei per islet, and islet cell size were unaffected by the $G\alpha_{i2}$ deficiency. This study provides causal evidence for the involvement of $G\alpha_{i2}$ in the positive regulation of plasma insulin levels and glucose tolerance in vivo and islet insulin secretion in vitro. Although PTx-based studies in mice and isolated islets assumed an inhibitory role for all $G\alpha_i$ and $G\alpha_o$ proteins, our data surprisingly indicate that $G\alpha_{i2}$ proteins rather stimulate than inhibit the secretion of insulin by regulating L-arginine- and L-ornithine-induced insulin secretion in vitro and in vivo.

Currently, the upstream regulator of $G\alpha_{i2}$ is unknown. Two recent studies describe mouse models lacking the nutrient receptor GPCR family C group 6 member A (GPRC6A), which is a receptor for L-amino acids (35), in particular those with basic side chains (24, 36). Whereas Smajilovic et al. (31) found evidence for the expression of GPRC6A in pancreatic islets but found no evidence that the L-arginine-induced insulin secretion was affected by GPRC6A deletion, Pi et al. (25, 26) reported that GPRC6A mediates osteocalcin- and L-arginine-induced insulin secretion from β-cells in global and β-cell-specific GPRC6A-deficient mouse models. Our data from global and

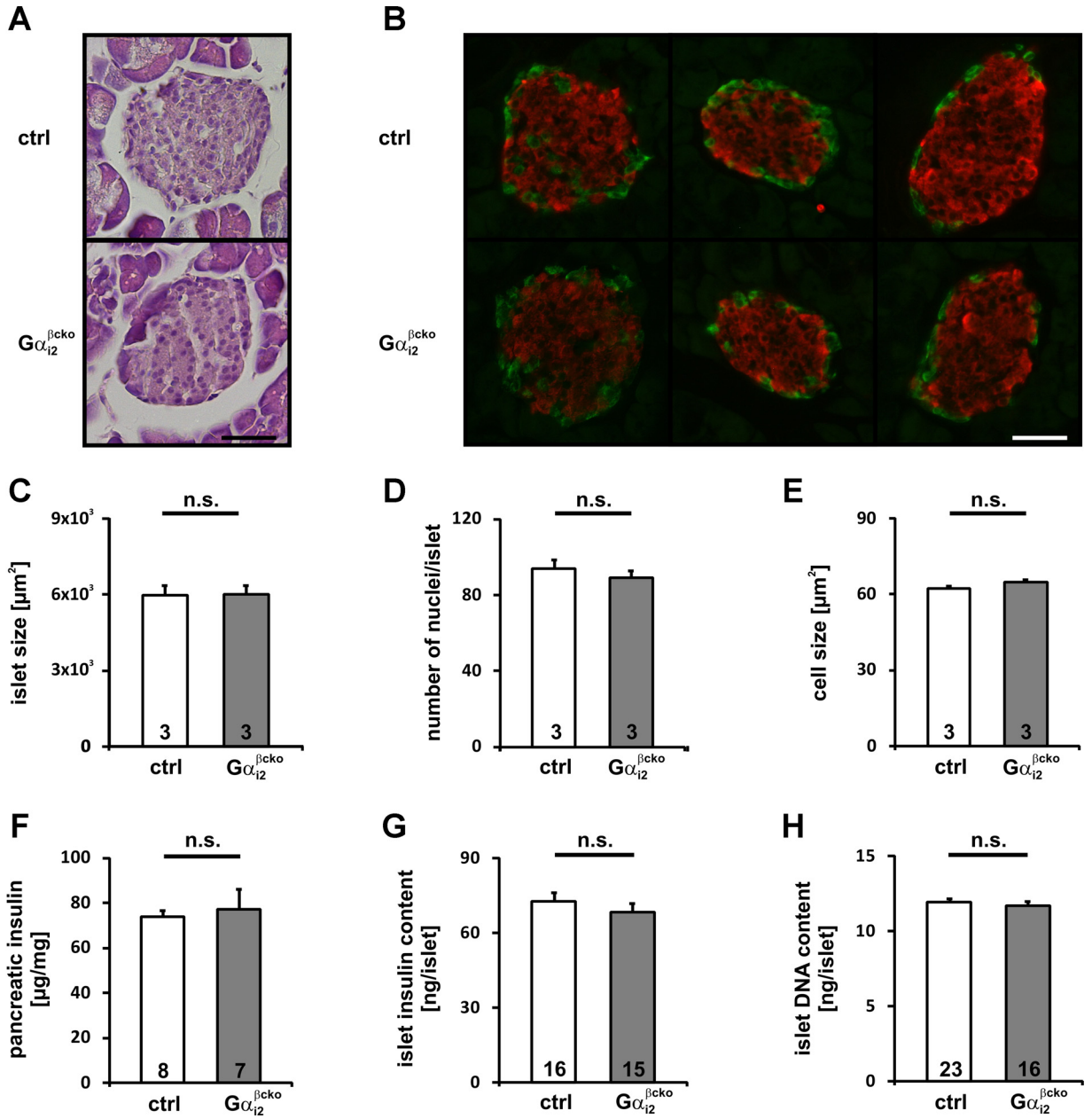

Fig. 6. Morphometric analyses of $G\alpha_{i2}^{\beta cko}$ islets. *A*: representative H&E-stained ctrl and $G\alpha_{i2}^{\beta cko}$ islets. *B*: representative sections of pancreata from ctrl and $G\alpha_{i2}^{\beta cko}$ mice visualized by immunofluorescence with anti-insulin (red) and anti-glucagon (green) antibodies. Scale bar, 40 µm. Islet size (*C*), number of nuclei per islet (*D*), and mean cell size (*E*) of ctrl and $G\alpha_{i2}^{\beta cko}$ islets were equal. Morphometric analyses were performed in 6-wk-old male mice. In total, 381 control and 455 $G\alpha_{i2}^{\beta cko}$ islets derived from 3 mice per genotype were quantified for this evaluation. Whole pancreas insulin content (*F*), islet insulin (*G*), and islet DNA content (*H*) of ctrl and $G\alpha_{i2}^{\beta cko}$ mice did not differ between genotypes. Islet insulin or islet DNA content was determined following static incubation experiments (*n*, number of triplicates).

β-cell-specific $G\alpha_{i2}$-deficient mice showing significantly decreased plasma insulin levels following glucose challenge are consistent with those from global and β-cell-deficient GPRC6A mice (25). Future studies will have to clarify whether the concordant findings in the GPRC6A-deficient and $G\alpha_{i2}$-deficient mice are mechanistically connected.

$G\alpha_{i2}^{\beta cko}$ islets secreted significantly less insulin upon co-stimulation with glucose and L-ornithine or L-arginine. These findings are complementary to recent studies analyzing global and β-cell-specific gene-targeted $G\alpha_o$ mice (42). Those authors suggest that $G\alpha_o$ is a strong inhibitor of insulin secretion, since its ablation produced a strong stimulatory effect on insulin release. $G\alpha_o$ has been implicated in regulating insulin vesicle docking on β-cell membranes (42) and in inhibiting insulin release via signaling through galanin- and SST-activated GPCRs (32, 34). In line with these findings, herein we find significant inhibition of insulin secretion by SST in both control and $G\alpha_{i2}^{\beta cko}$ islets. Of course, we are aware of the fact that these findings conflict with the expected functions for $G\alpha_{i2}$ based on PTx experiments, a pan inhibitor of $G\alpha_i$ and $G\alpha_o$ proteins and therefore a strong stimulator of insulin secretion. However, clonidine, an α_2-adrenoreceptor agonist, completely inhibited

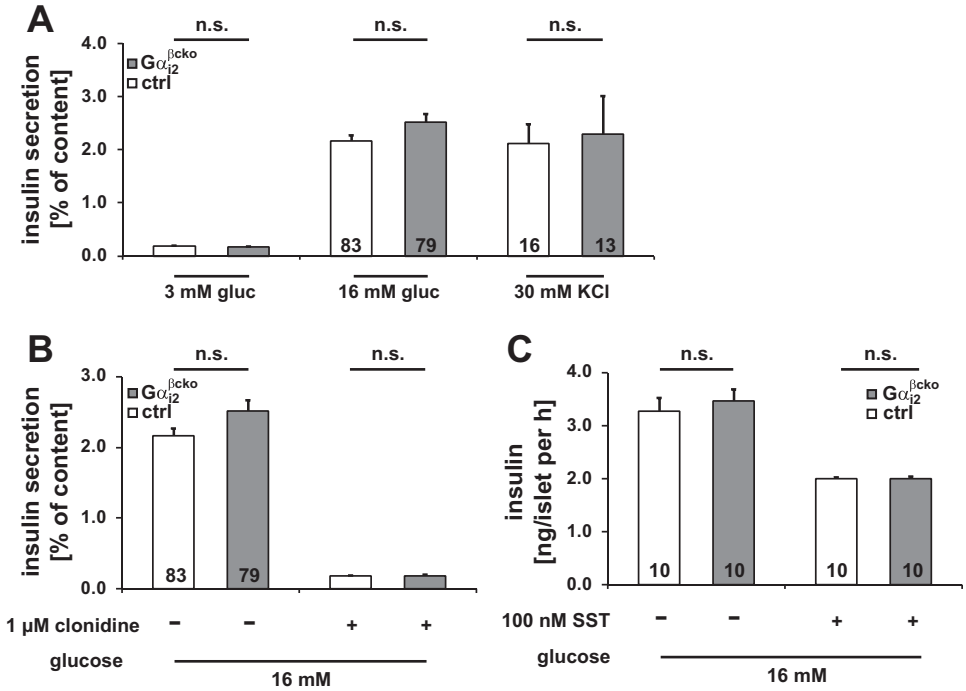

Fig. 7. Insulin secretion experiments in ctrl and $G\alpha_{i2}^{\beta cko}$ islets. *A*: glucose-induced insulin secretion measured at 3 and 16 mM glucose and 30 mM KCl (3 mM glucose: ctrl $n = 60$; $G\alpha_{i2}^{\beta cko}$ $n = 59$; n, number of incubations). Inhibitory effect of clonidine (*B*) and SST (*C*) on glucose-induced insulin secretion (1 μM clonidine: ctrl $n = 15$; $G\alpha_{i2}^{\beta cko}$ $n = 14$) was not affected by deletion of $G\alpha_{i2}$ in β-cells. All conditions were performed in islet preparations derived from ≥3 animals per genotype.

insulin release in control and $G\alpha_{i2}^{\beta cko}$ islets. Thus, our data set provides additional evidence that $G\alpha_{i2}$ is not involved in the α_2-adrenoreceptor-mediated signaling. The inhibition of the inhibitory $G\alpha_o$-driven pathway by PTx may be the major cause of PTx-induced insulin release from islets.

Moreover, the PTx effect was negligible in β-cell-specific $G\alpha_o$-deficient animals (42). This suggests that, despite the fact that $G\alpha_i$ proteins being expressed in islet cells and ADP-ribosylated by PTX, $G\alpha_o$ is the major target for PTx stimulating insulin secretion, thereby making an inhibitory role of $G\alpha_{i2}$ very unlikely (42). A single study shows an inhibitory role for $G\alpha_{i2}$ in ghrelin-dependent inhibition of insulin secretion based on an antisense oligonucleotide approach (5). Our data demonstrate that deletion of $G\alpha_{i2}$ resulted in normal glucose-induced insulin secretion per se, but stimulatory effects of amino acids like L-arginine and L-ornithine on insulin secretion were diminished in $G\alpha_{i2}^{\beta cko}$ islets. Moreover, $G\alpha_{i2}^{\beta cko}$ mice exhibit impaired glucose tolerance caused by significantly decreased plasma insulin levels. This is also in contrast to data obtained from PTx-based studies, strengthening again the fact that $G\alpha_{i2}$ is an important stimulator of insulin secretion. This view is also supported by the failure of in vivo-applied L-ornithine to provoke a rise in insulin plasma levels in β-cell-specific $G\alpha_{i2}$-deficient animals, which was seen in littermate controls.

Our detailed analysis of G protein levels in the $G\alpha_{i2}$ gene-targeted mouse lines corroborates significant upregulation of $G\alpha_{i3}$ (about 45%) in the global $G\alpha_{i2}$-deficient mice, which has also been described for other tissues and organs (6, 17, 39, 40). In contrast to the global $G\alpha_{i2}$-deficient islets, the $G\alpha_{i3}$ protein levels were unchanged in $G\alpha_{i2}^{\beta cko}$ islets. Since plasma insulin levels are reduced in both gene-targeted mouse lines, $G\alpha_{i3}$ is obviously not able to compensate for the lack of $G\alpha_{i2}$. This confirms previous reports on specific and distinct functions of $G\alpha_{i2}$ and $G\alpha_{i3}$ (8, 10, 17). Reduced Gβ expression levels parallel deletion of $G\alpha_{i2}$. Heterotrimeric G proteins are stoichiometrically composed of Gα and Gβγ, which dissociate selectively during the activation cycle. The stabilization of Gα and Gβγ in the trimeric complex is likely a major mechanism for the maintenance of the stoichiometric equivalence between Gα and Gβγ in the cell (9, 18). We found Gβ levels significantly reduced in both global and β-cell-specific $G\alpha_{i2}$-deficient

Table 1. *Insulin secretion evoked by 3 mM glucose concentration and in the presence of the respective substances*

		Ctrl			$G\alpha_{i2}^{\beta cko}$			
		Secreted insulin			Secreted insulin			
Condition		%Insulin content	SE	n	%Insulin content	SE	n	P
3 mM glucose		0.189	± 0.02	28	0.174	± 0.02	27	n.s.
3 mM glucose	1 μM clonidine	0.153	± 0.05	5	0.064	± 0.01	4	n.s.
	100 nM SST	0.176	± 0.05	4	0.141	± 0.04	3	n.s.
	200 mM palmitate	0.324	± 0.04	2	0.470	± 0.02	2	n.s.
	100 μM L-leucine	0.292	± 0.05	3	0.329	± 0.06	3	n.s.
	10 mM L-arginine	0.075	± 0.02	3	0.143	± 0.03	4	n.s.
	10 mM L-ornithine	0.150	± 0.02	6	0.039	± 0.01	5	n.s.

n.s., not significant; n, set of triplicates.

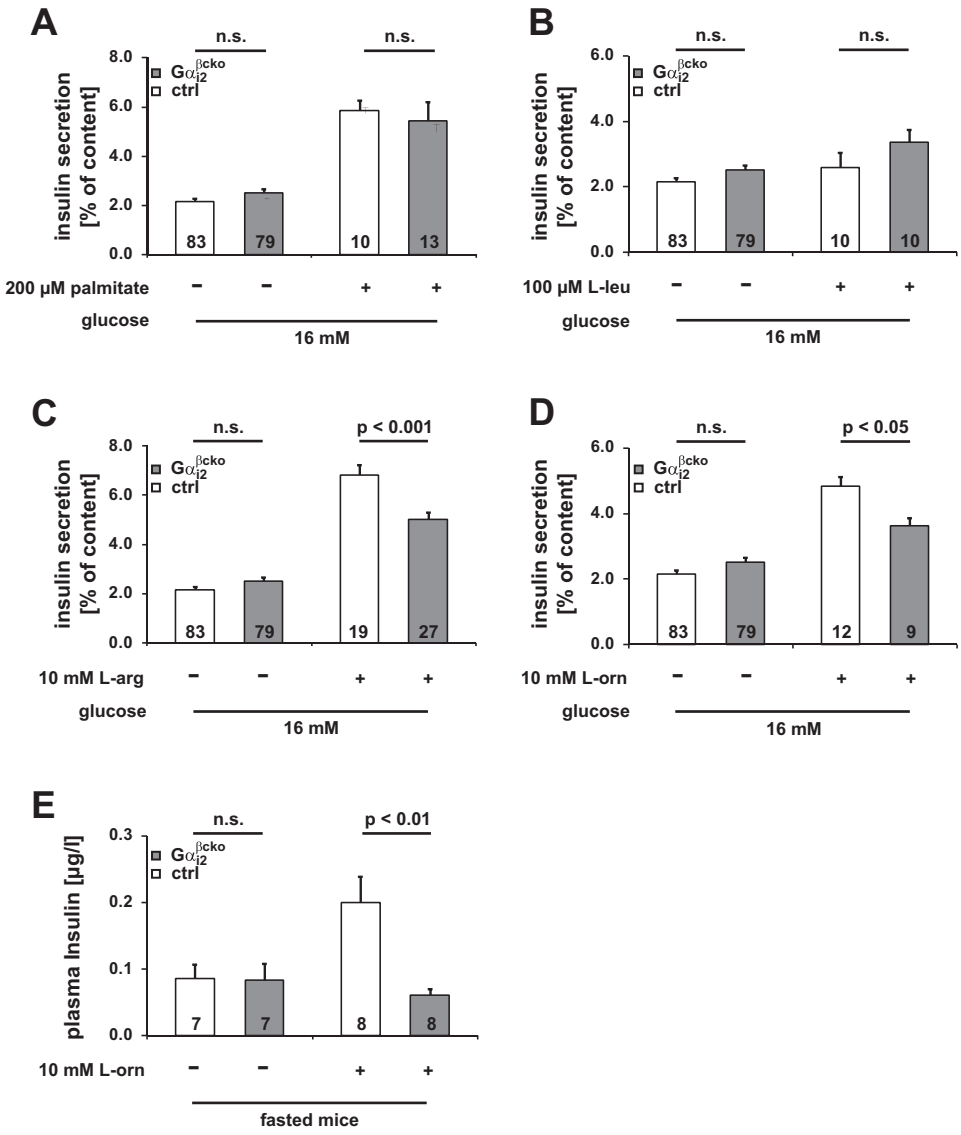

Fig. 8. Effect of different stimuli on insulin secretion experiments in ctrl and $G\alpha_{i2}^{\beta cko}$ islets. Stimulatory effect of palmitic acid (*A*), and L-leucine (*B*) was not affected by deletion of $G\alpha_{i2}$ in β-cells. Potentiating effect of L-arginine (*C*) and L-ornithine (*D*) on insulin secretion was significantly reduced in $G\alpha_{i2}^{\beta cko}$ (*n*, number of incubations). All conditions were performed in islet preparations derived from ≥3 animals per genotype. *E*: plasma insulin levels before and 1 min after ip injection of L-ornithine.

mouse lines, with a more pronounced reduction of Gβ levels in $G\alpha_{i2}^{\beta cko}$ islets (see Fig. 3*E*) than in $G\alpha_{i2}^{-/-}$ islets (see Fig. 2*E*). It is reasonable to assume that the differences in the extent of reduction between $G\alpha_{i2}^{-/-}$ and $G\alpha_{i2}^{\beta cko}$ islets result from the selective upregulation of $G\alpha_{i3}$ protein in $G\alpha_{i2}^{-/-}$ animals only, as $G\alpha_{i3}$ also requires Gβ as counterpart for the stabilization. This is in line with results from approaches using shRNA to downregulate Gβ isoforms in HeLa cells, which was accompanied with parallel reduced Gα protein levels in this cell line. Vice versa, several studies show that shRNA against $G\alpha_{i2}$ resulted in decreased $G\beta_1$, $G\beta_2$, and $G\beta_4$ protein levels (18, 39, 40). It has to be noted that $G\beta_2$ is the predominant isoform in islets, which has been reported only for human placenta (23). Signaling by the $G\alpha_i$ family of "inhibitory" proteins is complex. $G\alpha_i$ proteins can activate or inhibit many other effectors through either the GTP-bound $G\alpha_i$ or the released Gβγ dimer upon activation. Therefore, it is important to note that both the abolished signaling of $G\alpha_{i2}$ via direct interaction partners and the signaling via Gβγ complexes may equally contribute to the observed phenotypes of our gene-targeted mice.

The reduced insulin levels during glucose tolerance tests were observed in global and β-cell-specific $G\alpha_{i2}$-deficient mice. Differences in glucose were visible only in $G\alpha_{i2}^{\beta cko}$ animals, not in mice globally lacking $G\alpha_{i2}$. Wang et al. (34) also analyzed blood glucose levels of global $G\alpha_{i2}$-deficient mice, which were, similar to our studies, unaltered upon glucose challenge. However, those authors did not study β-cell-specific $G\alpha_{i2}$ mutants, and they did not report the plasma insulin levels during the glucose challenge. A reduced body weight of $G\alpha_{i2}^{-/-}$ mice has been reported (29) and is also observed in our mouse line. A lean phenotype improves glucose tolerance per se, which might explain similar blood glucose levels but simultaneously decreased plasma insulin levels in global $G\alpha_{i2}$ mutants. In line with this, $G\alpha_{i2}$-deficient mice showed improved insulin tolerance, arguing for the improved peripheral insulin sensitivity. The lean phenotype was not observed in β-cell-specific $G\alpha_{i2}$ mutants. Control and mutant animals showed similar weight gain. However, $G\alpha_{i2}^{\beta cko}$ animals showed impaired glucose tolerance caused by impairment of the β-cells' ability to secrete the required amount of insulin. The differences in

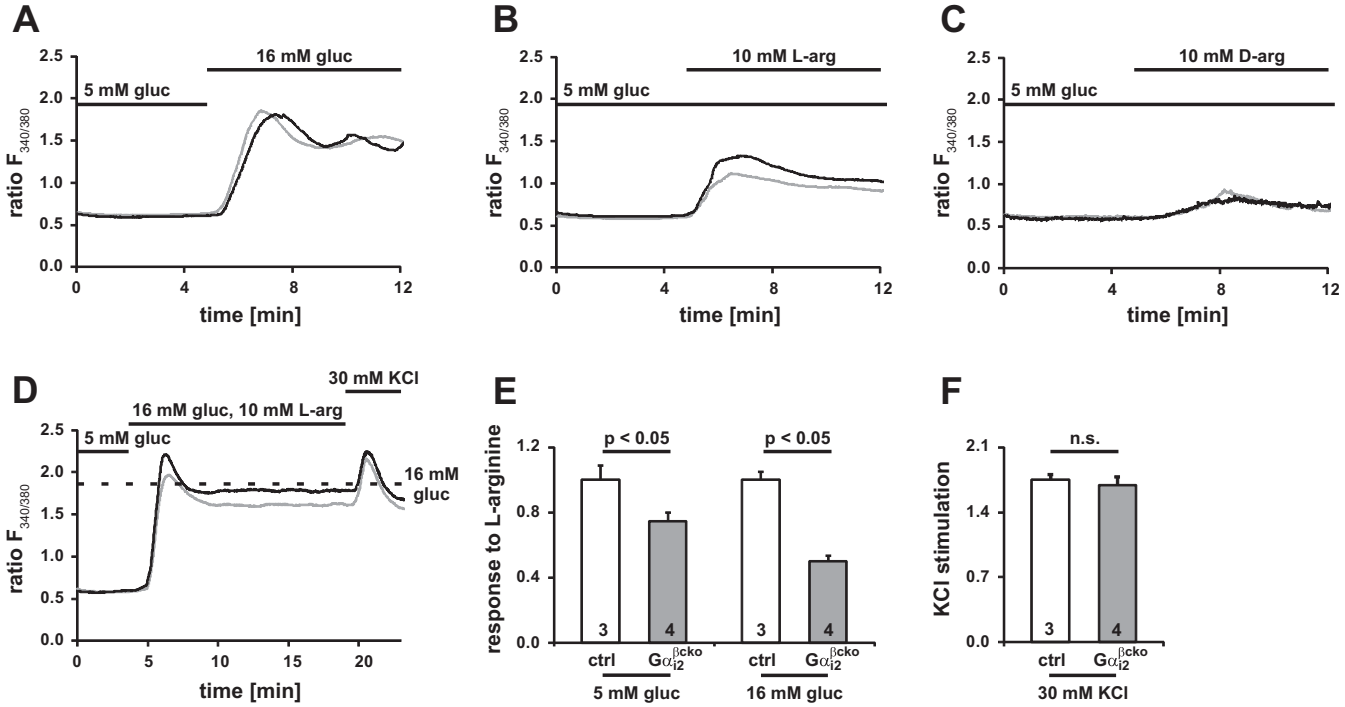

Fig. 9. Calcium measurements in dispersed ctrl and $G\alpha_{i2}^{\beta cko}$ β-cells. Representative $[Ca^{2+}]_i$ ratios (ctrl, black line; $G\alpha_{i2}^{\beta cko}$, gray line) elicited by 16 mM glucose (*A*), 10 mM L-arginine at 5 mM glucose (*B*), or 10 mM D-arginine at 5 mM glucose (*C*) (*A*, ctrl $n = 3$ mice, 465 cells; $G\alpha_{i2}^{\beta cko}$ $n = 4$ mice, 298 cells; *B*, ctrl $n = 7$ mice, 606 cells; $G\alpha_{i2}^{\beta cko}$ $n = 5$ mice, 687 cells; *C*, $n = 3$ mice, 447 cells; $G\alpha_{i2}^{\beta cko}$ $n = 3$ mice, 413 cells). *D*: representative $[Ca^{2+}]_i$ response to 16 mM glucose in combination with 10 mM L-arginine and final application of KCl (ctrl $n = 3$ mice, 585 cells; $G\alpha_{i2}^{\beta cko}$ $n = 4$ mice, 687 cells). Dotted line indicates maximum response to 16 mM glucose shown in *A*. *E*: maximum response to L-arginine was significantly reduced by 25% at 5 mM and 44% at 16 mM glucose concentrations in $G\alpha_{i2}^{\beta cko}$ β-cells compared with ctrl β-cells, whereas the response is the difference between the maxima obtained with glucose plus L-arginine (*D*) minus the maxima obtained with glucose alone (*A*); $n = 3$–4 animals per group. *F*: maximum depolarization evoked by 30 mM KCl ($n = 3$–4 animals per group). All recordings were started at 5 mM glucose with additions as shown on the figure.

blood glucose levels between global and β-cell-specific $G\alpha_{i2}$ mice imply that the metabolic functions of $G\alpha_{i2}$ in the body are broader than previously thought. $G\alpha_{i2}$ signaling might play a role for glucose homeostasis in liver, white adipose tissue, or skeletal muscle as well, but $G\alpha_{i2}$ signaling is also important for efficient pancreatic β-cell function. Thus, we propose that the diminished plasma insulin levels indeed result from islet $G\alpha_{i2}$ dysfunction, caused by an impaired potentiating effect of L-arginine and L-ornithine in pancreatic β-cells. In addition, the reduced increases of intracellular calcium levels upon L-arginine treatment in $G\alpha_{i2}^{\beta cko}$ β-cells strengthen the finding that $G\alpha_{i2}$ stimulates insulin secretion in a calcium-dependent manner. However, it remains elusive whether regulation of intracellular calcium release and/or extracellular calcium influx is disturbed by the $G\alpha_{i2}$ deletion.

In conclusion, the experiments presented identify $G\alpha_{i2}$ as an endogenous positive modulator of the L-arginine- and L-ornithine-induced stimulation of insulin secretion, which may be an important feedback mechanism for fine-tuning amino acid plasma levels and insulin secretion. Interestingly, the PTx-sensitive $G\alpha_{i2}$ isoform was identified as a stimulator of insulin secretion. This strengthens the hypothesis that $G\alpha_o$ and $G\alpha_i$ proteins have distinct, nonredundant, and importantly opposite roles in pancreatic β-cells: *1*) $G\alpha_o$ isoforms function as inhibitors of insulin secretion and are the main isoform being responsible for the dysregulated insulin release upon PTx treatment; and *2*) $G\alpha_{i2}$ isoforms stimulate insulin secretion upon L-arginine and L-ornithine treatment.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: V.L., A. Schürmann, C.H., and B.N. conception and design of research; V.L., K.F., A.N., M.R., and A. Schönsiegel performed experiments; V.L., K.F., M.R., and A. Schönsiegel analyzed data; V.L., K.F., L.B., A. Schürmann, C.H., and B.N. interpreted results of experiments; V.L. and K.F. prepared figures; V.L., C.H., and B.N. drafted manuscript; V.L., K.F., A.N., L.B., A. Schürmann, C.H., and B.N. edited and revised manuscript; V.L., K.F., A.N., M.R., A. Schönsiegel, L.B., A. Schürmann, C.H., and B.N. approved final version of manuscript.

REFERENCES

1. **Ahren B.** Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes. *Nat Rev Drug Discov* 8: 369–385, 2009.
2. **Ashcroft FM, Proks P, Smith PA, Ammala C, Bokvist K, Rorsman P.** Stimulus-secretion coupling in pancreatic β-cells. *J Cell Biochem* 55 *Suppl*: 54–65, 1994.
3. **Birnbaumer L.** G proteins in signal transduction. *Annu Rev Pharmacol Toxicol* 30: 675–705, 1990.
4. **Clapham DE, Neer EJ.** G protein βγ subunits. *Annu Rev Pharmacol Toxicol* 37: 167–203, 1997.
5. **Dezaki K, Kakei M, Yada T.** Ghrelin uses Gαi2 and activates voltage-dependent K^+ channels to attenuate glucose-induced Ca^{2+} signaling and insulin release in islet β-cells: novel signal transduction of ghrelin. *Diabetes* 56: 2319–2327, 2007.
6. **Dizayee S, Kaestner S, Kuck F, Hein P, Klein C, Piekorz RP, Meszaros J, Matthes J, Nürnberg B, Herzig S.** $G\alpha_{i2}$- and $G\alpha_{i3}$-specific regulation of voltage-dependent L-type calcium channels in cardiomyocytes. *PloS one* 6: e24979, 2011.
7. **Exner T, Jensen ON, Mann M, Kleuss C, Nürnberg B.** Posttranslational modification of Ga_{o1} generates Ga_{o3}, an abundant G protein in brain. *Proc Natl Acad Sci USA* 96: 1327–1332, 1999.
8. **Ezan J, Lasvaux L, Gezer A, Novakovic A, May-Simera H, Belotti E, Lhoumeau AC, Birnbaumer L, Beer-Hammer S, Borg JP, Le Bivic A, Nürnberg B, Sans N, Montcouquiol M.** Primary cilium migration depends on G-protein signalling control of subapical cytoskeleton. *Nat Cell Biol* 15: 1107–1115, 2013.
9. **Gilman AG.** G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* 56: 615–649, 1987.
10. **Gohla A, Klement K, Nürnberg B.** The heterotrimeric G protein G_{i3} regulates hepatic autophagy downstream of the insulin receptor. *Autophagy* 3: 393–395, 2007.
11. **Gulbenkian A, Schobert L, Nixon, Tabachnick II.** Metabolic effects of pertussis sensitization in mice and rats. *Endocrinology* 83: 885–892, 1968.
12. **Herrera PL, Orci L, Vassalli JD.** Two transgenic approaches to define the cell lineages in endocrine pancreas development. *Mol Cell Endocrinol* 140: 45–50, 1998.
13. **Hurowitz EH, Melnyk JM, Chen YJ, Kouros-Mehr H, Simon MI, Shizuya H.** Genomic characterization of the human heterotrimeric G protein α, β, and γ subunit genes. *DNA Res* 7: 111–120, 2000.
14. **Katada T, Ui M.** Effect of in vivo pretreatment of rats with a new protein purified from Bordetella pertussis on in vitro secretion of insulin: role of calcium. *Endocrinology* 104: 1822–1827, 1979.
15. **Katada T, Ui M.** In vitro effects of islet-activating protein on cultured rat pancreatic islets. Enhancement of insulin secretion, adenosine 3′:5′-monophosphate accumulation and ^{45}Ca flux. *J Biochem* 89: 979–990, 1981.
16. **Klose C, Straub I, Riehle M, Ranta F, Krautwurst D, Ullrich S, Meyerhof W, Harteneck C.** Fenamates as TRP channel blockers: mefenamic acid selectively blocks TRPM3. *Br J Pharmacol* 162: 1757–1769, 2011.
17. **Köhler D, Devanathan V, Bernardo de Oliveira Franz C, Eldh T, Novakovic A, Roth JM, Granja T, Birnbaumer L, Rosenberger P, Beer-Hammer S, Nürnberg B.** Gαi2- and Gαi3-deficient mice display opposite severity of myocardial ischemia reperfusion injury. *PloS one* 9: e98325, 2014.
18. **Krumins AM, Gilman AG.** Targeted knockdown of G protein subunits selectively prevents receptor-mediated modulation of effectors and reveals complex changes in non-targeted signaling proteins. *J Biol Chem* 281: 10250–10262, 2006.
19. **Lee JY, Ristow M, Lin X, White MF, Magnuson MA, Hennighausen L.** RIP-Cre revisited, evidence for impairments of pancreatic β-cell function. *J Biol Chem* 281: 2649–2653, 2006.
20. **Leiss V, Friebe A, Welling A, Hofmann F, Lukowski R.** Cyclic GMP kinase I modulates glucagon release from pancreatic α-cells. *Diabetes* 60: 148–156, 2011.
21. **Leopoldt D, Harteneck C, Nürnberg B.** G proteins endogenously expressed in Sf 9 cells: interactions with mammalian histamine receptors. *Naunyn Schmiedebergs Arch Pharmacol* 356: 216–224, 1997.
22. **Minetti GC, Feige JN, Bombard F, Heier A, Morvan F, Nürnberg B, Leiss V, Birnbaumer L, Glass DJ, Fornaro M.** $G\alpha_{i2}$ signaling is required for skeletal muscle growth, regeneration, and satellite cell proliferation and differentiation. *Mol Cell Biol* 34: 619–630, 2014.
23. **Nürnberg B, Harhammer R, Exner T, Schulze RA, Wieland T.** Species- and tissue-dependent diversity of G-protein β-subunit phosphorylation: evidence for a cofactor. *Biochem J* 318: 717–722, 1996.
24. **Pi M, Chen L, Huang MZ, Zhu W, Ringhofer B, Luo J, Christenson L, Li B, Zhang J, Jackson PD, Faber P, Brunden KR, Harrington JJ, Quarles LD.** GPRC6A null mice exhibit osteopenia, feminization and metabolic syndrome. *PloS one* 3: e3858, 2008.
25. **Pi M, Wu Y, Lenchik NI, Gerling I, Quarles LD.** GPRC6A mediates the effects of L-arginine on insulin secretion in mouse pancreatic islets. *Endocrinology* 153: 4608–4615, 2012.
26. **Pi M, Wu Y, Quarles LD.** GPRC6A mediates responses to osteocalcin in beta-cells in vitro and pancreas in vivo. *J Bone Miner Res* 26: 1680–1683, 2011.
27. **Plummer NW, Spicher K, Malphurs J, Akiyama H, Abramowitz J, Nürnberg B, Birnbaumer L.** Development of the mammalian axial skeleton requires signaling through the $G\alpha_i$ subfamily of heterotrimeric G proteins. *Proc Natl Acad Sci USA* 109: 21366–21371, 2012.
28. **Regard JB, Kataoka H, Cano DA, Camerer E, Yin L, Zheng YW, Scanlan TS, Hebrok M, Coughlin SR.** Probing cell type-specific functions of G_i in vivo identifies GPCR regulators of insulin secretion. *J Clin Invest* 117: 4034–4043, 2007.
29. **Rudolph U, Finegold MJ, Rich SS, Harriman GR, Srinivasan Y, Brabet P, Boulay G, Bradley A, Birnbaumer L.** Ulcerative colitis and adenocarcinoma of the colon in $G\alpha_{i2}$-deficient mice. *Nat Genet* 10: 143–150, 1995.
30. **Schulz N, Kluth O, Jastroch M, Schürmann A.** Minor role of mitochondrial respiration for fatty-acid induced insulin secretion. *Int J Mol Sci* 14: 18989–18998, 2013.
31. **Smajilovic S, Clemmensen C, Johansen LD, Wellendorph P, Holst JJ, Thams PG, Ogo E, Brauner-Osborne H.** The L-α-amino acid receptor GPRC6A is expressed in the islets of Langerhans but is not involved in L-arginine-induced insulin release. *Amino Acids* 44: 383–390, 2013.
32. **Tang G, Wang Y, Park S, Bajpayee NS, Vi D, Nagaoka Y, Birnbaumer L, Jiang M.** G_{o2} G protein mediates galanin inhibitory effects on insulin release from pancreatic β-cells. *Proc Natl Acad Sci USA* 109: 2636–2641, 2012.
33. **Tonack S, Tang C, Offermanns S.** Endogenous metabolites as ligands for G protein-coupled receptors modulating risk factors for metabolic and cardiovascular disease. *Am J Physiol Heart Circ Physiol* 304: H501–H513, 2013.
34. **Wang Y, Park S, Bajpayee NS, Nagaoka Y, Boulay G, Birnbaumer L, Jiang M.** Augmented glucose-induced insulin release in mice lacking G_{o2}, but not G_{o1} or G_i proteins. *Proc Natl Acad Sci USA* 108: 1693–1698, 2011.
35. **Wellendorph P, Brauner-Osborne H.** Molecular cloning, expression, and sequence analysis of GPRC6A, a novel family C G-protein-coupled receptor. *Gene* 335: 37–46, 2004.
36. **Wellendorph P, Johansen LD, Jensen AA, Casanova E, Gassmann M, Deprez P, Clement-Lacroix P, Bettler B, Brauner-Osborne H.** No evidence for a bone phenotype in GPRC6A knockout mice under normal physiological conditions. *J Mol Endocrinol* 42: 215–223, 2009.
37. **Wettschureck N, Offermanns S.** Mammalian G proteins and their cell type specific functions. *Physiol Rev* 85: 1159–1204, 2005.
38. **Wicksteed B, Brissova M, Yan W, Opland DM, Plank JL, Reinert RB, Dickson LM, Tamarina NA, Philipson LH, Shostak A, Bernal-Mizrachi E, Elghazi L, Roe MW, Labosky PA, Myers MG, Jr., Gannon M, Powers AC, and Dempsey PJ.** Conditional gene targeting in mouse pancreatic β-cells: analysis of ectopic Cre transgene expression in the brain. *Diabetes* 59: 3090–3098, 2010.
39. **Wiege K, Ali SR, Gewecke B, Novakovic A, Konrad FM, Pexa K, Beer-Hammer S, Reutershan J, Piekorz RP, Schmidt RE, Nürnberg B, Gessner JE.** $G\alpha_{i2}$ is the essential $G\alpha_i$ protein in immune complex-induced lung disease. *J Immunol* 190: 324–333, 2013.
40. **Wiege K, Le DD, Syed SN, Ali SR, Novakovic A, Beer-Hammer S, Piekorz RP, Schmidt RE, Nürnberg B, Gessner JE.** Defective macrophage migration in $G\alpha_{i2}$- but not $G\alpha_{i3}$-deficient mice. *J Immunol* 189: 980–987, 2012.
41. **Zawalich WS, Zawalich KC.** Regulation of insulin secretion by phospholipase C. *Am J Physiol Endocrinol Metab* 271: E409–E416, 1996.
42. **Zhao A, Ohara-Imaizumi M, Brissova M, Benninger RK, Xu Y, Hao Y, Abramowitz J, Boulay G, Powers AC, Piston D, Jiang M, Nagamatsu S, Birnbaumer L, Gu G.** $G\alpha_o$ represses insulin secretion by reducing vesicular docking in pancreatic beta-cells. *Diabetes* 59: 2522–2529, 2010.