Delineation of glutamate pathways and secretory responses in pancreatic islets with β-cell–specific abrogation of the glutamate dehydrogenase

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ABSTRACT **In pancreatic β-cells, glutamate dehydrogenase (GDH) modulates insulin secretion, although its function regarding specific secretagogues is unclear. This study investigated the role of GDH using a β-cell–specific GDH knockout mouse model, called β*Glud1*$^{-/-}$. The absence of GDH in islets isolated from β*Glud1*$^{-/-}$ mice resulted in abrogation of insulin release evoked by glutamine combined with 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid or l-leucine. Reintroduction of GDH in β*Glud1*$^{-/-}$ islets fully restored the secretory response. Regarding glucose stimulation, insulin secretion in islets isolated from β*Glud1*$^{-/-}$ mice exhibited half of the response measured in control islets. The amplifying pathway, tested at stimulatory glucose concentrations in the presence of KCl and diazoxide, was markedly inhibited in β*Glud1*$^{-/-}$ islets. On glucose stimulation, net synthesis of glutamate from α-ketoglutarate was impaired in GDH-deficient islets. Accordingly, glucose-induced elevation of glutamate levels observed in control islets was absent in β*Glud1*$^{-/-}$ islets. Parallel biochemical pathways, namely alanine and aspartate aminotransferases, could not compensate for the lack of GDH. However, the secretory response to glucose was fully restored by the provision of cellular glutamate when β*Glud1*$^{-/-}$ islets were exposed to dimethyl glutamate. This shows that permissive levels of glutamate are required for the full development of glucose-stimulated insulin secretion and that GDH plays an indispensable role in this process.**

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INTRODUCTION

Glutamate dehydrogenase (GDH) is a highly conserved mitochondrial enzyme encoded by *GLUD1* (Michaelidis *et al.*, 1993) that catalyzes the reversible reaction α-ketoglutarate + NH_3 + NADH ↔ glutamate + NAD^+ (Hudson and Daniel, 1993). In eukaryotes, GDH exhibits complex allosteric regulation by leucine, pyridine, adenine, and guanine nucleotides (Fisher, 1985; Smith *et al.*, 2001). GDH is mainly expressed in brain, kidney, liver, pancreas, and lymph nodes. While it catalyzes the same reaction in every tissue, its function regarding metabolic homeostasis and its preferred directional flux varies according to specific organs, nutrient state, allosteric regulation, and redox and energy states of mitochondria (Frigerio *et al.*, 2008). In pancreatic β-cells, the importance of GDH in insulin secretion was recognized long ago (Sener and Malaisse, 1980). Activating mutations of GDH have been associated with hypoglycemia in infants and young children (Stanley *et al.*, 1998). GDH can play an anaplerotic role, generating α-ketoglutarate (α-KG) to feed the tricarboxylic acid (TCA) cycle, or a cataplerotic role, generating glutamate at the expense of α-ketoglutarate (Owen *et al.*, 2002). Therefore GDH might play a role in a glucose-induced amplifying pathway through generation of glutamate (Maechler and Wollheim, 1999; Hoy *et al.*, 2002) and/or as an amino acid sensor triggering insulin release upon

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Abbreviations used: α-KG, α-ketoglutarate; β*Glud1*$^{-/-}$, β-cell–specific GDH knockout mice; Ad-GDH, adenovirus encoding for GDH; ALAT, alanine aminotransferase; AOA, aminooxyacetate; ASAT, aspartate aminotransferase; AUC, area under the curve; BCATm, mitochondrial branched-chain aminotransferase; BCH, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid; CoA, coenzyme A; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; GC-MS, gas chromatography–mass spectrometry; GDH, glutamate dehydrogenase; HPLC, high-performance liquid chromatography; KIC, α-ketoisocaproate; KRBH, Krebs Ringer bicarbonate HEPES buffer; TCA, tricarboxylic acid.

glutamine stimulation in conditions of GDH allosteric activation (Sener *et al.*, 1981b; Fahien *et al.*, 1988; Li *et al.*, 2006).

The preferential flux direction of GDH within the β-cell is still debated. Most studies have investigated GDH function through increasing activity using the allosteric activator leucine or its nonmetabolized analogue 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH; Sener *et al.*, 1982; Panten *et al.*, 1984; Fahien *et al.*, 1988). Alternatively, GDH activity can be increased by means of overexpression of the enzyme, an approach that has been combined with allosteric activation of the enzyme (Carobbio *et al.*, 2004). In other studies, an activating mutation of GDH associated with a hyperinsulinism syndrome (Stanley *et al.*, 1998) has been expressed in β-cells of transgenic mice, rendering their islets glutamine-responsive in terms of insulin secretion (Li *et al.*, 2006). Taken together, these gain-of-function approaches demonstrated that glutamine can be turned into a secretagogue upon enhanced GDH activity, although glucose-stimulated insulin secretion is not significantly modified (Sener *et al.*, 1982; Carobbio *et al.*, 2004; Li *et al.*, 2006).

In a limited number of studies, GDH activity was reduced in insulin-secreting cells using inhibitors (Bryla *et al.*, 1994; Yang *et al.*, 2003) or an antisense approach (Maechler *et al.*, 2006). Green tea polyphenols were also shown to inhibit GDH and to reduce insulin release when islets were stimulated with glutamine plus BCH, although not upon glucose stimulation (Li *et al.*, 2006). Moreover, GDH activity can be reduced when the enzyme is ADP-ribosylated by the mammalian Sir2 homologue SIRT4, thereby inhibiting insulin secretion (Haigis *et al.*, 2006; Ahuja *et al.*, 2007).

In this study, we investigated the role of GDH in pancreatic islets isolated from β-cell–specific GDH knockout mice, called β*Glud1*$^{-/-}$ (Carobbio *et al.*, 2009). These mice develop normally, and they exhibit normoglycemia, whereas their insulin levels are reduced (Carobbio *et al.*, 2009). The aim of the present study was to characterize the insulin secretory pattern of β*Glud1*$^{-/-}$ islets, thereby determining the putative role of GDH in the amplifying pathway and its requirement in the responses to glucose versus glutamine stimulations. Moreover, we investigated the complex equilibrium between closely associated amino acids and contribution of related biochemical routes.

RESULTS

Kinetics of glucose-stimulated insulin secretion in β*Glud1*$^{-/-}$ islets

In control islets, 11.8 and 22.8 mM glucose stimulated insulin secretion 1.8-fold and 3.9-fold over basal release, respectively (Figure 1A). In GDH knockout islets isolated from β*Glud1*$^{-/-}$ mice, the secretory responses were reduced by 48% at 11.8 mM glucose and by 50% at 22.8 mM glucose compared with control islets (Figure 1, A and B). Basal insulin release at 2.8 mM glucose was not affected by the lack of GDH, and total islet insulin contents were similar between the two groups (Supplemental Figure S1). These data demonstrate that GDH is required for the full development of glucose-stimulated insulin secretion.

We then tested whether β*Glud1*$^{-/-}$ islets were intrinsically unable to develop a complete secretory response. To this end, glucose was first directly switched from basal 2.8 mM to stimulatory 22.8 mM, showing again a secretory response reduced by half in β*Glud1*$^{-/-}$ islets compared with controls (Figure 1C; area under the curve [AUC]: 832 ± 37 vs. 1171 ± 311 ng/15 min, respectively; –53%; $p < 0.02$). Then glucose was raised further to supraphysiological concentrations (30 mM). Under such extreme conditions, β*Glud1*$^{-/-}$ islets could secrete insulin to levels approaching those of control islets (AUC: 2557 ± 346 vs. 1761 ± 348 ng/15 min, respectively; NS). This suggests that GDH-independent, glucose-derived additive factors could partially compensate for the absence of this enzyme at very high glucose concentrations.

Glutamine-induced insulin secretion in β*Glud1*$^{-/-}$ islets rescued by GDH ectopic expression

On its own, glutamine is not an efficient secretagogue, unless it is combined with an allosteric activator of GDH, such as BCH. Such an experimental maneuver allows complete glutamine oxidation with NH_4^+ release (Sener *et al.*, 1981b) and elevation of the necessary Ca^{2+} signal (Gao *et al.*, 1999). As shown in Figure 2A, insulin secretion was poorly increased in control islets in the presence of 5 mM glutamine, whereas further addition of 10 mM BCH resulted in a robust sevenfold secretory response versus basal release (AUC: 2321 ± 905 vs. 330 ± 72 ng/15 min, respectively; $p < 0.01$; Figure S2). In β*Glud1*$^{-/-}$ islets, the absence of GDH resulted in complete abrogation of glutamine responses, both in the absence and in the presence of BCH (Figure 2A). The glutamine response was also tested in a static insulin secretion assay in the presence of leucine. Leucine exhibits dual effects on mitochondrial activation, since it is a natural GDH allosteric activator and is additionally metabolized, feeding the TCA cycle with its own carbons (Figure 2B). These properties of leucine can be tested separately by using either its nonmetabolized analogue BCH for GDH activation (see above) or its deamination product α-ketoisocaproate (KIC) for direct metabolic contribution. It is noteworthy that KIC tested alone fails to stimulate insulin release (Figure S3), unless it is combined with another metabolite, for instance, glutamine (Sener *et al.*, 1981a). In control islets, 10 mM leucine plus 2 mM glutamine induced a 15.3-fold response, whereas a poor 3.6-fold increase in insulin release was observed in β*Glud1*$^{-/-}$ islets, corresponding to 71% inhibition ($p < 0.01$; see Figure 2C). The response to 10 mM KIC plus 2 mM glutamine was 18.5-fold in control islets and 19.2-fold in β*Glud1*$^{-/-}$ islets. These data show that the KIC response was fully preserved in β*Glud1*$^{-/-}$ islets, incidentally demonstrating that, beside GDH abrogation, the TCA cycle machinery was functional.

Transduction of β*Glud1*$^{-/-}$ islets with an adenovirus encoding for GDH (Ad-GDH) fully restored the secretory response to glutamine combined with BCH (Figure 2D). Of note, β*Glud1*$^{-/-}$ islets transduced with Ad-GDH became responsive to glutamine alone, an effect attributed to the resulting overexpression of GDH, as reported previously (Carobbio *et al.*, 2004). These data show that the glutamine response is completely dependent on GDH, without compensatory pathways.

We also measured insulin secretion in the in situ pancreatic perfusion preparation (Figure S4). This model confirmed abrogation of the secretory response evoked by the combination of 5 mM glutamine plus 10 mM BCH in β*Glud1*$^{-/-}$ mice versus controls (–64%; $p = 0.03$).

The amplifying pathway tested in β*Glud1*$^{-/-}$ islets

The classical experimental procedure to reveal a glucose-evoked amplifying pathway requires clamping of $[Ca^{2+}]_i$ at permissive levels; this is achieved by depolarization of β-cells with KCl in the presence of diazoxide, which holds K_{ATP} channels open (Gembal *et al.*, 1992). At basal glucose, Ca^{2+}-induced insulin release stimulated by KCl was observed both in control and in β*Glud1*$^{-/-}$ islets, while the amplitude was slightly reduced in knockout islets ($p = 0.05$; Figure 3, A and B). In control islets, further addition of 22.8 mM glucose induced amplification of the Ca^{2+} signal with strong and sustained secretory response. The amplifying pathway was not induced in

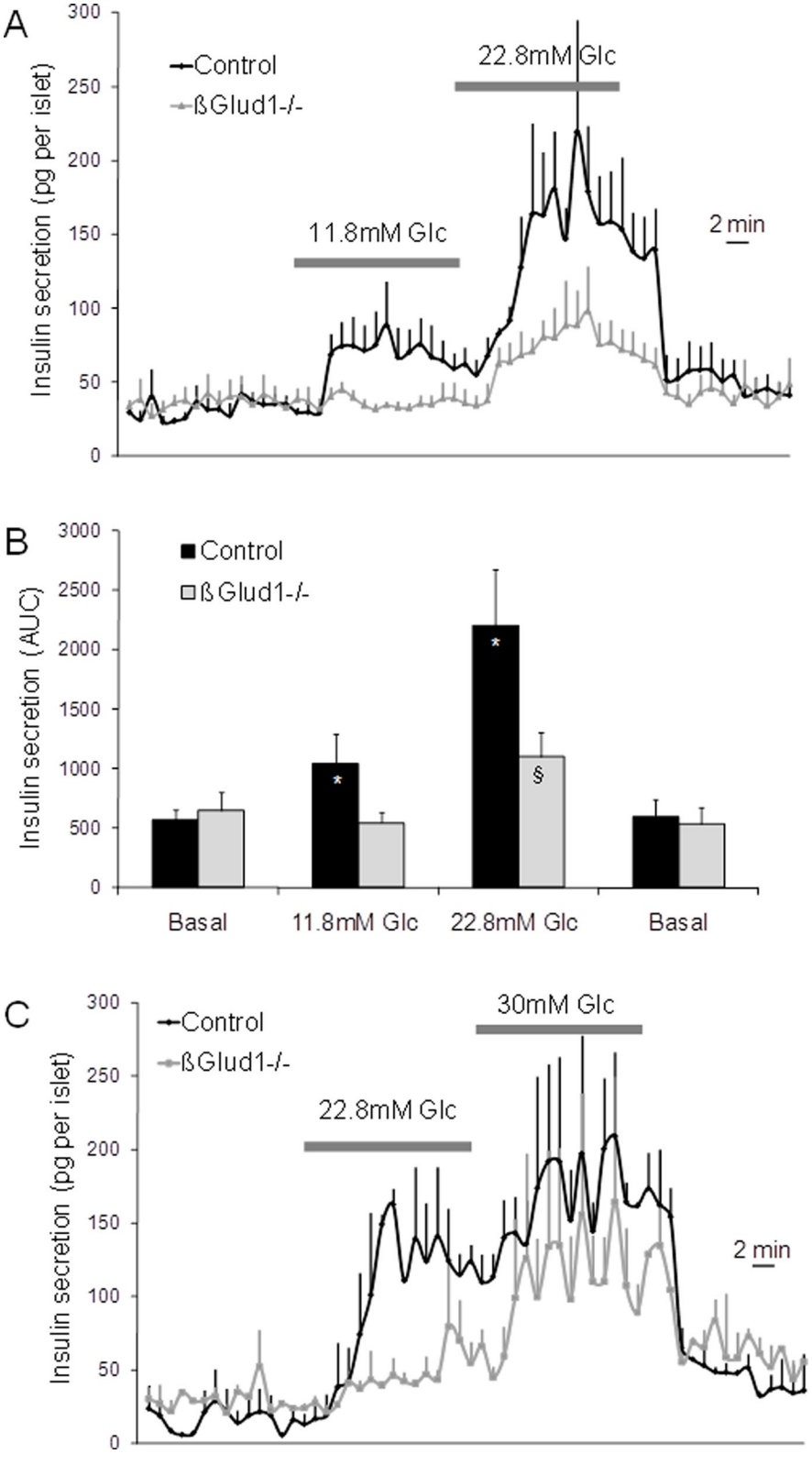

FIGURE 1: Kinetics of glucose-stimulated insulin secretion in β*Glud1*$^{-/-}$ islets. After an overnight culture in RPMI-1640 medium, islets isolated from control and β*Glud1*$^{-/-}$ knockout mice were handpicked and perifused with KRBH at 2.8 mM glucose (Basal) before 15-min stimulations with the indicated glucose (Glc) concentrations. (A) Islets were stimulated with 11.8 and 22.8 mM glucose. (B) Quantification of insulin secretion shown in (A) expressed as AUC. (C) Islets were stimulated with 22.8 and 30 mM glucose. Values are means ± SE of four independent experiments. *, $p < 0.05$ vs. basal of corresponding genotype; §, $p < 0.05$ vs. control under corresponding stimulation condition.

β*Glud1*$^{-/-}$ islets, which secreted much less insulin during the stimulation period versus controls (–72%; $p = 0.01$; Figure 3, A and B). This shows that GDH is required for the development of the amplifying pathway.

Ca^{2+} levels and glutamate sensitivity of β*Glud1*$^{-/-}$ islets

Elevation of cytosolic Ca^{2+} is required for insulin exocytosis, although it is not sufficient for the full development of the glucose response. To determine whether the reduction in glucose-stimulated insulin secretion observed in β*Glud1*$^{-/-}$ islets was associated with modifications of the $[Ca^{2+}]_i$ response, we measured this parameter using fura-2 fluorescence. As shown in Figure 4A, control and β*Glud1*$^{-/-}$ islets responded similarly to 22.8 mM glucose stimulation, both in terms of kinetic and amplitude of the responses.

On glucose stimulation, both ATP and Ca^{2+} increases are preserved in β*Glud1*$^{-/-}$ islets (see Carobbio *et al*. [2009] and Figure 4A, respectively), indicating that the triggering pathway does not rely on GDH activity. Conversely, the amplifying pathway is deficient in GDH knockout β-cells (Figure 3A). We then tested whether a lack of glutamate, secondary to GDH deletion, could explain the reduced secretory response in β*Glud1*$^{-/-}$ islets. Islets were stimulated with 22.8 mM glucose in the absence or the presence of dimethyl glutamate, a cell membrane–permeable glutamate precursor (Figure 5A). In control islets, insulin secretion was stimulated 12-fold by 22.8 mM glucose, while the response was 55% lower in β*Glud1*$^{-/-}$ islets ($p < 0.01$; Figure 4B). Addition of dimethyl glutamate fully restored the secretory response of β*Glud1*$^{-/-}$ islets, whereas it did not exhibit additive effects in controls.

Respective roles of GDH and aminotransferases in glucose-stimulated insulin secretion

Because impaired glucose-stimulated insulin secretion in β*Glud1*$^{-/-}$ islets could be rescued by provision of cellular glutamate, we measured concentrations of glutamate and related metabolites (Figure 5A) in response to glucose. In control islets, four amino acids were increased by 22.8 mM glucose stimulation: glutamate (twofold) and its amidation product glutamine (3.7-fold), aspartate (1.5-fold), and taurine (1.8-fold); see Table 1. In β*Glud1*$^{-/-}$ islets, glucose stimulation failed to increase these amino acids, resulting in lower islet contents of glutamate (–41%) and aspartate (–32%) versus controls. Glutamate decarboxylation catalyzed by glutamate decarboxylase (GAD) forms γ-aminobutyric acid (GABA). Interestingly, the GABA/glutamate ratio was about twofold higher in glucose-stimulated β*Glud1*$^{-/-}$ islets versus controls, in both the absence and

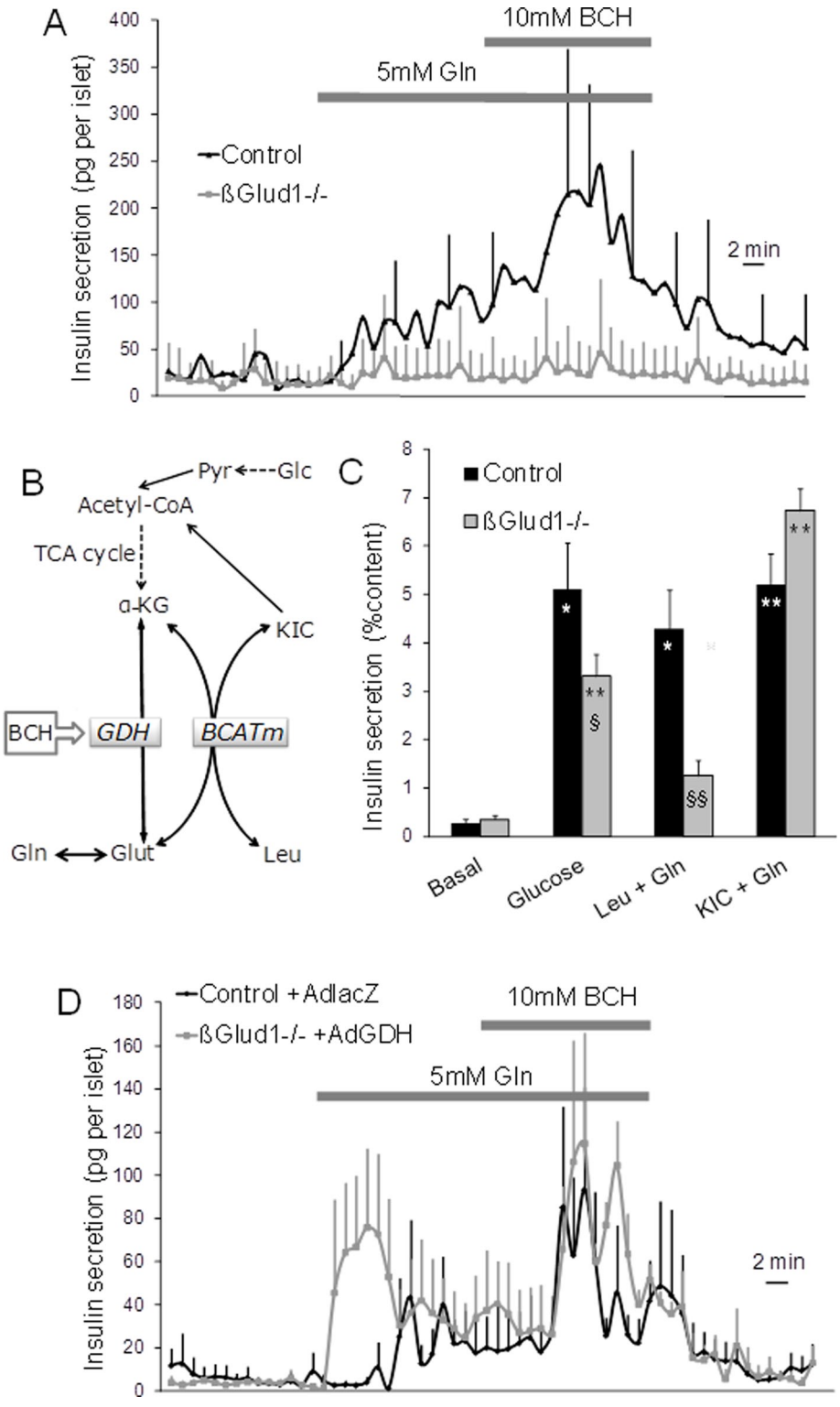

FIGURE 2: Requirement of GDH for secretory responses of glutamine combined with L-leucine and derivatives in control and β*Glud1*$^{-/-}$ islets. After an overnight culture in RPMI-1640 medium, islets isolated from control and β*Glud1*$^{-/-}$ mice were handpicked and perifused with KRBH at 2.8 mM glucose (Basal). (A) Islets were sequentially stimulated for 15 min with 5 mM of glutamine (Gln) and Gln plus 10 mM BCH. (B) L-Leucine (Leu) is deaminated to KIC by BCATm, transferring the amino group to α-KG and thereby producing glutamate (Glut). Glutamate can also be produced by deamidation of Gln. KIC can generate acetyl-CoA, the latter being also produced by pyruvate (Pyr), a cytosolic product of glucose (Glc) catabolism. GDH can be allosterically activated by L-leucine or its nonmetabolized analogue BCH. (C) Secretory responses of β*Glud1*$^{-/-}$ islets to L-leucine and KIC. Insulin secretion was tested in islets over a 1-h incubation period at basal and 22.8 mM glucose, and at basal plus 2 mM glutamine with either 10 mM L-leucine or KIC. (D) Islets isolated from control and β*Glud1*$^{-/-}$ mice were transduced with Ad-lacZ and Ad-GDH adenoviruses, respectively. After overnight culture, islets were perifused with KRBH at 2.8 mM glucose (Basal) and then stimulated for 15 min with 5 mM Gln and 15 min with Gln plus 10 mM BCH. (B and C) Values are means ± SE of three independent experiments for each group. *, $p < 0.05$, **, $p < 0.01$ vs. basal of corresponding genotype; §, $p < 0.05$, §§, $p < 0.01$ control under corresponding stimulation condition; ##, $p < 0.01$ vs. 22.8 mM glucose of corresponding genotype.

presence of glutamine (Table 1). This suggests that, in the absence of GDH, there was increased production of GABA via GAD using glutamate as a substrate.

Glutamate can be formed from the TCA cycle intermediate α-ketoglutarate through GDH. Alternatively, glutamate can arise from glutamine deamidation or from transamination of α-ketoglutarate, with alanine and aspartate as amino group donors, generating pyruvate and oxaloacetate, respectively (Figure 5A). To test the putative contribution of the respective alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) to cellular glutamate levels, we inhibited these enzymes with aminooxyacetate (AOA). As shown in Figure 5B, stimulation with 22.8 mM glucose increased insulin secretion 4.6-fold in control islets, while the response was 44% lower in β*Glud1*$^{-/-}$. Glutamate concentrations were increased twofold in control islets stimulated with 22.8 mM glucose (Figure 5C). In β*Glud1*$^{-/-}$ islets, glutamate levels at 22.8 mM glucose were 41% lower compared with controls, a magnitude similar to the reduced insulin release measured in the same islets (Figure 5B). The presence of 2 mM AOA did not modify the secretory response to glucose stimulation in either control or β*Glud1*$^{-/-}$ islets. AOA partially reduced the glucose-induced elevation of islet glutamate contents, indicating some contribution of aminotransferases in this process (Figure 5C). Of note, glutamate synthesis from α-ketoglutarate by GDH requires ammonia as a cosubstrate, and ammonia was not added in the stimulation buffer. However, the increment in cellular glutamate measured in control islets upon glucose stimulation would require only hundreds of picomoles of ammonia, probably contributed by the cellular milieu.

Regarding aspartate levels, there was a 1.5-fold increase upon glucose stimulation in control islets (Figure 5D). GDH knockout

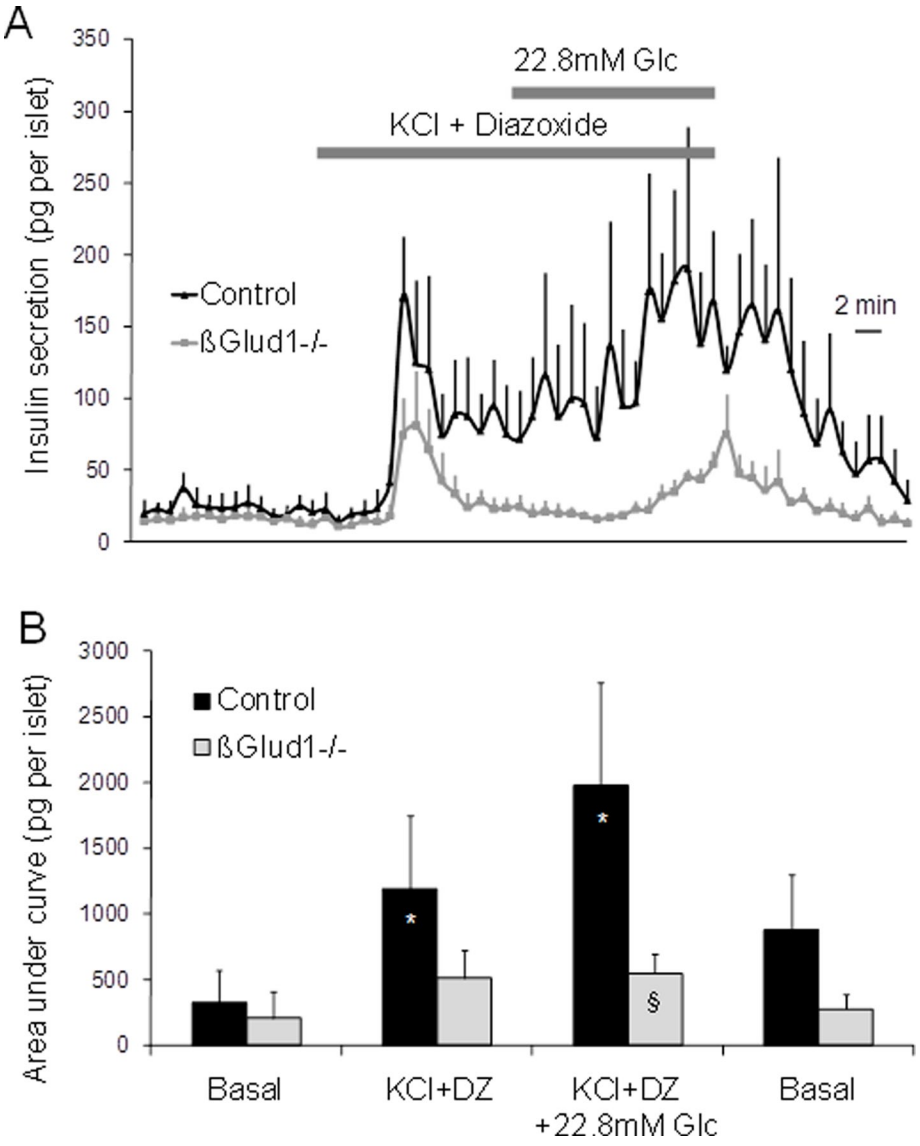

FIGURE 3: The amplifying pathway of the secretory response tested in the absence of GDH in β*Glud1*$^{-/-}$ islets. (A) After an overnight culture in RPMI-1640 medium, islets isolated from control and β*Glud1*$^{-/-}$ mice were handpicked and perifused with KRBH at 2.8 mM glucose (Basal). Then islets were stimulated for 15 min with 30 mM KCl plus 250 μM diazoxide (DZ) before elevation of glucose (Glc) concentration to 22.8 mM for another 15-min period. (B) Quantification of insulin secretion shown in (A) expressed as AUC. Values are means ± SE of three independent experiments. *, $p < 0.05$ vs. basal of corresponding genotype; §, $p < 0.05$ vs. control under corresponding stimulation condition.

impaired glucose-induced increases in aspartate levels as observed in β*Glud1*$^{-/-}$ islets compared with controls (–32%). Measurements of alanine revealed that glucose stimulation did not modify concentrations of this amino acid in control islets, whereas addition of AOA decreased islet alanine contents by 40% (Figure 5E). In β*Glud1*$^{-/-}$ islets kept at nonstimulatory 2.8 mM glucose, alanine was 32% lower compared with control islets, possibly indicating the use of this amino acid for maintenance of the basal glutamate pool in GDH-null islets through a transamination reaction.

Contribution of glucose-derived carbons for de novo glutamate and aspartate synthesis

Overall, measurements of islet amino acid contents (Table 1) pointed to GDH as an important player contributing to glutamate and aspartate levels upon glucose stimulation (Figure 6, A–C).To further investigate the contribution of GDH to the active synthetic pathway of glutamate and aspartate evoked by glucose, we incubated isolated islets in medium containing 22.8 mM [U-^{13}C]-glucose. [U-^{13}C]glucose is metabolized to [1,2-^{13}C]acetyl-coenzyme A (CoA) which may condense with oxaloacetate. After successive steps in the TCA cycle [4,5-^{13}C]α-ketoglutarate is formed, which may undergo reductive amination by GDH to generate [4,5-^{13}C]glutamate (metabolite with two labeled carbons, M+2). Alternatively, [4,5-^{13}C]α-ketoglutarate can be further metabolized in the TCA cycle to oxaloacetate, which may be transaminated to double-labeled (M+2) aspartate. We observed that the net synthesis of glutamate from α-ketoglutarate was reduced by 23% in β*Glud1*$^{-/-}$ islets, while aspartate labeling was similar to controls (Figure 6D).

Effects of glutamine on glutamate levels and insulin secretion in β*Glud1*$^{-/-}$ islets

Glutamine can enter into β-cells, in which it is efficiently converted to glutamate via glutaminase. However, further catabolism requires allosteric activation of GDH in order to feed the TCA cycle. Consequently, glutamine-induced elevation of cellular glutamate is not sufficient to promote insulin secretion (Bertrand *et al.*, 2002; Fernandez-Pascual *et al.*, 2004). This is shown here by the addition of 1 mM glutamine at basal glucose, which did not stimulate insulin release (Figure 7A), unlike the slight response evoked by 5 mM glutamine tested in islet perifusion system (Figure 2A). Glucose stimulation of β*Glud1*$^{-/-}$ islets resulted in a blunted secretory response and an absence of a glutamate increase (Figure 7, A and B). Exposure of β*Glud1*$^{-/-}$ islets to glutamine markedly increased glutamate concentrations and restored glucose-stimulated insulin secretion to levels similar to those of control islets. In these conditions, glucose contributed to the rise in Ca^{2+} (Figure 4A), while glutamine provided elevated glutamate (Figure 7B). Therefore both glutamine and dimethyl glutamate (Figure 4B) can serve as a glutamate precursor to restore glutamate pools and insulin secretion in glucose-stimulated GDH-deficient islets.

In control islets exposed to glutamine at basal glucose, aspartate and alanine levels were increased 2.9-fold and twofold, respectively (Table 1). In β*Glud1*$^{-/-}$ islets, the same glutamine and glucose conditions failed to significantly increase aspartate and alanine levels, indicating that GDH is required for such pathways.

DISCUSSION

The present study characterized the amino acid profile and the secretory responses of GDH-deficient β-cells in islets isolated from

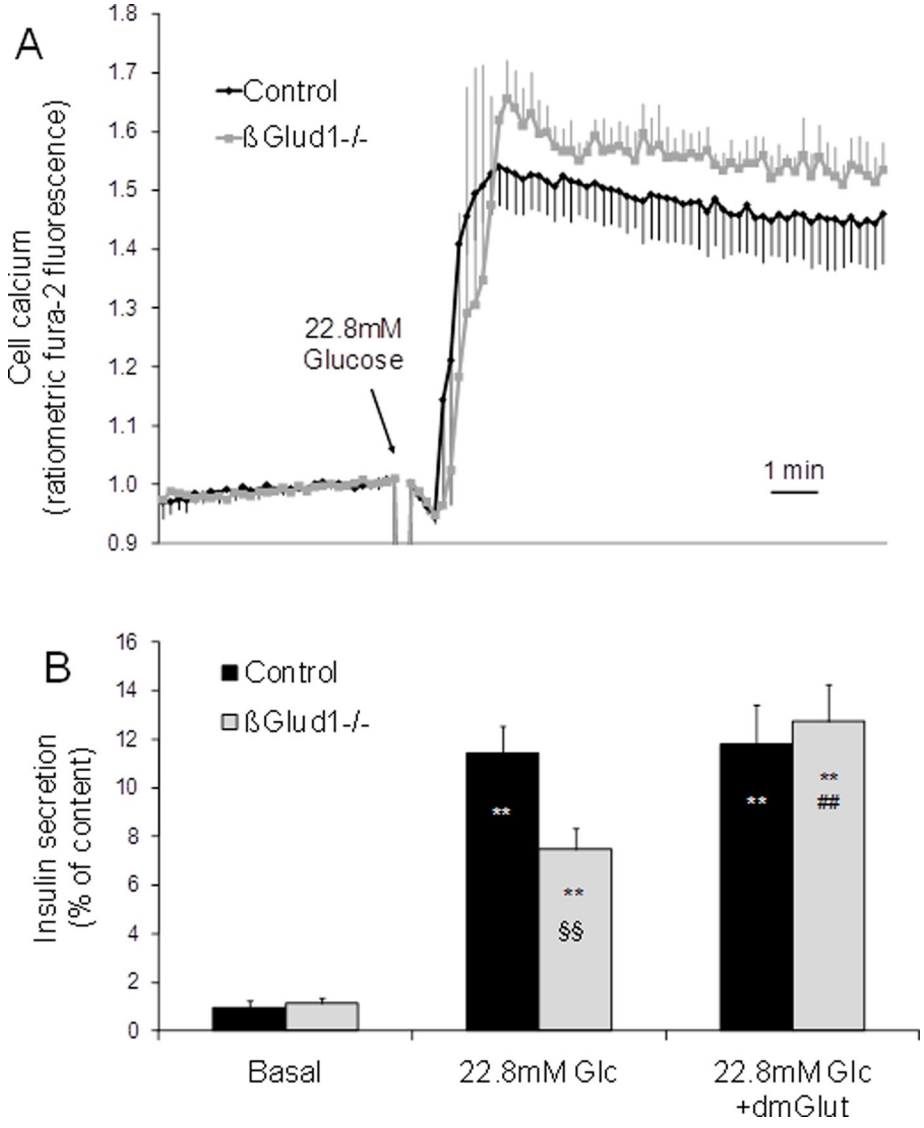

FIGURE 4: Cellular calcium changes and glutamate sensitivity of β*Glud1*$^{-/-}$ islets. Islets were isolated from control and β*Glud1*$^{-/-}$ mice and kept in culture before experiments. (A) Cellular calcium levels were monitored in islets loaded with fura-2 and placed in KRBH with 2.8 mM glucose before stimulation with 22.8 mM glucose. (B) Insulin secretion was tested in islets over a 1-h incubation period at 2.8 mM (Basal) and 22.8 mM (Glc) glucose supplemented with 5 mM dimethyl glutamate (dmGlut) where indicated. Values are means ± SE of six independent mice for each group. **, $p < 0.01$ vs. basal of corresponding genotype; §§, $p < 0.01$ vs. control under corresponding stimulation condition; ##, $p < 0.01$ vs. 22.8 mM Glc of corresponding genotype.

β*Glud1*$^{-/-}$ mice. This offered the opportunity of investigating the role of GDH in the secretory response of different secretagogues and its putative requirement for the development of the amplifying pathway. First, we found evidence that secretion induced by a rise in intracellular Ca^{2+} evoked by KCl was not significantly modified in β*Glud1*$^{-/-}$ islets compared with controls, although we observed a trend toward lower insulin release. Second, glucose-stimulated insulin secretion was reduced by half in GDH-null β-cells, correlating with impaired glutamate formation, while the rise in Ca^{2+} was preserved. In the absence of GDH, the amplifying pathway failed to develop upon glucose stimulation in β*Glud1*$^{-/-}$ islets. Finally, insulin secretion induced by glutamine required both expression and allosteric activation of GDH, as demonstrated in β*Glud1*$^{-/-}$ islets treated with BCH or leucine. Collectively these data show that GDH differently and specifically contributes to secretory responses of the main secretagogues.

Regarding leucine, we also tested its deamination product KIC in order to discriminate between GDH allosteric activation properties (tested with BCH), as well as leucine's contribution to the TCA cycle activation with its own carbons (tested with KIC; see Figure S3). In the presence of glutamine, preservation of the KIC response, but not that of leucine, in β*Glud1*$^{-/-}$ islets suggested that GDH is required for transamination of leucine to KIC by mitochondrial branched-chain aminotransferase (BCATm). BCATm is necessary for insulin secretion evoked by KIC and glutamine, as shown in islets from mice lacking this enzyme (Zhou *et al.*, 2010). In the BCATm reaction, the TCA cycle intermediate α-ketoglutarate is used as an acceptor of the amino group transferred from leucine, thereby forming glutamate. To refill the TCA cycle, GDH might serve as anaplerotic enzyme in these specific conditions, completing a futile cycle enabling efficient leucine deamination. This might explain the weak leucine response in GDH-deficient islets, while combination of KIC plus glutamine maintained a robust secretory response.

Unlike glucose, glutamine alone does not stimulate insulin secretion under normal conditions (Malaisse *et al.*, 1980). Glutamine becomes a secretagogue only when GDH is allosterically activated, for instance, by BCH (Panten *et al.*, 1984), or in the case in which the enzyme carries an activating mutation (Li *et al.*, 2006) responsible for a hyperinsulinism syndrome (Stanley *et al.*, 1998). The present study demonstrates that the secretory response evoked by the combination of glutamine plus BCH (or leucine) is totally GDH-dependent and there is no alternative compensatory pathway. Although glutamine deamidation produces glutamate, further catabolism through GDH is required for induction of insulin secretion. Therefore elevation of intracellular glutamate levels is not sufficient to promote insulin exocytosis without the necessary Ca^{2+} increase to permissive levels, as shown previously in permeabilized insulin-secreting cells (Maechler and Wollheim, 1999). Elevation of cytosolic Ca^{2+} requires ATP generation secondary to mitochondrial metabolism through TCA cycle activation, an effect achieved by glucose alone, but not by glutamine, unless it is combined with GDH allosteric activators (Sener *et al.*, 1981b). As opposed to glutamine, glucose stimulation promotes elevation of both cytosolic Ca^{2+} (Figure 4A) and cellular glutamate levels (Figure 5C). Of note, glutamine has been reported to remain a weak secretagogue, even upon KCl depolarization (Bertrand *et al.*, 2002; Fernandez-Pascual *et al.*, 2004), suggesting that other signals are still missing in these experimental conditions. Alternatively, glutamine-derived glutamate might be preferentially converted to GABA, in accordance with observations in rat islets showing that in the absence

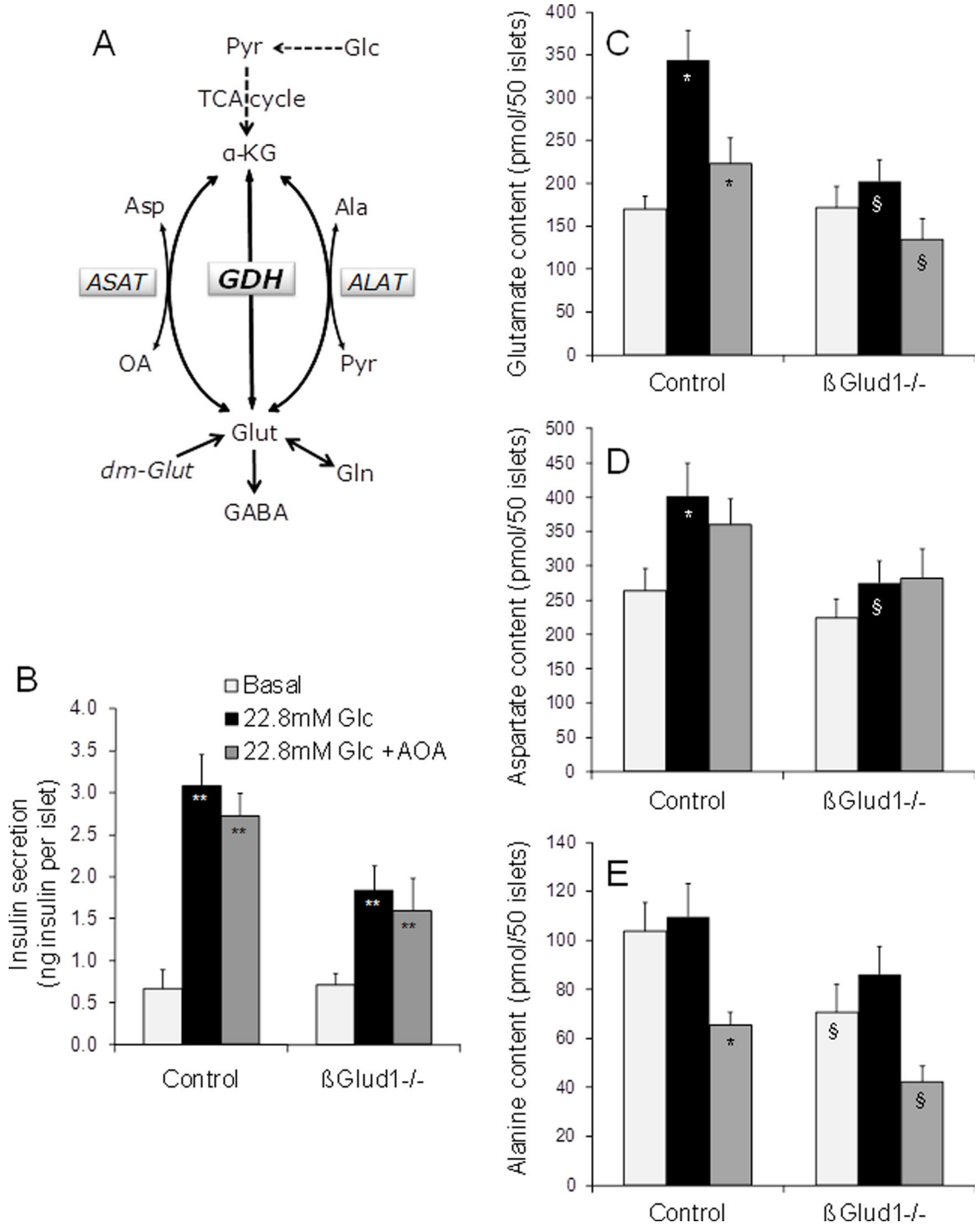

FIGURE 5: Respective roles of GDH and aminotransferases in glucose-stimulated insulin secretion. (A) GDH and aminotransferases (ASAT and ALAT) connect glutamate (Glut) and the TCA cycle intermediate α-KG. ASAT uses either oxaloacetate (OA) or aspartate (Asp) as a cosubstrate and ALAT uses either pyruvate (Pyr) or alanine (Ala), along with Glut or α-KG, respectively. Glutamate can also arise from glutamine (Gln) deamidation. (B–E) After an overnight culture in RPMI-1640 medium, islets isolated from control and β*Glud1*$^{-/-}$ mice were handpicked and preincubated for 1 h in glucose- and glutamine-free RPMI-1640 medium. Then islets were incubated for 1 h at 2.8 mM (Basal) and 22.8 mM (Glc) glucose in the absence or presence of 2 mM AOA. At the end of the assay period, supernatants were collected to measure insulin secretion (B) and islets were collected in 5% (wt/vol) 5-sulfosalicylic acid before determination of amino acid concentrations by HPLC (C–E). Values are means ± SE of six independent mice for each group. *, $p < 0.05$, **, $p < 0.01$ vs. basal of corresponding genotype; §, $p < 0.05$, §§, $p < 0.01$ vs. control under corresponding stimulation condition; #, $p < 0.01$ vs. 22.8 mM Glc of corresponding genotype.

of any other substrates, glutamine is dose-dependently decarboxylated independently of mitochondrial metabolism (Fernandez-Pascual *et al.*, 2004).

Failure of glutamate generation upon glucose stimulation in GDH-deficient islets could be rescued by exposure to glutamate precursors. Indeed, provision of cellular glutamate to β*Glud1*$^{-/-}$

Amino acid	Group	Basal 2.8 mM glucose	Glucose 22.8 mM	Glc 22.8 mM + AOA	Basal + 1 mM glutamine	Glc 22.8 mM + glutamine
Alanine (Ala)	Control	103.8 ± 11.5	109.6 ± 13.5	65.3 ± 5.3**	203.1 ± 45.4**	147.5 ± 20.8
	β*Glud1*$^{-/-}$	70.6 ± 11.5*	86.2 ± 11.2	42.3 ± 6.3*	92.4 ± 14.7*	137.6 ± 28.1
Arginine (Arg)	Control	125.3 ± 31.0	86.8 ± 22.4	30.4 ± 5.5	222 ± 59.5	189.9 ± 60.6
	β*Glud1*$^{-/-}$	82.8 ± 20.6	78.3 ± 19.1	30.3 ± 3.9	148.6 ± 21.5	151.2 ± 20.0
Aspartate (Asp)	Control	263.7 ± 32.8	402.0 ± 47.2**	359.6 ± 38.0	776.2 ± 211.9**	453.6 ± 146.7
	β*Glud1*$^{-/-}$	224.6 ± 27.1	275.5 ± 31.5*	282.1 ± 42.2	407.0 ± 128.6*	428.6 ± 80.2
γ-aminobutyric acid (GABA)	Control	78.9 ± 10.9	101.3 ± 18.0	87.7 ± 12.7	210.9 ± 73.1**	62.2 ± 11.7***
	β*Glud1*$^{-/-}$	105.2 ± 21.6	108 ± 16.6	67.4 ± 15.8	169.6 ± 33.7	146.9 ± 29.0*
Glutamate (Glu)	Control	170.3 ± 15.1	343.8 ± 34.7**	223.2 ± 30.6**	1848.0 ± 215.2**	1592.9 ± 167.6**
	β*Glud1*$^{-/-}$	172.2 ± 24.6	202.9 ± 24.6*	134.4 ± 24.4*	1389.3 ± 232.3**	1881.2 ± 256.5**
Glutamine (Gln)	Control	41.3 ± 16.2	151.2 ± 63.1**	35.4 ± 2.7	22246 ± 1730**	24192 ± 2689**
	β*Glud1*$^{-/-}$	29.3 ± 9.7	98.0 ± 36.2	15.1 ± 3.0*	17221 ± 2888**	20798 ± 1910**
Glycine (Gly)	Control	264.6 ± 76.5	356.9 ± 99.9	96.2 ± 9.7	942.0 ± 382.4**	594 ± 106.3
	β*Glud1*$^{-/-}$	221.9 ± 59.5	271.4 ± 68.1	89.3 ± 23.3	456.3 ± 114.1	334.7 ± 63.6
Histidine (His)	Control	24.8 ± 2.3	31.9 ± 7.5	18.9 ± 3.5	74.5 ± 43.7**	19 ± 5.5
	β*Glud1*$^{-/-}$	24.4 ± 5.3	22.2 ± 4.2	20.1 ± 12.8	25.3 ± 1.3*	44.5 ± 12.3
Serine (Ser)	Control	217.8 ± 25.6	254.4 ± 54.9	129.5 ± 20.5	446.0 ± 120.5**	334.7 ± 26.8
	β*Glud1*$^{-/-}$	196.2 ± 35.1	200.2 ± 32.5	146.3 ± 67.7	252.6 ± 29.5	371.2 ± 103.6
Taurine (Tau)	Control	314.1 ± 37.5	571.4 ± 66.4**	468.1 ± 24.8**	626.1 ± 184	450.2 ± 138.3
	β*Glud1*$^{-/-}$	318.6 ± 74.0	398.7 ± 61.0	333.7 ± 53.4	406.3 ± 94.3	433.8 ± 56.1
Tyrosine (Tyr)	Control	34.6 ± 3.3	86.8 ± 22.4	20.9 ± 4.4	32.1 ± 10	23.3 ± 4.0
	β*Glud1*$^{-/-}$	29.6 ± 5.6	78.3 ± 19.1	20.3 ± 6.2	16.9 ± 2.6	15.4 ± 3.5

After an overnight culture, isolated islets were preincubated for 1 h in glucose- and glutamine-free RPMI-1640 medium. Then islets were incubated for 1 h at 2.8 mM (basal) and 22.8 mM glucose with or without 2 mM AOA or 1 mM glutamine. Values (in picomoles per 50 islets) are means ± SE of six independent mice. *, $p < 0.05$ vs. control under corresponding stimulation condition; **, $p < 0.05$ vs. basal of corresponding phenotype; ***, $p < 0.05$ vs. basal plus glutamine of corresponding phenotype.

TABLE 1: Amino acid levels in islets from control and β*Glud1*$^{-/-}$ mice stimulated with glucose and glutamine.

islets, adding either dimethyl glutamate or glutamine, fully restored the secretory response to glucose (Figures 4B and 7A, respectively). This is in agreement with replenishment of cytosolic glutamate in β-cells down-regulating the mitochondrial glutamate carrier GC1 and thereby restoring insulin secretion (Casimir *et al.*, 2009a). Recently, bioinformatic analysis of gene expression in mouse islets versus other murine tissues identified ornithine aminotransferase as a new "disallowed gene" in pancreatic islets (Pullen *et al.*, 2010). This enzyme, which converts glutamate to ornithine, is selectively down-regulated in β-cells, preventing dissipation of the glutamate pool (Pullen *et al.*, 2010). Altogether, these observations established that permissive levels of glutamate are necessary for the full development of the secretory response to glucose stimulation. In other words, intracellular glutamate would render insulin granules exocytosis-competent. The cytosolic target of glutamate might be the insulin granule itself, as several studies by different groups have shown the requirement of glutamate uptake by secretory vesicles for insulin exocytosis (Maechler and Wollheim, 1999; Hoy *et al.*, 2002; Eto *et al.*, 2003; Storto *et al.*, 2006; Gammelsaeter *et al.*, 2011). Present data point to GDH as the enzyme in charge of a glucose-induced increase in cellular glutamate, with aminotransferases playing no significant role in this process. Moreover, β*Glud1*$^{-/-}$ islets reveal that GDH is required for development of the amplifying pathway.

On the basis of present data, we designed a model for glutamate-related pathways (Figure 6). When control islets are stimulated with glucose, glutamate and aspartate levels are increased along with insulin secretion (Figure 6B). Measurements of carbon fluxes using [U-^{13}C]glucose labeling and gas chromatography–mass spectrometry (GC-MS) analysis revealed that GDH contributes to the net synthesis of glutamate from α-ketoglutarate upon glucose stimulation. The lower glutamate enrichment in glucose carbons observed in β*Glud1*$^{-/-}$ islets was not due to lower transaminase activity, since the labeling was not reduced in aspartate. In contrast, the slight tendency toward an increased labeling of aspartate in β*Glud1*$^{-/-}$ islets may illustrate that ^{13}C labeling was retained in the TCA cycle when the flow of carbons toward glutamate was hindered by the lack of GDH.

Inhibition of transaminases does not change the secretory response, but it lowers aspartate levels in control islets, indicating that ALAT works in the direction of α-ketoglutarate formation upon glucose stimulation. Significantly, our study shows increased aspartate and unchanged alanine levels upon glucose stimulation, while others reported a decrease and an increase, respectively (Li *et al.*, 2003,

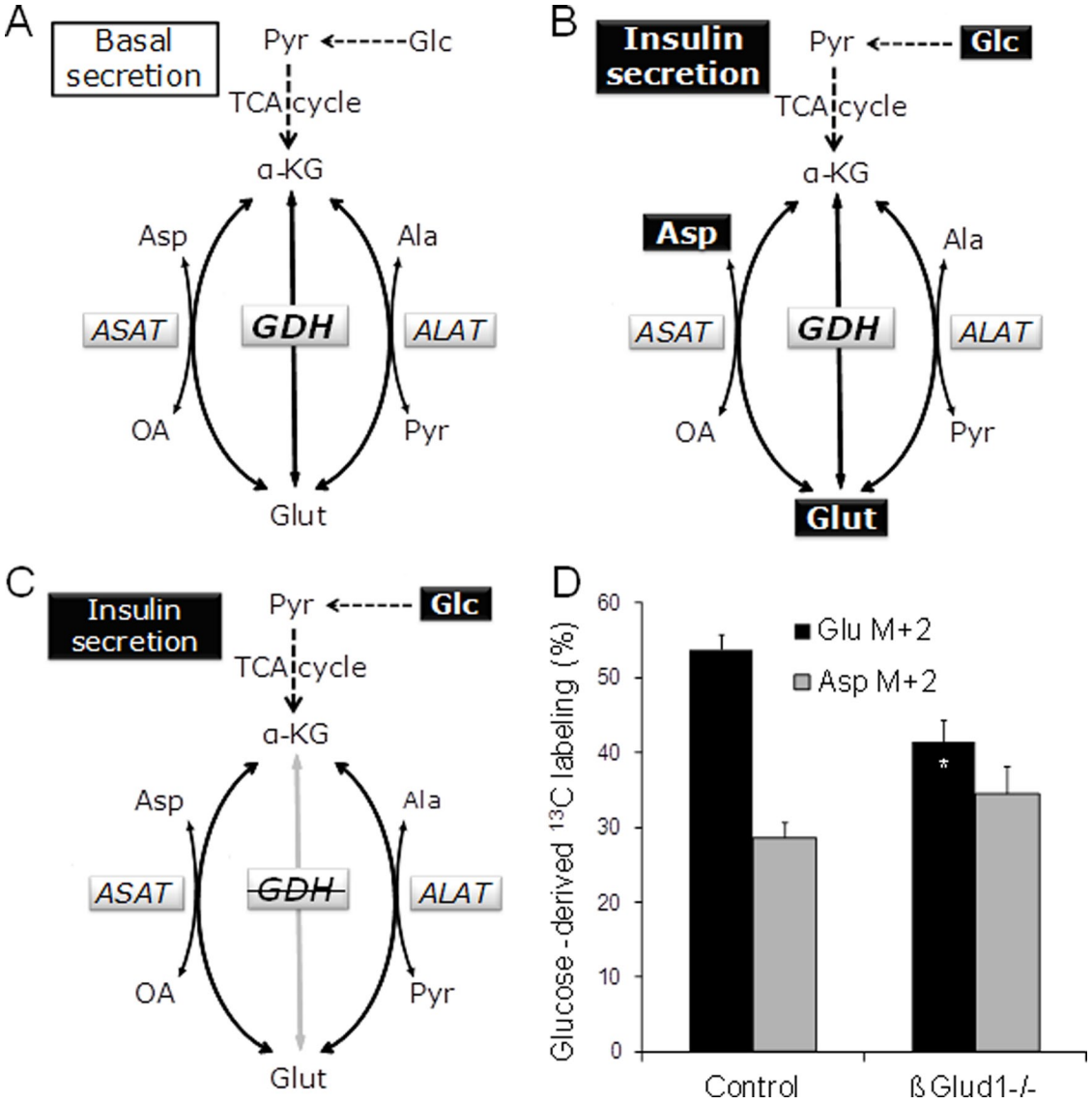

FIGURE 6: Contribution of glucose and GDH for glutamate and aspartate synthesis. (A–C) A model for the role of GDH in glutamate-related pathways. GDH and aminotransferases (ASAT and ALAT) are three enzymes connecting glutamate (Glut) to the TCA cycle intermediate α-KG. Depending on its flux direction, ASAT uses either oxaloacetate (OA) or aspartate (Asp) as a cosubstrate and ALAT uses either pyruvate (Pyr) or alanine (Ala), along with Glut or α-KG, respectively. (A) Basal insulin release is observed at 2.8 mM glucose (Glc), accompanied by basal levels of glutamate, aspartate, and alanine. (B) A level of 22.8 mM glucose (**Glc**) stimulates insulin secretion and results in increased levels of both glutamate and aspartate. (C) In the absence of GDH (~~*GDH*~~), glucose stimulation fails to increase glutamate and aspartate, and the secretory response is blunted. (D) Isolated islets were incubated in medium containing 22.8 mM [U-^{13}C]glucose for 1 h. Next islets were extracted, lyophilized, reconstituted, and derivatized before GC-MS analysis of isotopic enrichment in glutamate and aspartate. The quantification of double labeling (M+2) was calculated as percent of the pool of unlabeled metabolite. Values are means ± SD, $n = 6$. *, $p < 0.05$ vs. Glu M+2 of corresponding genotype.

2006). These discrepancies might be explained by preincubation conditions resulting in different amino acid pools when glucose stimulation is initiated. For instance, in studies by Li *et al.* (2003, 2006) mouse islets were preincubated with 10 mM glutamine, thereby filling the cellular glutamine/glutamate pool before stimulation. We opted for fuel depletion, that is, both glucose- and glutamine-free preincubation medium, before stimulation with one or the other metabolite. Discrepancies might also be explained by species specificities. Indeed, using the rat-derived insulin-secreting cell line INS-1E, we previously reported decreased aspartate and increased alanine upon glucose stimulation (Carobbio *et al.*, 2004). More recently, no change in aspartate levels was recorded in glucose-stimulated rat islets when preincubated in the absence of nutrients (Pizarro-Delgado *et al.*, 2009). It is intriguing that these two amino acids, aspartate in particular, exhibit high variability between studies. Aspartate participates in both amino acid metabolism and the glucose-induced NADH mitochondrial shuttle (Casimir *et al.*, 2009b). This might render its cellular levels highly sensitive to nutrient states. Of note, while 4 mM AOA was shown to inhibit glucose-stimulated insulin secretion in rat islets (Casimir *et al.*, 2009b), we did not observe such an effect at 2 mM AOA in mouse islets. This is consistent with previous studies reporting stronger effects of AOA in rat (MacDonald, 1982; Malaisse *et al.*, 1982) versus mouse (Eto *et al.*, 1999; Ravier *et al.*, 2000) islets. Thus the present results confirm

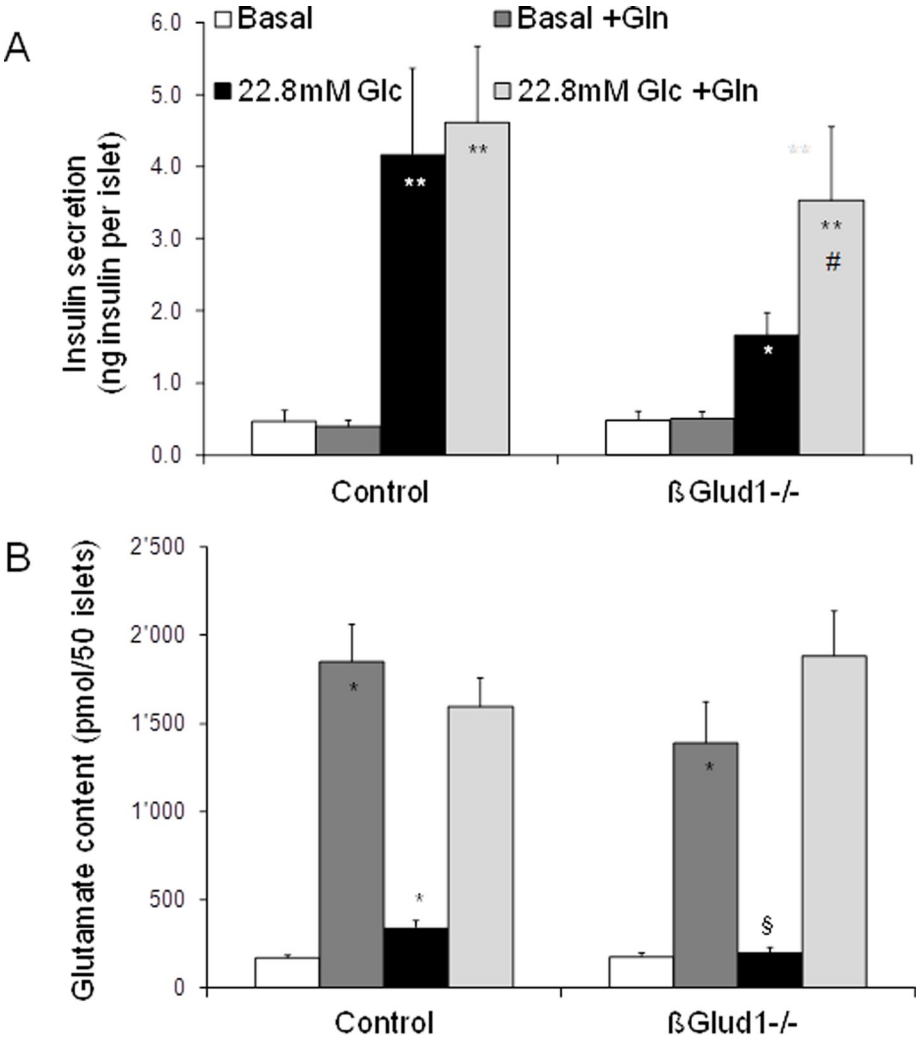

FIGURE 7: Effects of glutamine on glutamate levels and insulin secretion in β*Glud1*$^{-/-}$ islets. After an overnight culture in RPMI-1640 medium, islets isolated from control and β*Glud1*$^{-/-}$ mice were handpicked and preincubated for 1 h in glucose- and glutamine-free RPMI-1640 medium. Then, islets were incubated for 1 h at 2.8 mM (Basal) and 22.8 mM (Glc) glucose in the absence or presence of 1 mM glutamine (Gln). At the end of the assay period, supernatants were collected to measure insulin secretion (A), and islets were collected in 5% (wt/vol) 5-sulfosalicylic acid before determination of amino acid concentrations by HPLC (B). Values are means ± SE of six independent mice for each group. *, $p < 0.05$, **, $p < 0.01$ vs. basal of corresponding genotype; §, $p < 0.05$, §§, $p < 0.01$ vs. control under corresponding stimulation condition; #, $p < 0.05$ vs. 22.8 mM glucose of corresponding genotype.

that glucose-stimulated insulin secretory response of mouse islets is not affected by AOA at 2 mM (Figure 5B), while at 5 mM there are either no (Eto *et al.*, 1999) or marginal (Ravier *et al.*, 2000) inhibitory effects. The reason for such a species difference is unknown.

The fact that the rise in glutamate evoked by glucose is not affected by transaminase inhibition suggests that this amino acid is mainly contributed by an alternative enzyme connecting the TCA cycle to glutamate, namely GDH. In the absence of GDH, glucose stimulation can no longer increase glutamate and aspartate (Figure 7D). This observation further points to GDH as the key enzyme for the generation of glutamate. Moreover, transaminases are not able to compensate for the lack of GDH in β*Glud1*$^{-/-}$ islets. Data show that GDH is also required for aspartate formation during glucose stimulation. The "GABA-shunt," active at high glucose levels, is an alternative pathway for glutamate metabolism. According to this model (Pizarro-Delgado *et al.*, 2009), glutamate exported out of mitochondria is decarboxylated to GABA. Next importing GABA back into mitochondria results in α-ketoglutarate–dependent transamination, favoring the formation of the TCA cycle intermediate succinate (Pizarro-Delgado *et al.*, 2010). Alternatively, GABA can be released from the β-cell and exert an autocrine positive feedback loop, as observed in human islets (Braun *et al.*, 2010). However, rodent insulin-secreting cells do not exhibit similar sensitivity, as shown in rat and mouse islets (Gilon *et al.*, 1991) and insulinoma INS-1E cells (Rubi *et al.*, 2001).

According to previous and present results, we favor the following model for GDH regulation in β-cells: 1) at basal glucose, a low energy state might induce moderate glutamate consumption to maintain basal ATP levels; 2) upon glucose stimulation inducing insulin secretion, GDH would work in the cataplerotic direction, generating glutamate from α-ketoglutarate enriched by glucose-derived carbons; 3) in the pathological situation of hyperinsulinism syndrome, the GDH-activating mutation renders the enzyme anaplerotic and the β-cell glutamine-responsive, thereby triggering inappropriate insulin release even at low glucose.

In conclusion, this study demonstrates that GDH is essential for sustained insulin release evoked by glucose. The lack of GDH prevents glucose-induced glutamate generation, with transaminases failing to play a compensatory role. Provision of cellular glutamate is sufficient to restore the full development of glucose-stimulated insulin secretion, indicating that permissive levels of glutamate are required in the amplifying pathway.

MATERIALS AND METHODS

Generation and genotyping of β-cell–specific GDH knockout mouse (β*Glud1*$^{-/-}$)

Female transgenic mice containing the exon 7 of *Glud1* flanked by two lox/P sites, previously generated in the lab (Carobbio *et al.*, 2009), were crossed with male mice expressing Cre recombinase under the control of the rat insulin promoter (Rip-Cre mice; Herrera, 2000), in order to achieve the recombination of GDH specifically within β-cells. Animals were maintained on a mixed (C57BL/6J × 129/Sv) genetic background. Both male and female animals were studied for some representative parameters, and control islets were isolated from nonknockout mice from the same litters. Mouse breeding and handling was carried out in our local certified animal facility according to procedures that were approved by the animal care and experimentation authorities of the Canton of Geneva. Transgenic animals were genotyped by PCR on genomic DNA extracted from tail biopsies (Carobbio *et al.*, 2009).

Pancreatic islet isolation and adenoviral treatment

Mouse pancreatic islets were isolated by collagenase digestion (collagenase P; Roche, Rotkreuz, Switzerland), as described

previously (Pralong *et al.*, 1994), and were cultured free-floating in RPMI-1640 medium supplemented with 5% (vol/vol) heat-inactivated fetal calf serum, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, and 50 µM 2-mercaptoethanol. For rescue experiments, control islets were infected with Ad-lacZ, and ectopic expression of *hGLUD1* was achieved by transducing islets isolated from β*Glud1*$^{-/-}$ mice with the recombinant adenovirus overexpressing GDH (pAd-GDH), as detailed previously (Carobbio *et al.*, 2004). Transductions were done by infection of islets on the day of isolation with 1 µl of purified virus/1 ml of media for 2 h (corresponding to approximately 40 plaque-forming units per cell), and islets were used the next day for secretion assays. Transient correction of GDH deficiency did not induce apparent changes in islet morphology.

Insulin secretion and measurements

For static incubations, mouse islets were maintained for 2 h in glucose-free and glutamine-free RPMI-1640 medium and then washed in Krebs Ringer bicarbonate HEPES buffer (KRBH, containing [in mM]: 135 NaCl, 3.6 KCl, 10 HEPES, pH 7.4, 5 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, 0.1% bovine serum albumin) containing basal (2.8 mM) glucose concentration. Then batches of 40–50 islets were handpicked and incubated for 1 h at 37°C with the indicated secretagogues. At the end of the assay period, islets were put on ice, and the supernatant was collected to measure insulin secretion by radioimmunoassay (Linco, St. Charles, MO). Values were expressed per number of islets.

For islet perifusions, 10 handpicked islets were each put in a 250-µl chamber and thermostated at 37°C (Brandel, Gaithersburg, MD). The flux was set at 0.5 ml/min, and fractions were collected every min after a 30-min washing period at basal 2.8 mM glucose. Islets were then stimulated for 15 min with the indicated secretagogues. At the end of the assay period, supernatants were collected to measure insulin. All compounds used for insulin secretion were obtained from Sigma-Aldrich (Buchs, Switzerland).

For in situ pancreatic perfusion, the pancreas was perfused ex vivo in anesthetized mice as described previously (Maechler *et al.*, 2002) with a 1.5 ml/min perfusion rate.

Measurement of amino acid levels

After stimulation with the different secretagogues to measure insulin secretion, islets were resuspended in 35% (wt/vol) 5-sulfosalicylic acid to extract amino acids. Following overnight incubation at 4°C, samples were frozen at –80°C, and amino acid measurements were done by reverse-phase high-performance liquid chromatography (HPLC) after derivatization with *O*-phthalaldehyde (Bustamante *et al.*, 2001).

[U-^{13}C]glucose incubations

Islets were isolated, cultured, and preincubated as described above. Batches of 300 islets were then transferred to new tubes containing KRBH-BSA buffer with 22.8 mM [U-^{13}C]glucose and incubated for 1 h at 37°C. The incubation was terminated by putting islet tubes in ice-cold water before removal of media and islet extraction using 70% vol/vol ethanol. The extracted islets were lyophilized, reconstituted in water, and derivatized with *N*-methyl-*N*-(tert-butyldimethylsilyl)trifluoroacetamide (Sigma-Aldrich) in the presence of 15% *N*′-*N*-dimethyl formamide modified after the method of Mawhinney *et al.* (1986) before GC-MS analysis of isotopic enrichment in glutamate and aspartate. The GC-MS system consisted of a Shimadzu GC-2010 gas chromatograph linked to a Shimadzu GC–MS-Q2010plus mass spectrometer (Shimadzu Corporation, Tokyo, Japan). The percent labeling was corrected for natural abundance of the isotope by subtracting the mass distribution of a standard containing the relevant metabolites. The quantification of double labeling (M+2) was calculated as percent of the pool of metabolite being unlabeled.

Ca^{2+} measurements

After isolation, mouse islets were kept overnight in RPMI-1640 medium before being transferred onto glass coverslips; this was followed by another overnight culture to let islets settle and adhere. Islets were then loaded with 3 µM Fura2-AM (Molecular Probes, Eugene, OR) for 1 h in 2.8 mM glucose KRBH at 37°C and washed before transfer to the microscope (Nikon Eclipse Ti, Egg, Switzerland). Ratiometric measurements of fura-2 fluorescence were performed with 340/380-nm filters for excitation and a 510-nm filter for emission, and the signal was acquired using MetaMorph system software (Molecular Devices, Downingtown, PA).

Statistical analysis

Statistics were done using the SPSS 15.0 statistical package (SPSS, Chicago, IL). Unless indicated, data are represented as the means ± SE for at least three independent experiments performed in triplicate. Differences between β*Glud1*$^{-/-}$ and control were assessed by the two-tailed unpaired *t* test for single comparison or by one-way analysis of variance analysis, using a post hoc multiple comparison procedure (Fischer's least-significant difference method). Results were considered statistically significant at $p < 0.05$.

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