Deletion of Glutamate Dehydrogenase in β-Cells Abolishes Part of the Insulin Secretory Response Not Required for Glucose Homeostasis*§

Stefania Carobbio, Francesca Frigerio, Blanca Rubi, Lauрéne Vetterli, Maria Bloksgaard, Asllan Gjinovci, Shirin Pournourmohammadi, Pedro L. Herrera, Walter Reith, Susanne Mandrup, and Pierre Maechler

From the 1Department of Cell Physiology and Metabolism, and the 2Department of Genetic Medicine and Development, the 3Department of Pathology and Immunology, Geneva University Medical Centre, 1 rue Michel-Servet, 1211 Geneva 4, Switzerland and the 4Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark

Insulin exocytosis is regulated in pancreatic β-cells by a cascade of intracellular signals translating glucose levels into corresponding secretory responses. The mitochondrial enzyme glutamate dehydrogenase (GDH) is regarded as a major player in this process, although its abrogation has not been tested yet in animal models. Here, we generated transgenic mice, named βGlud1+/−, with β-cell-specific GDH deletion. Our results show that GDH plays an essential role in the full development of the insulin secretory response. In situ pancreatic perfusion revealed that glucose-stimulated insulin secretion was reduced by 37% in βGlud1+/−. Furthermore, isolated islets with either constitutive or acute adenovirus-mediated knock-out of GDH showed a 49 and 38% reduction in glucose-induced insulin release, respectively. Adenovirus-mediated re-expression of GDH in βGlud1−/− islets fully restored glucose-induced insulin release. Thus, GDH appears to account for about 40% of glucose-stimulated insulin secretion and to lack redundant mechanisms. In βGlud1−/− mice, the reduced secretory capacity resulted in lower plasma insulin levels in response to both feeding and glucose load, while body weight gain was preserved. The results demonstrate that GDH is essential for the full development of the secretory response in β-cells. However, maximal secretory capacity is not required for maintenance of glucose homeostasis in normo-caloric conditions.

Pancreatic β-cells produce the hormone insulin that is essential for glucose homeostasis. Upon nutrient stimulation, elevation of cytosolic calcium in the β-cell is the primary and necessary signal for insulin exocytosis (1). Then, increasing the magnitude of the secretory response requires amplification of the calcium signal supported by metabolism-derived additive factors (2). The enzyme glutamate dehydrogenase (GDH, EC 1.4.1.3) has been proposed to participate to the development of the secretory response. GDH is a homohexamer located in the mitochondrial matrix that catalyzes the reversible reaction: α-ketoglutarate + NH₄ + NAD(P)H ⇌ glutamate + NAD(P)⁺; inhibited by GTP and activated by ADP (3, 4). Regarding β-cell, allosteric activation of GDH by L-leucine or its non-metabolized analogue BCH has triggered most of the attention over the last three decades (5).

To date, the role of GDH in β-cell function remains unclear and debated. Specifically, GDH might play a role in glucose-induced amplifying pathway through generation of glutamate (6, 7). GDH is also an amino acid sensor triggering insulin release upon glutamine stimulation in conditions of GDH allosteric activation (8–10). Recently, the importance of GDH has been further highlighted by studies showing that SIRT4, a mitochondrial ADP-ribosyltransferase, down-regulates GDH activity and thereby modulates insulin secretion (11, 12).

GDH is encoded by a well-conserved 45-kb gene named GLUD1, which is organized into 13 exons (13). A decade ago, clinical data and associated genetic studies revealed GDH as a key enzyme for the control of insulin secretion. Indeed, mutations rendering GDH more active are responsible for a hyperinsulinism syndrome (14). Mutations producing a less active, or even nonactive, GDH enzyme have not been reported, leaving open the question if such mutations would be either lethal or asymptomatic. Despite these numerous reports on GDH and insulin secretion, abrogation of GDH in β-cells has not been tested in animal models until now. As a consequence, it is not yet established if GDH is in fact required for normal β-cell function and glucose homeostasis.

Here, we generated transgenic mice lacking GDH specifically in β-cells and questioned the putative requirement of GDH for β-cell function as well as the consequences of GDH abrogation on glucose homeostasis.

EXPERIMENTAL PROCEDURES

Transgene Cloning—By screening a RPCI-22 mouse 129 BAC library (MRC Genome Resource Facility, The Hospital of Sick

* This study was supported by the Swiss National Science Foundation (to P. M., W. R., and P. L. H.), an EPDF/Novo Nordisk research grant (to P. M.), the Dr. Max Cloetta Foundation (to P. M.), the Danish Natural Science Research Council and Health Science Research Council (to S. M.), the Juvenile Diabetes Research Foundation (to P. H.), and Fondation Romande Diabetes Research Council and Health Science Research Council (to S. M.), the Dr. Max Cloetta Foundation (to P. M.), an EFSD/Novo Nordisk research grant (to P. M.), and an EFSD Research Grant (to S. M.).

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

§ To whom correspondence should be addressed. Tel.: 41-22-379-55-54; E-mail: Pierre.Maechler@medecine.unige.ch.

© 2009 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Deletion of GDH in β-Cells

Children, Toronto, Canada) with a cDNA probe corresponding to human GLUD1 exons 6–8, we obtained two fragments, respectively, of 6 and 10 kb comprising the sequence of Glud1 exon 7. Successively, three DNA fragments of the 129 SvJ Glud1 locus were inserted into the cloning sites of the targeting vector. They comprised 3 kb of the region upstream Glud1 exon 7 (short arm), 0.6 kb of the region containing Glud1 exon 7, and 5.6 kb of the region downstream Glud1 exon 7 (long arm). The three fragments were obtained by PCR using a RPCI-22 129 BAC clone as template and ligated into the KStoxPfrtNeoBS (3'-5') vector (15). The resulting targeting construct contained two flox sites flanking Glud1 exon 7, one frt-flanked neomycin resistance cassette, and two homology arms.

Generation of Glud1floxt/lox Mouse—The targeting vector (15 μg) was linearized at the single SacII site and electroporated into three batches of 1.7 × 107 R1 ES cells, a cell line derived from (129/Sv × 129/Sv-Cp)F1 3.5-day blastocysts (16). Cells were then cultured in the presence of G418 (0.7 mg/ml) for 6 days. Homologous recombination events were identified by PCR and Southern blot analyses, see supporting information. Correctly targeted ES cell clones were injected into C57BL/6j blastocysts and implanted into recipient females (Karolinska Institutet, Stockholm, Sweden). Resulting chimeric mice (F0) were backcrossed to C57BL/6j mice and germline transmission into F1 was assessed by PCR and Southern blot analyses. The Glud1floxt/lox mouse line was obtained following deletion of the frt-flanked neomycin resistance cassette by crossing heterozygous with Flpe deleter mice (17).

Generation βGlud1−/− Mouse—Glud1 floxed animals were crossed with mice expressing the Cre recombinase under a rat insulin promoter (18). Heterozygous βGlud1−/+ were then bred with homozygous Glud1floxt/lox to obtain homozygous βGlud1−/− mice. Mice were maintained for 6 and 10 kb comprising the sequence of Glud1 exon 7, and 3 kb of the region upstream Glud1 exon 7, one flanked neomycin resistance cassette, and two homology arms.

Genotyping and Immunoblotting—Transgenic animals were identified by PCR on genomic DNA extracted from tail biopsies (Genelute Mammalian Genomic DNA kit, Sigma, Buchs, Switzerland). Primers (purchased from Microsynth GmbH, Baglach, Switzerland) used to genotype floxed mice were: 5'-TGTAATGTGTCTGTGTCAAC and 5'-CTAAGGCACCCA-GACAGTGG (86-bp fragment). Primers to genotype RIP-Cre mice were: 5'-TAAGGCTAAGAGGTGT and 5'-TCCA-TGTTGATACAGGGAC (350-bp fragment). GDH protein was analyzed by immunoblotting as described (19) on 13% SDS-PAGE that was run using 10 μg of proteins from pancreatic islet extract or standards of purified bovine GDH (Roche Applied Science, Rotkreuz, Switzerland).

Immunohistochemistry—Adult mouse pancreata were harvested in cold phosphate-buffered saline and treated overnight at 4 °C in 4% paraformaldehyde before embedding in paraffin and 5-μm thick tissue sections were mounted on adhesive-coated slides. Pancreata sections were incubated with a diluted primary antibody for 2 h at room temperature, and with an appropriate Cy3- (Jackson ImmunoResearch Laboratories, Inc, WestGrove, PA) or ALEXA-conjugated (Molecular Probes, Inc, Eugene, OR) anti-IgG serum for 1 h. The antibodies and their dilution used in the present analysis were as follows: guinea pig anti-insulin (Dako, Carpinteria, CA, dilution 1/400), rabbit anti-glucagon (Dako, Carpinteria, CA; dilution 1/100). Sections were analyzed on a Zeiss Axiopt microscope equipped with an AxioCAM color CCD camera (Carl Zeiss, Feldbach, Switzerland). Islet morphology was analyzed to discriminate between organized versus disorganized islets. As a criterion, islets with more than 10% α-cells present in the core of the islets were considered as disorganized.

Pancreatic Islets Isolation and Adenoviral Treatment—Mouse pancreatic islets were isolated by collagenase digestion as described previously (19) and cultured free-floating in RPMI 1640 medium before use. For rescue experiments, ectopic expression of hGLUD1 was achieved by transducing islets isolated from βGlud1−/− with the recombinant adenovirus Ad-GDH over a 90-min period as detailed previously (19) and islets were used the next day for secretion assays and control immunoblotting. For acute in vitro GDH knock-out, islets isolated from control and transgenic Glud1floxt/lox mice were subjected to adenoviral treatment for 90 min with 0.4 μl/ml of the recombinant adenovirus rAdInsPNCre, thereby enabling expression of nuclear-localized Cre recombinase specifically in β-cells (20). Islets transduced with rAdInsPNCre were kept in culture for 3 days before insulin secretion assays.

Insulin Secretion and Measurements—Prior to the experiments, islets were maintained for 2 h in glucose-free culture medium. For static incubations, islets were then washed and preincubated further in glucose-free Krebs–Ringer bicarbonate HEPES buffer (KRBH, containing in mM: 135 NaCl, 3.6 KCl, 10 HEPES (pH 7.4), 5 NaHCO3, 0.5 NaH2PO4, 0.5 MgCl2, 1.5 CaCl2, 0.1% bovine serum albumin). Then, batches of 10 islets were hand-picked and incubated as described (21) for 30 min at 37 °C at basal (2.8 mM) glucose and stimulated with 22.8 mM glucose or 5 mM glutamine plus 10 mM BCH (2-aminobicyclo-[2,2,1]heptane-2-carboxylic acid). At the end of the assay period, islets were resuspended in acid-EtOH to determine their insulin contents. For islets perfusions (19), 10 hand-picked islets were put per chamber of 250 μl volume, thermostatted at 37 °C (Brandel, Gaithersburg, MD). The flux was set at 0.5 ml/min, and fractions were collected every minute after a 20-min washing period at basal 2.8 mM glucose. In situ pancreatic perfusion was performed in anesthetized mice as described (22) with a 1.5 ml/min perfusion rate. Pancreatic insulin content was determined in mouse pancreata that were first frozen in liquid nitrogen, then homogenized, resuspended in cold acid ethanol, and left at 4 °C for 48 h with sonication after 24 h. Insulin concentration in these different collected samples was measured by radioimmunoassay (Linco Research Inc., St. Charles, MO).

A bioluminescent assay kit (Roche Applied Science) was used to measure ATP levels in batches of 10 isolated islets following a 30-min incubation period at 37 °C in the presence of 2.8 and 22.8 mM glucose.

The enzymatic activity of GDH in isolated islets was measured as described (19) under the oxidative deamination direction as NADH fluorescence excited at 340 nm and measured at
Deletion of GDH in β-Cells

Generation of Transgenic Mice—We generated transgenic mice with β-cell-specific conditional abrogation of GDH using the loxP-flp-recombinase strategy. The targeting vector carried Glud1 (NCBI Access No. P26443) exon 7, comprising the NADH binding site essential for GDH activity (13), flanked by two loxP sites (Fig. 1A). We cloned 5′ and 3′ regions flanking exon 7 as 3-kb and 5.6-kb fragments, respectively. The targeting vector also contained a neo cassette (positive marker) flanked by two FLP/FRT sites. The resulting founder mice were crossed to obtain F1 mice carrying a mutant Glud1 genomic locus containing the exon 7 flanked by loxP sites and the neo cassette (Glud1-neo-lox). Next, excision of the neo cassette by crossing F1 with Flp deleter mice gave rise to mice carrying intact Glud1 exon 7 flanked by loxP sites only (Glud1lox/lox).

Constitutive β-Cell-specific GDH Knock-out—Glud1lox/lox mice were crossed with mice expressing the Cre recombinase under the control of an insulin promoter (RIP), generating mice with β-cell-specific GDH knock-out (BGlud1−/−). Allele analysis in different tissues revealed that the 278-bp knock-out tolerance test, overnight fasted mice were injected intraperitoneally with human recombinant insulin (0.75 units/kg body weight, Novo Nordisk Pharma AG, Künschâdt, Switzerland) and glucose levels determined (Glucotrend) in blood collected from the tail vein at the indicated time intervals. Insulin resistance was estimated using the homeostasis model assessment index (HOMA-IR) calculated as follows: fasting glucose (mM) × fasting insulin (milliunits/ml)/22.5 (23–25).

Circulating glucagon and insulin levels from fed and fasted mice were determined on multiplex suspension array system (Bio-Plex, Bio-Rad Laboratories, Reinach, Switzerland) using LincoPlex kits for mouse hormones (Linco Research Inc.).

RESULTS

Glucose Tolerance and Insulin Release Tests—Overnight (15 h) fasted mice were injected intraperitoneally with glucose (3 g/kg body weight). Whole blood was collected from tail vein at times 0, 15, 30, 60, 120 min for glucose level measurements using a glucometer (Glucotrend, Roche Applied Science). Additionally, plasma insulin levels were determined from time 0 and 15-min blood sampling using an ultrasensitive mouse insulin ELISA (Mercodia AB, Uppsala, Sweden). For the insulin
genomic map of wild-type Glud1 locus

FIGURE 1. Conditional Glud1 targeting with β-cell-specific constitutive Glud1 knock-out. A, upper panel shows a partial genomic map of wild-type Glud1 locus from exon 5 (E5) to 11 (E11). Individual letters represent restriction sites (B, BamHI; N, Nhel). The lower panel shows the targeted locus after excision of the neomycin-resistance selection cassette (Neo, dark green rectangle) that is flanked by two frt sites (light green rectangle); and replacement of the exon 7 (red rectangle) that is flanked by two loxP sites (orange arrowhead). B, transgenic Glud1+/mice were crossed with RIP-Cre mice resulting in constitutive β-cell-specific Glud1 knock-out animals (BGlud1−/−). Tissues collected from these animals were analyzed by PCR to detect knock-out (278 bp) and intact lox/lox (828 bp) alleles in different organs. Lanes show molecular weight markers (M); genomic DNA from the tail (T), brain (Br), liver (Li), pancreas (Pa), muscle (Mu), kidney (Ki); and negative control (C) without DNA. The 278-bp knock-out allele was detected in pancreas only (n=3). C, immunoblot analysis was performed by migrating in SDS-PAGE 10 μg per lane of proteins extracted from isolated islets. GDH bands, contributed by both β- and non-β-cells, were much lower in BGlud1−/− (−/−) islets compared with control (+/+) and the lower nonspecific band exhibited similar intensity. As GDH standard (std), 50 ng of purified GDH were loaded. D, enzymatic activity of GDH in islets isolated from control and BGlud1−/− mice (n=4, *, p < 0.05 versus Control).
Deletion of GDH in β-Cells

![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)

FIGURE 2. Phenotype of Glud1<sup>−/−</sup> mice exhibiting normal growth and reduced β-cell secretory responses. A, body weights over 4-13 weeks of age for male controls (black curve, n = 4-27) and Glud1<sup>−/−</sup> mice (gray curve, n = 3-18). Body weights were similar at birth. B, weights of organs collected at 13 weeks of age show no significant difference between controls (black bars) and Glud1<sup>−/−</sup> (gray bars) mice (n = 5 for each genotype). BAT, brown adipose tissue; Epidid.F, epididymal fat. C, assessment of β-cell function using the in situ pancreatic perfusion preparation in control (black trace) and Glud1<sup>−/−</sup> (gray trace) mice. Glucose concentration was raised in the perfused medium from basal 2.8 mM to stimulatory 22.8 mM for a 30-min period (n = 4 independent experiments). D, analysis of the two phases of glucose-stimulated insulin secretion from the in situ pancreatic perfusion presented in panel C. Summations of insulin release during the first 5 min of glucose stimulation correspond to the 1st phase and the remaining 25 min to the 2nd phase of the glucose response (*, p < 0.05 and **, p < 0.001 versus phase-matched control group).

### TABLE 1

| Glucagon (pmol/l) | Control<sup>+</sup> | Glud1<sup>−/−</sup> | p Value<sup>+</sup> |
|-------------------|-------------------|-------------------|-------------------|
| Fast              | 26.3 ± 10.1       | 27.8 ± 9.9        | 0.91              |

| Insulin (pmol/l) | Control<sup>+</sup> | Glud1<sup>−/−</sup> | p Value<sup>+</sup> |
|-------------------|-------------------|-------------------|-------------------|
| Fed               | 143.3 ± 27.4<sup>+</sup> | 65.2 ± 10.5<sup>+</sup> | 0.002              |
| Fast              | 29.3 ± 6.2        | 24.7 ± 5.0        | 0.12              |

<sup>+</sup>Mice of 5-7 months of age.

<sup>+</sup>p < 0.05 versus fasted mice of corresponding genotype; n = 4–7.

<sup>+</sup>p < 0.05 versus control.

allele was detected in pancreas and not in other tissues (Fig. 1B).

In particular, we did not detect homologous recombination in the brain following a cross with the RIP-Cre transgenic line used in our study (18), unlike what was reported using another RIP-Cre mouse line (26). At the protein level, immunoblotting following SDS-PAGE on isolated islets (Fig. 1C) showed efficient targeted GDH knock-out, although the lowest unspecific band was unchanged. Some residual expression could be contributed by the non-β-cells.

GDH enzymatic activity was reduced by 60% (p < 0.05) in islets isolated from Glud1<sup>−/−</sup> mice compared with controls (Fig. 1D). The remaining activity corresponds approximately to the proportion of non-β-cells composing the islets. As control mice, we used Glud1<sup>lox/lox</sup> animals obtained from the same litters as Glud1<sup>−/−</sup> mice to optimize standardization of the genetic background between the two groups.

Phenotype and Pancreatic Secretory Responses of β-Cell GDH Knock-out Mice—Surprisingly, β-cell-specific GDH knock-out mice were not diabetic, no glucosuria was detected, and the growth and organ weights were normal compared with control mice (Fig. 2, A and B).

Based on the unexpected normal development of the animals, despite deletion of GDH in β-cells, we measured insulin secretion in the in situ pancreatic perfusion preparation (Fig. 2C), a model that integrates paracrine and neuronal effectors of the β-cell. This physiological model demonstrated clear reduction of the secretory response evoked by 22.8 mM glucose in Glud1<sup>−/−</sup> mice versus controls; both during the first (−48%, p < 0.01) and the second (−37%, p < 0.05) phases (Fig. 2D). The data show that abrogation of the mitochondrial enzyme GDH in β-cells limits both the phase of secretion commonly associated with the amplifying pathway (second phase) as well as the triggering pathway (first phase).

In good agreement with basal versus stimulated insulin secretion measured in the perfused pancreas (Fig. 2C), circulating insulin concentrations were not significantly different in the fasted state, but the increase associated with the fed state was reduced by 55% (p < 0.05) in Glud1<sup>−/−</sup> mice (Table 1).

Islet Phenotype of Constitutive β-Cell-specific GDH Knock-out—Insulin secretory defects have been associated with modified islet organization in general and infiltration of α-cells within the core of the islet in particular (27). Accordingly, we analyzed islet cell distribution by immunostaining on pancreatic sections (Fig. 3, A and B). Most of the control islets (70%) exhibited the expected α- and β-cell distribution, i.e. insulin β-cells in the core of the islet and glucagon α-cells forming its periphery. In Glud1<sup>−/−</sup> animals, the percentage of disorganized islets was as high as 67%, revealing α-cells mixed with β-cells in the core of the islet (Fig. 3C). It should be noted that RIP-Cre transgene does not by itself promote changes in islet morphology (18). Pancreatic insulin contents were not affected in Glud1<sup>−/−</sup> animals compared with controls (124.1 ± 24.9 and 125.7 ± 9.1 ng/g pancreas of wet mass, respectively), and islets were similarly distributed in the whole pancreas. Functions of the α-cells were apparently not affected by GDH knock-out in β-cells, as revealed by similar plasma glucagon levels between the two groups under fasting (stimulatory) conditions (Table 1).

A primary function of mitochondrial metabolism is to produce ATP that is necessary in β-cells for triggering (first phase)
Deletion of GDH in β-Cells

Secretory Responses of Isolated Islets with Constitutive β-Cell GDH Knock-out—Kinetics of secretory responses were analyzed in perfusion experiments on islets isolated from βGlud1−/− compared with controls. Basal insulin release measured at 2.8 mM glucose was not affected by the absence of GDH. When stimulated with 22.8 mM glucose, control and βGlud1−/− islets exhibited glucose-stimulated insulin release (Fig. 4A). However, constitutive abrogation of GDH in β-cells resulted in markedly reduced secretory responses, both during first phase (−68%, p < 0.01) and second sustained phase (−49%, p < 0.05). These data demonstrate that GDH is essential for the full development of the secretory response.

To test if the reduced glucose-evoked insulin release observed in βGlud1−/− islets was a direct consequence of the absence of GDH, we reintroduced GDH by means of adenoviral transduction (Fig. 4B). Insulin secretion stimulated with 22.8 mM glucose was increased 5.7-fold in control islets (p < 0.01) and only 2.0-fold in βGlud1−/− islets (p < 0.05). Following ectopic re-expression of GDH in βGlud1−/− islets, amplitude of the secretory response was rescued to similar levels compared with controls (4.9-fold, p < 0.05). As expected from a previous study (19), overexpression of GDH did not significantly modify glucose-induced insulin release compared with control islets (Fig. 4B).

Secretory Responses of Isolated Islets with Acute in Vitro β-Cell GDH Knock-out—To investigate whether abrogation of GDH could induce compensatory pathways in the β-cells, we also performed acute in vitro knockout in islets of transgenic floxed mice. Islets isolated from Glud1lox/lox mice were transduced with a recombinant adenovirus (AdInspNCre) expressing the Cre recombinase specifically in β-cells (20). PCR analysis demonstrated in vitro homologous recombination resulting in the knock-out of GDH in β-cells of islets isolated from transgenic Glud1lox/lox mice but not in islets from wild-type animals (Fig. 5A).

In control islets, stimulatory glucose concentration (22.8 mM) induced a 4.9-fold (p < 0.05) increase in insulin secretion compared with basal release (Fig. 5B). The secretory response

and maintaining (second phase) the secretory response. At this point, it could be hypothesized that β-cell GDH knock-out would impair the primary function of mitochondrial activation that is to generate ATP and thereby inhibit glucose-stimulated insulin secretion. ATP levels were slightly lower under basal glucose concentrations (2.8 mM) in islets of βGlud1−/− mice. However, upon glucose stimulation (22.8 mM), islet ATP concentrations increased similarly between the two groups (Fig. 3D). GDH knock-out did not affect glucose-induced ATP generation. Interestingly, data suggest that maintenance of basal housekeeping ATP levels under low glucose conditions might be normally contributed by GDH-dependent metabolism.

FIGURE 3. Islet phenotype of constitutive β-cell GDH knock-out. A, immunostaining of representative islets from control (left panel) and βGlud1−/− (right panel) mice using antibodies directed against insulin to detect β-cells (green) and against glucagon to reveal α-cells (red). In control animals, the majority of islets should be organized with α-cells surrounding the core of the structure formed by β-cells. B, immunostaining of representative pancreatic sections from control (left panel) and βGlud1−/− (right panel) mice using an antibody directed against insulin to detect β-cells (green) and against glucagon to reveal α-cells (red). Bar graph shows quantification as relative percentage of organized versus disorganized islets in control (black bars) and βGlud1−/− (gray bars) pancreata. Scale bar, 50 μm; n = 3 independent analyses; *, p < 0.05. C, bar graph shows islet ATP levels following a 30-min incubation period at basal 2.8 mM (Glc 2.8) to stimulatory 22.8 mM (Glc 22.8) glucose concentrations (n = 4); *, p < 0.05 versus control Glc 2.8; §, p < 0.05 versus corresponding genotype matched Glc 2.8.
Deletion of GDH in β-Cells

**A**

![Graph showing insulin secretion over time](image)

**B**

![Graph showing insulin secretion levels](image)

**FIGURE 4.** *Constitutive β-cell-specific Glut1 knock-out impairs insulin secretion that is rescued by GDH ectopic re-expression.* A, after an overnight culture, islets isolated from the two groups were hand-picked and distributed into perfusion chambers. Insulin release was measured in the effluent of chambers perfused first with basal 2.8 mM glucose before stimulation for 20 min with 22.8 mM glucose. Compared with controls, islets isolated from βGlut1−/− mice exhibited marked reduction of the secretory responses during first phase (−68%, p < 0.01) and second sustained phase (−49%, p < 0.05). n = 7, 8, islets isolated from βGlut1−/− mice were transduced with adenovirus AdGDH carrying hGLUD1 to restore GDH expression. Insulin secretion was measured over a 15-min period at basal 2.8 mM (Glc 2.8) and stimulatory 22.8 mM glucose (Glc 22.8) on batches of 10 islets. *, p < 0.05 versus control Glc 22.8; §, p < 0.05 versus corresponding group at Glc 2.8 (n = 3).

**FIGURE 5.** *Acute in vitro β-cell-specific Glut1 knock-out impairs insulin secretion.* A, acute β-cell Glut1 knock-out assessed by PCR analysis of genomic DNA obtained from islets isolated from wild-type (WT) and transgenic Glut1lox/lox (Tg) mice 3 days after a 90-min transduction period with rAdInsPNCre adenovirus. The PCR 726-bp product corresponds to the wild-type allele in control islets. In Tg islets, the 828-bp fragment shows intact lox/lox allele contributed by non-β-cells as well as residual non-transduced β-cells. Successful homologous recombination is revealed by the lane showing the 278-bp band, indicating absence of Glut1 exon 7 in Tg islets. B, three days after acute in vitro β-cell-specific Glut1 knock-out, islets were washed, preincubated, and then incubated for 30 min at basal (2.8 mM) and stimulated with 22.8 mM glucose (Glc) or a mixture of 5 mM glutamine plus 10 mM BCH at basal glucose (Gln + BCH). n = 3 independent experiments; *, p < 0.04 versus basal Glc; §, p < 0.03 versus stimulus-matched Control.

Evoked by 22.8 mM glucose was reduced by 38% (p < 0.02) in islets with acute abrogation of GDH. These results are in accordance with knockdown of GDH by antisense approach in insulinoma INS-1E cells (2). Insulin release was also stimulated by a mixture commonly used to specifically induce GDH-dependent insulin secretion (5), *i.e.* 5 mM glutamine plus the l-leucine analogue BCH (10 mM) serving as an allosteric activator of GDH. The resulting amino acid dependent secretory response had a magnitude of 3.2-fold (p < 0.005) in control islets, that was inhibited by 47% (p < 0.03) in knock-out islets.

As both acute (Fig. 5) and constitutive (Fig. 4) knock-outs exhibited similar secretion impairments, we can conclude that redundant mechanisms do not exist at the cellular level.

**Glucose Homeostasis in β-Cell GDH Knock-out**—Parameters related to the control of glucose homeostasis were measured at 2, 5, and 12 months of age. After a 15-h fasting period, glycemia were similar to controls in βGlut1−/− mice of all tested ages. Intraperitoneal glucose tolerance tests (ipGTT) were performed on 2- and 5-month-old mice. Despite the severe reduction in glucose-stimulated insulin secretion in βGlut1−/− animals (Fig. 2, C and D), blood glucose excursions were similar to controls (Fig. 6, A and E). At 2 months of age, plasma insulin levels measured 15 min following intraperitoneal glucose injection were reduced by 38% (p < 0.001) in βGlut1−/− mice (Fig. 6C). When performing an insulin tolerance test (ITT) in 2-month-old mice, blood glucose lowering was faster in βGlut1−/− mice compared with controls (Fig. 6B). Low circulating insulin concentrations associated with accelerated glucose clearance suggested higher sensitivity of peripheral tissues. However, ITT performed in 5-month-old mice did not show significant differences between knock-outs and controls (Fig. 6F). To further investigate insulin sensitivity in 2- and 5-month-old mice, we calculated the HOMA index as a surrogate of euglycemic clamp test (28). As expected, insulin resistance increased with age in both groups (Fig. 6D) and βGlut1−/− mice exhibited similar HOMA-IR compared with controls.

It should be noticed that RIP-Cre transgenic mice used in the present study (18) exhibit normal glucose homeostasis (see Fig. 6, A and E and Refs. 29, 30), as opposed to one particular RIP-Cre line (31, 32) that has been associated with either glucose intolerance or diabetes (33).

In old animals of 12 months of age, both control and knock-out animals exhibited signs of glucose intolerance. Return to
Deletion of GDH in β-Cells

Mitochondrial metabolism is crucial for the coupling of glucose recognition to insulin exocytosis in β-cells (2). In this context, importance of GDH has been well recognized in the past (5, 9, 34), although recent studies revisited its specific role and regulation in the β-cell (10, 11, 19, 35, 36). GDH might raise insulin release by participating to the amplifying pathway (6, 7) and/or by relaying signals of protein abundance to mitochondria (8–10). The present study was not designed to detail the amplifying pathway in particular, but rather investigated the consequences of the lack of GDH on β-cell insulin secretory response and on resulting glucose homeostasis.

The first conclusion of the present study is that GDH is necessary for the full development of the secretory response. The absence of this mitochondrial enzyme sets a limit (about 60%) to insulin release evoked by optimal glucose concentrations. Secondly, half of the amplitude obtained with optimal glucose-stimulated insulin secretion is not required to maintain glucose homeostasis. The βGlud1−/− mice demonstrate that partially reduced β-cell secretory response is asymptomatic under conditions of normo-calorie feeding, pending metabolic adaptations. The fact that β-cell function was limited but not abrogated might explain the observed preservation of normal animal weight gain, not requiring maximal development of the secretory response when fed a normal diet.

FIGURE 6. Glucose homeostasis in βGlud1−/− mice at 2, 5, and 12 months of age. A, E, G, following an overnight fast, control and βGlud1−/− mice were subjected to an intraperitoneal glucose tolerance test (ipGTT). Mice were challenged with 3 g of glucose per kg of body weight and glycaemia determined over a 2-h period. Control, black curve; βGlud1−/−, gray curve. A, 2-month-old mice, control n = 23, βGlud1−/− n = 16; E, 5-month-old mice, control n = 15, βGlud1−/− n = 9; G, 12-month-old mice, control n = 7, βGlud1−/− n = 5. C, plasma samples were collected during the ipGTT from A for determination of circulating insulin levels at fasting low glycaemia compared with early elevated glycaemia 15 min after glucose injection. Control n = 14; βGlud1−/− mice n = 8; §, p < 0.001 versus corresponding 2-month-old group, **, p < 0.005 versus Control at corresponding time point. D, insulin resistance was estimated in 2- and 5-month-old mice using the homeostasis model assessment index (HOMA-IR). §, p < 0.01 versus corresponding group at time 0. B, F, H, an insulin tolerance test served to assess insulin sensitivity of peripheral tissues by measuring blood glucose concentrations over a 60-min period after intraperitoneal injection in fasted mice of 0.75 units insulin per kg of body weight. B, 2-month-old mice, control n = 5, βGlud1−/− n = 7; F, 5-month-old mice, control n = 12, βGlud1−/− n = 8; H, 12-month-old mice, control n = 4, βGlud1−/− n = 3, *, p < 0.05 versus Control at corresponding time points.

normoglycemia was slower in βGlud1−/− mice (Fig. 6G). Indeed, 120 min following intraperitoneal glucose injection, blood glucose levels were 15.0 ± 2.0 mM in controls (n = 7) and 19.9 ± 4.0 mM in GDH-null mice (n = 5). Insulin tolerance test revealed similar insulin sensitivity between the groups in 12-month-old animals (Fig. 6H).

Taken together, data on glucose homeostasis in βGlud1−/− mice demonstrate that limited β-cell secretory response does not impair normal growth when animals are fed ad libitum a normo-calorie diet. Such physiological adaptation might be less efficient in old mice, along with development of age-dependent glucose intolerance (Fig. 6G).

DISCUSSION

Mitochondrial metabolism is crucial for the coupling of glucose recognition to insulin exocytosis in β-cells (2). In this context, importance of GDH has been well recognized in the past (5, 9, 34), although recent studies revisited its specific role and regulation in the β-cell (10, 11, 19, 35, 36). GDH might raise insulin release by participating to the amplifying pathway (6, 7) and/or by relaying signals of protein abundance to mitochondria (8–10). The present study was not designed to detail the amplifying pathway in particular, but rather investigated the consequences of the lack of GDH on β-cell insulin secretory response and on resulting glucose homeostasis.

The first conclusion of the present study is that GDH is necessary for the full development of the secretory response. The absence of this mitochondrial enzyme sets a limit (about 60%) to insulin release evoked by optimal glucose concentrations. Secondly, half of the amplitude obtained with optimal glucose-stimulated insulin secretion is not required to maintain glucose homeostasis. The βGlud1−/− mice demonstrate that partially reduced β-cell secretory response is asymptomatic under conditions of normo-calorie feeding, pending metabolic adaptations. The fact that β-cell function was limited but not abrogated might explain the observed preservation of normal animal weight gain, not requiring maximal development of the secretory response when fed a normal diet.
Deletion of GDH in β-Cells

The absence of GDH in β-cells inhibited glucose-stimulated insulin secretion. Results indicate that there are no alternative pathways or mechanisms compensating for the lack of GDH as both acute in vitro and constitutive GDH knock-outs resulted in similar reduction of the secretory response. Importantly, β-cells of βGlud1−/− mice were still able to partially achieve glucose-stimulated insulin secretion, although the amplitude was shifted down. Both first and second phases were similarly impaired, suggesting that GDH-dependent amplification of the secretory response would be involved in both phases. Therefore, our data reveal that GDH is not necessary for glucose recognition per se as secretion was indeed initiated up to a certain level, although the amplitude was strongly limited.

Pancreatic islet cells are organized with β-cells forming the core of the structure and most of glucagon secreting α-cells distributed at the surface of the islet (37). Insulin secretory defects have been associated with modified islet architecture in general and in particular with infiltration of α-cells within the core of the islet (27, 38). Such phenotype is reported here for βGlud1−/− mice exhibiting reduced secretory responses, although total pancreatic islet distribution and insulin content were not affected. This observation further suggests that β-cell function could participate to islet architecture.

The pancreatic β-cell has evolved over millions of years toward high performance to fully optimize energy storage during occasional short periods of food abundance to resist starvation periods. In the light of present and previous results, one can speculate that the amplifying pathway in β-cell appeared as a signal of exceptional abundance, induced in conditions of high nutrient supply to optimize storage of energy exceeding immediate requirements by the organism. Noteworthy, investigators typically study the amplifying pathway in experimental conditions where insulin release is evoked by highest physiological glucose concentrations. Such experimental paradigm might not reflect ordinary requirement of β-cell function in individuals with intact β-cells in particular with infiltration of α-cells within the core of the islet (27, 38).

ACKNOWLEDGMENTS—We thank Gaelle Chaffard and Clarissa Bartley (Geneva) for expert technical assistance, Jennifer Skaug at the MRC Genome Resource Facility (Toronto) for screening BAC library, Johannes Wilbertz at Karolinska Institutet (Stockholm) for blastocyst injection and generation of chimera, Lelio Orci (Geneva) for helpful discussions. The groups of P. M. and P. L. H. are members of the Geneva Programme for Metabolic Disorders. The authors declare that they have no competing financial interests.

REFERENCES

1. Henquin, J. C. (2000) Diabetes 49, 1751–1760
2. Maechler, P., Carobbio, S., and Rubi, B. (2006) Int. J. Biochem. Cell Biol. 38, 696–709
3. Hudson, R. C., and Daniel, R. M. (1993) Comp. Biochem. Physiol. B 106, 767–792
4. Frigerio, F., Casimir, M., Carobbio, S., and Maechler, P. (2008) Biochim. Biophys. Acta 1777, 965–972
5. Sener, A., and Malaisse, W. J. (1980) Nature 288, 187–189
6. Maechler, P., and Wollheim, C. B. (1999) Nature 402, 685–689
7. Hoy, M., Maechler, P., Efanon, A. M., Wollheim, C. B., Berggren, P. O., and Gromada, J. (2002) FEBS Lett. 531, 199–203
8. Sener, A., Malaisse-Lagae, F., and Malaisse, W. J. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 5460–5464
9. Fahien, L. A., MacDonald, M. J., Kniotek, E. H., Mertz, R. J., and Fahien, C. M. (1988) J. Biol. Chem. 263, 13610–13614
10. Li, C., Matter, A., Kelly, A., Petty, T. J., Najafi, H., MacMullen, C., Daikhin, Y., Nissim, I., Lazarow, A., Kwagh, J., Collins, H. W., Hsu, B. Y., Yudkoff, M., Matschinsky, F. M., and Stanley, C. A. (2006) J. Biol. Chem. 281, 15064–15072
11. Haigis, M. C., Mostoslavsky, R., Haigis, K. M., Fahie, K., Christodoulou, D. C., Murphy, A. J., Valenzuela, D. M., Yancopoulos, G. D., Karow, M., Blander, G., Wolberger, C., Proli, T. A., Weindruch, R., Alt, F. W., and Guarente, L. (2006) Cell 126, 941–954
12. Abuja, N., Schwer, B., Carobbio, S., Waltregny, D., North, B. J., Cas-tronovo, V., Maechler, P., and Verdin, E. (2007) J. Biol. Chem. 282, 33583–33592
13. Michaelidis, T. M., Tzimagiorgis, G., Moschonas, N. K., and Papametheakis, I. (1993) Genomics 16, 150–160
14. Stanley, C. A., Lieu, Y. K., Hsu, B. Y., Burlina, A. B., Greenberg, C. R., Hopwood, N. J., Perlman, K., Bhamra, B. H., Zammarchi, E., and Poncz, M. (1998) Engl. J. Med. 338, 1352–1357
15. Bonneau, E., Touka, M., AitLounis, A., Baas, D., Barras, E., Ucla, C., Moreau, A., Flamant, F., Dubruille, R., Couble, P., Collignon, J., Durand, B., and Reith, W. (2004) Mol. Cell. Biol. 24, 4417–4427
16. Nagy, A., Rossant, J., Nagy, R., Abramow-Newley, W., and Roeder, J. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8424–8428
17. Rodriguez, C. I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A. F., and Dynmec, S. M. (2000) Nat. Genet. 25, 139–140
18. Herrera, P. L. (2000) Development 127, 2317–2322
19. Carobbio, S., Ishihara, H., Fernandez-Pascual, S., Bartley, C., Martin-Del-Rio, R., and Maechler, P. (2004) Diabetologia 47, 266–276
20. Ishihara, H., Maechler, P., Gjinovic, A., Herrera, P. L., and Wollheim, C. B. (2003) Nat. Cell Biol. 5, 330–335
21. Rubi, B., del Arco, A., Bartley, C., Satrustegui, J., and Maechler, P. (2004) J. Biol. Chem. 279, 55659–55666
22. Maechler, P., Gjinovic, A., and Wollheim, C. B. (2002) Diabetes 51, 599–602
23. Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F., and Turner, R. C. (1985) Diabetologia 28, 412–419
24. Konrad, D., Rudich, A., and Schoenle, E. J. (2007) Diabetologia 50, 833–839
25. Xia, Z., Sniderman, A. D., and Cianflone, K. (2002) J. Biol. Chem. 277, 45874–45879
26. Ristow, M., Mulder, H., Pomplun, D., Schulz, T. J., Muller-Schmehl, K., Krause, A., Fle, M., Puccio, H., Muller, J., Isken, F., Spranger, J., Muller-
Wieland, D., Magnuson, M. A., Mohlig, M., Koenig, M., and Pfeiffer, A. F. (2003) J. Clin. Invest. 112, 527–534
27. Gorogawa, S., Fujitani, Y., Kaneto, H., Hazama, Y., Watada, H., Miyamoto, Y., Takeda, K., Akira, S., Magnuson, M. A., Yamazaki, Y., Kajimoto, Y., and Hori, M. (2004) Biochem. Biophys. Res. Commun. 319, 1159–1170
28. Ye, F., Tao, R., Cong, W., Tian, J., and Liu, Q. (2008) J. Biochem. Biophys. Methods 70, 978–984
29. Produit-Zengaffinen, N., Davis-Lameloise, N., Perreten, H., Becard, D., Gjinovci, A., Keller, P. A., Wollheim, C. B., Herrera, P., Muzzin, P., and Assimacopoulos-Jeannet, F. (2007) Diabetologia 50, 84–93
30. Hashimoto, N., Kido, Y., Uchida, T., Asahara, S., Shigeyama, Y., Matsuda, T., Takeda, A., Tsuchihashi, D., Nishizawa, A., Ogawa, W., Fujimoto, Y., Okamura, H., Arden, K. C., Herrera, P. L., Noda, T., and Kasuga, M. (2006) Nat. Genet. 38, 589–593
31. Postic, C., Shiota, M., Niswender, K. D., Jetton, T. L., Chen, Y., Moates, J. M., Shelton, K. D., Lindner, J., Cherrington, A. D., and Magnuson, M. A. (1999) J. Biol. Chem. 274, 305–315
32. Gannon, M., Shiota, C., Postic, C., Wright, C. V., and Magnuson, M. (2000) Genesis 26, 139–142
33. Lee, J. Y., Ristow, M., Lin, X., White, M. F., Magnuson, M. A., and Hennighausen, L. (2006) J. Biol. Chem. 281, 2649–2653
34. Panen, U., Zielmann, S., Langer, J., Zunker, B. J., and Lenzen, S. (1984) Biochem. J. 219, 189–196
35. Argmann, C., and Auwerx, J. (2006) Cell 126, 837–839
36. Liu, C., Allen, A., Kwag, J., Diliba, N. M., Qin, W., Najafi, H., Collins, H. W., Matschinsky, F. M., Stanley, C. A., and Smith, T. J. (2006) J. Biol. Chem. 281, 10214–10221
37. Orci, L., and Unger, R. H. (1975) Lancet 2, 1243–1244
38. Wang, M. Y., Grayburn, P., Chen, S., Ravazzola, M., Orci, L., and Unger, R. H. (2008) Proc. Natl. Acad. Sci. U. S. A. 105, 6139–6144