SIDT2 is involved in the NAADP-mediated release of calcium from insulin secretory granules

Guoying Chang¹, Rui Yang¹, Yanan Cao², Aifang Nie², Xuefan Gu¹ and Huiwen Zhang¹

¹Department of Pediatric Endocrinology and Genetic Metabolism, Xinhua Hospital, Shanghai Institute for Pediatric Research, Shanghai Jiao Tong University School of Medicine, Shanghai, China

²Shanghai Clinical Center for Endocrine and Metabolic Diseases, Shanghai Key Laboratory for Endocrine Tumors, Rui-Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Correspondence
should be addressed
to H Zhang or X Gu
Email
huiwenzhang@yahoo.com or
gu_xuefan@163.com

Abstract

The *Sid2* global knockout mouse (*Sid2*^{−/−}) has impaired insulin secretion. The aim of this study was to assess the role of SIDT2 protein in glucose-induced insulin secretion in primary cultured mouse β-cells. The major metabolic and electrophysiological steps of glucose-induced insulin secretion of primary cultured β-cells from *Sid2*^{−/−} mice were investigated. The β-cells from *Sid2*^{−/−} mice had normal NAD(P)H responses and K_{ATP} and K_V currents. However, they exhibited a lower [Ca²⁺]_i peak height when stimulated with 20 mM glucose compared with those from WT mice. Furthermore, it took a longer time for the [Ca²⁺]_i of β-cell from *Sid2*^{−/−} mice to reach the peak. Pretreatment with ryanodine or 2-aminoethoxydiphenyl borate (2-APB) did not change [Ca²⁺]_i; the response pattern to glucose in *Sid2*^{−/−} cells. Extraordinarily, pretreatment with bafilomycin A1 (Baf-A1) led to a comparable [Ca²⁺]_i increase pattern between these two groups, suggesting that calcium traffic from the intracellular acidic compartment is defective in *Sid2*^{−/−} β-cells. Bath-mediated application of 50 nM nicotinic acid adenine dinucleotide phosphate (NAADP) normalized the [Ca²⁺]_i response of *Sid2*^{−/−} β-cells. Finally, glucose-induced CD38 expression increased to a comparable level between *Sid2*^{−/−} and WT islets, suggesting that *Sid2*^{−/−} islets generated NAADP normally. We conclude that *Sid2* is involved in NAADP-mediated release of calcium from insulin secretory granules and thus regulates insulin secretion.

Key Words

- ▶ insulin secretory granule
- ▶ *Sid2*
- ▶ Ca²⁺
- ▶ NAADP
- ▶ CD38

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Introduction

Lysosomal proteomic analyses suggest that, in addition to the well-known lysosomal membrane proteins, e.g. LAMP1, LAMP2, some less-abundant proteins without clear physiological function reside on lysosomal membranes, including SIDT2 (Bagshaw *et al.* 2005, Chapel *et al.* 2013, Zhang *et al.* 2007). As a member of the SID1 transmembrane family (Elhassan *et al.* 2012), SIDT2 was presumed to be

a small RNA membrane transporter. However its exact function has not been explored experimentally.

Currently, no known human diseases are caused by *SIDT2* gene mutations. To explore its pathophysiological function, we generated a global knockout *Sid2*^{−/−} mouse model, which exhibited age-dependent increased plasma glucose levels and impaired glucose tolerance (Gao *et al.*

2013). Islets from *Sidt2*^{-/-} mice cultured *in vitro* produced less insulin when stimulated with a high concentration of glucose or a depolarizing concentration of KCl.

Under physiological conditions, glucose-induced insulin release depends on rapid glucose uptake and metabolism in the pancreatic β -cells, which results in increased cytosolic ATP/ADP, sequential closure of ATP-sensitive potassium channels (K_{ATP}) and depolarization of the cell membrane (Bratanova-Tochkova *et al.* 2002, Eliasson, *et al.* 1997, Gylfe *et al.* 2000). Such depolarization activates membrane voltage-gated Ca^{2+} channels and allows extracellular Ca^{2+} to enter the cytoplasm, which in turn induces Ca^{2+} release from intracellular stores. The resulting rise in $[Ca^{2+}]_i$ then triggers insulin secretory granule (ISG) exocytosis. Abnormalities in intracellular Ca^{2+} signals have been associated with impaired insulin secretion in both rodent and human pancreatic β -cells (Islam 2010).

Ca^{2+} -induced Ca^{2+} release (CICR) from intracellular stores is a critical step during insulin secretion in both INS cell line and mouse pancreatic β -cells (Kang *et al.* 2005, Kang and Holz 2003, Roderick *et al.* 2003, Varadi and Rutter 2004). Acidic organelles such as lysosomes and ISGs are the physiological sources of intracellular free Ca^{2+} (Christensen *et al.* 2002, Suckale and Solimena 2010), which are mobilized in response to the second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) (Galione *et al.* 2009, Kinnear *et al.* 2004, Yamasaki *et al.* 2004). Local Ca^{2+} release from the acidic stores is then amplified by ER Ca^{2+} release via a CICR-dependent manner (Patel *et al.* 2001). However, the NAADP receptor remains elusive (Calcraft *et al.* 2009, Morgan and Galione 2014, Wang *et al.* 2012). Two-pore channels (TPCs) were proposed as the long sought-after NAADP receptor in the intracellular acidic compartment (Calcraft *et al.* 2009, Ruas *et al.* 2015). However, study by other groups has suggested that TPCs are NAADP-independent highly selective Na^+ channels (Cang *et al.* 2013, Wang *et al.* 2012). More study on this pathway is imperative.

The aim of this study was to investigate the role of SIDT2 during glucose-induced insulin secretion. We show that SIDT2 is localized in ISGs, where it participates in NAADP-mediated ISG calcium release, playing a critical role in regulating glucose homeostasis.

Materials and methods

Animals

Generation of the *Sidt2*^{-/-} mice was described previously (Gao *et al.* 2013). All mice were housed in Animal Laboratory Center at Xinhua hospital, Shanghai, China. The Institutional Review Ethics Board of Xinhua Hospital approved all procedures involving mice. Male mice aged 6 months age were used in this study.

Islet isolation and primary cell culture

The islets were isolated as described previously (Lacy and Kostianovsky 1967). Briefly, islets were incubated in 0.05 mg/ml trypsin without phenol red for 3 min at 37°C. The cells were then centrifuged, and re-suspended in RPMI 1640 supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Single cells were then spread onto coverslips pre-treated with poly-L-lysine (15–30 kDa; Sigma). The cells began to form monolayers after 48 h of culture.

Sidt2 RNA analysis

Total RNA was extracted from the pancreatic islets. The forward and reverse primers of pair 1 were located within exons 17 and 18, respectively. Qualitative real-time RT-PCR (qPCR) using pair 1 was performed to analyze the residual RNA content. The forward and reverse primers of pair 2 were designed against regions in exons 2 and 5, respectively, whereas those of pair 3 were in exons 1 and 6. To confirm the deletion of exon 2 of *Sidt2* gene at the mRNA level, RT-PCR was performed using primer pairs 2 and 3. Amplicons from primer pair 3 were directly sequenced. The sequences of three primer pairs were shown in Table 1.

Western blotting analysis

A total of about 200 isolated islets were lysed in 100 μ l 1×RIPA buffer supplemented with protease inhibitors

Table 1 Oligonucleotide primers used to amplify *Sidt2* cDNA

Primers	Forward primer	Reverse primer	Length
Primer1	CTTCAACAACATCCTCAGCAAC	ATGGCGTAAAACAGACCAAAGT	161 bp
Primer2	GTGAATGTCCTGAACAAGCAGA	CTTGACAATTACCGAGTCCACA	360 bp
Primer3	AGCGAGCTGGTCAACATCTAC	GGTACATGCCAATGAAGGCTA	535 bp

(Beyotime, Shanghai, China). Total proteins were extracted and separated using a 10% SDS-PAGE gel followed by wet transfer onto PVDFs. The primary antibodies used were goat polyclonal anti-SIRT2 (1:300 dilution; Santa-Cruz Biotechnology), rabbit anti-CD38 (1:500 dilution; Novus Biologicals, Littleton, CO, USA), and mouse monoclonal anti-tubulin (1:1000 dilution; Beyotime). Signals were detected using HRP-conjugated secondary antibodies and ECL Detection Kit (Beyotime).

Immunofluorescence staining on pancreas sections and isolated primary β -cells

Five-micrometer paraffin-embedded pancreas sections were stained as described previously (Gao *et al.* 2013). Isolated primary β -cells were fixed with 4% paraformaldehyde for 20 min, permeabilized for 20 min in 0.2% Triton X-100 buffer, blocked with PBS containing 5% BSA, and subjected to double immunofluorescence staining. They were first incubated with anti-SIRT2 (1:200) and anti-insulin antibodies (1:100 dilution, Cell Signaling Technology). Then they were incubated with Alexa Fluor 488 donkey-anti-rabbit IgG and Alexa Fluor 594 chicken-anti-goat IgG (1:200 dilution, Molecular Probes). Images were visualized by confocal microscopy (Leica 710, Mannheim, Germany). The co-localization of SIRT2 with insulin was analyzed by Image Pro Plus 6.0 and expressed as Pearson's correlation coefficient, where P values > 0.5 indicated a significant correlation.

Assessment of NAD(P)H using a fluorimetric assay and an enzymatic recycling method

Suspension of dispersed islet cells (10^6 cells/ml) was in 96-well plates in a temperature controlled chamber in Krebs-Ringer Bicarbonate Buffer (KRBB) supplemented with 3 mM glucose. NAD(P)H fluorescence (340 nm excitation, 420 nm emission) was measured using a Hitachi F-2000 fluorescence spectrophotometer. The fluorescence intensity was represented as arbitrary units (AU).

Batches of 100 islets were incubated in 20 mM glucose for 10 min, and then extracted using RIPA buffer. The aliquots were heated at 60°C for 10 min to eliminate both NAD and NADP. NADPH levels were then determined by the enzymatic recycling method using NAD(P)H Assay Kit (AAT Bioquest, Sunnyvale, CA, USA). The mean changes in NADPH were expressed as a percentage of unstimulated batches.

Electrophysiological measurements of K_{ATP} and K_v currents in single islet β -cells

Whole-cell patch-clamp recordings in dispersed β -cells were conducted using a patch-clamp amplifier EPC10 (HEKA Elektronik, Lambrecht, Germany) as reported previously with some modification (Deng *et al.* 2014). The β -cells were identified by a cell capacitance > 5 pF (Barg *et al.* 2000). Stimulation and recordings were controlled by PULSE software. The patch electrodes were fabricated from borosilicate glass and had a resistance of 2.5–5 M Ω while filled with internal recording solution. Capacitive transients and series resistance errors were minimized before recording. All experiments were carried out at room temperature (22–25°C).

K_{ATP} currents were recorded by applying 10 mV hyper- and depolarizing voltage pulses (duration, 200 ms; pulse interval, 2 s) from a holding potential of -70 mV using the perforated patch whole-cell configuration. The extracellular solution consisted of (in mM) 138 NaCl, 5.6 KCl, 2.6 CaCl₂, 1.2 MgCl₂, and 5 HEPES (pH 7.4 with NaOH) and was supplemented with glucose as indicated. The pipette solution consisted of (in mM) 76 K₂SO₄, 10 KCl, 10 NaCl, 1 MgCl₂, 5 HEPES (pH 7.35 with KOH), and 0.24 mg/ml of the pore-forming antifungal agent amphotericin B. The voltage clamp was considered satisfactory when the series conductance was stable and greater than 35 nS.

K_v currents were recorded by a series of 10 mV voltage steps to the potentials between -40 and $+60$ mV from the holding potential -70 mV using standard whole-cell patch-clamp recording mode. The extracellular solution consisted of (in mM) 135 NaCl, 5.4 KCl, 1 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 3 glucose (pH 7.4 with NaOH). The pipette solution consisted of (in mM) 140 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 EGTA, and 3 MgATP (pH 7.3 with KOH).

[Ca²⁺]_i recording in single islet β -cells

[Ca²⁺]_i was measured by placing the coverslip on the stage of IX71 inverted microscope (Olympus) (Deng *et al.* 2014). The single islet cells were loaded with 4 μ M Fura 2-AM (Sigma) for 30 min at 37°C in KRBB containing 3 mM glucose, then they were washed with KRBB, and stimulated with 20 mM glucose. The single cells were illuminated by excitation at 340 nm (F340) and 380 nm (F380) using a monochromator (Till Photonics, Munich, Germany), and the emission signals were detected at 510 nm using an image-intensifying CCD camera (SensiCam, PCO, Kelheim, Germany). The images were

collected at 10 s intervals. The $[Ca^{2+}]_i$ was expressed as the ratio of F340/F380 (AU). The $[Ca^{2+}]_i$ peak height upon stimulation was calculated as the ratio of difference between the highest $[Ca^{2+}]_i$ value and the baseline. The time to the peak $[Ca^{2+}]_i$ (T_{peak}) was defined as a period from the point when the stimulation was applied to the point when the highest $[Ca^{2+}]_i$ value was obtained. The maximal rate of Ca^{2+} rise was defined as the max velocity increase in the $[Ca^{2+}]_i$ curves ($\max.d[Ca^{2+}]_i/dt$). Calcium channel blockers, including ryanodine and 2-APB, and the vacuolar H^+ -ATPase inhibitor Baf-A1 were purchased from Gene Operation (Ann Arbor, MI, USA). NAADP was obtained from Sigma. Calcium-free KRBB was prepared by omitting $CaCl_2$ from its composition. All the drugs were applied through a superfusion system.

Measuring the pH in ISGs and fibroblast lysosomes

The islet β -cells were stained with 2 μ M LysoSensor Green DND-189 (Molecular Probes) in 1 ml KRBB for 30 min at 37°C. Green fluorescence that accumulated in acidic organelles was imaged by Leica 710 confocal microscopy

(excitation 443 nm, emission 505 nm). The fluorescence intensity was also represented as AU.

Primary cultured fibroblasts isolated from mouse tail tip were loaded with the same pH indicator to measure the pH of lysosomes. The signals were observed by Leica 710 confocal microscopy and were detected using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using FloJo version 7.6.1 (Treestar, Ashland, OR, USA).

Statistical analysis

Results are expressed as means $\pm$ s.d. An independent-samples *t* test was used to evaluate differences between groups. Differences were statistically significant when $P \leq 0.05$.

Results

Expression and localization of SIDT2 in pancreatic β -cells

SIDT2 expression was observed in WT islets using pair 1, and RNA expression was reduced by 70% in *Sid2*^{-/-}

Figure 1

Analysis of SIDT2 RNA and protein expression in mouse islets. (A) qPCR analysis of *Sid2* expression. (B) RT-PCR products amplified using primer pairs 2 and 3. (C) Sequencing of the amplicon obtained with primer pair 3. (D) Western blotting of SIDT2 expression in INS-1 cells and islets. (E) Immunostaining of SIDT2 and insulin in mouse pancreatic islets (scale bars = 10, 10, and 5 μ m in the upper, middle, and lower panels, respectively). (F) Immunofluorescence images of SIDT2 and insulin in a β -cell from WT mouse (scale bar = 5 μ m).

islets (Fig. 1A). The *Sid2* gene-specific product obtained using pair 2 was amplified from the WT islets (Fig. 1B, lane 2), but not from *Sid2*^{-/-} islets (Fig. 1B, lane 3). The amplicon from *Sid2*^{-/-} islets using pair 3 (Fig. 1B, lane 5) was shorter than that obtained from WT islets (Fig. 1B, lane 4, WT, 535 bp; *Sid2*^{-/-}, 413 bp). Direct sequencing of the primer pair 3 amplicon confirmed the deletion of exon 2 in the *Sid2*^{-/-} islets, which was presumed to cause a premature stop codon at amino acid 83 of SIRT2 protein (Fig. 1C). Western blotting analysis revealed that SIRT2 was expressed endogenously in INS-1 cells and WT islets, whereas its expression was almost completely lost in *Sid2*^{-/-} islets (Fig. 1B). The SIRT2 signals colocalized with the insulin granules of β -cells in WT islets (Fig. 1E), as well as in single β -cells (Fig. 1F). The calculated Pearson's value for SIRT2 with insulin was 0.86 in isolated primary β -cells.

Normal NAD(P)H responses to glucose in *Sid2*^{-/-} β -cells

To assess whether SIRT2 deficiency affected glucose metabolism in β -cells, the NAD(P)H autofluorescence response to 20mM glucose was measured. The patterns of NAD(P)H responses to 20mM glucose in WT and *Sid2*^{-/-} β -cells were similar (Fig. 2A). Moreover, the autofluorescence levels under 3mM or 20mM glucose were comparable between these two groups ($n=10$ islets for each group, $P>0.05$, Fig. 2B). NAD(P)H increase to 20mM glucose in *Sid2*^{-/-} β -cells was not different from WT (50.1 ± 7.6 AU vs 49.8 ± 6.3 AU, WT vs *Sid2*^{-/-}, $P>0.05$, cells from 6 mice per group).

In addition, an enzymatic recycling method was used to measure the changes in NADPH in islet extracts. In WT islets, NADPH levels rose by $59\pm9.3\%$ after the addition of 20mM glucose. Similarly, a $62\pm13.6\%$ increase was observed in *Sid2*^{-/-} mouse islets (WT vs *Sid2*^{-/-}, $P>0.05$, $n=300$ islets from 6 mice per group, Fig 2C and Fig. 2D).

These data indicate that mitochondrial glycolytic function was normal in *Sid2*^{-/-} mouse islets.

Normal K_{ATP} and K_v currents in single β -cells from *Sid2*^{-/-} mice

The K_{ATP} current amplitudes were stable in 3mM glucose, which were inhibited markedly by 20mM glucose in both groups (Fig. 3A and Fig. 3B). The maximum steady-state outward currents were reduced by $65.1\pm4.13\%$ in WT and $59.3\pm7.9\%$ in *Sid2*^{-/-} β -cells on perfusion with 20mM glucose ($P>0.05$, $n=15$ cells).

K_v channels in pancreatic β -cells negatively regulate Ca^{2+} entry and insulin secretion (Su *et al.* 2001). The outward voltage-dependent K^+ currents in *Sid2*^{-/-} β -cells were similar to those in WT β -cells, and could be antagonized by 20mM glucose (Figs 3C and Fig. 3E). The peak outward K_v currents were reduced from 1127.4 ± 97.6 pA/pF (3mM glucose) to 459.2 ± 60.6 pA/pF (20mM glucose) in WT β -cells ($P<0.01$, $n=20$ cells), and from 1159.7 ± 95.8 to 544.4 ± 51.1 pA/pF in *Sid2*^{-/-} β -cells ($P<0.01$, $n=20$ cells). The reduction in K^+ current by 20mM glucose was statistically significant in both groups when the membrane potential was clamped at 0mV ($n=20$ cells, Figs 3D and Figs 3F).

Attenuated and delayed glucose-stimulated Ca^{2+} response of *Sid2*^{-/-} β -cells

Upon stimulation with 20mM glucose, the $[Ca^{2+}]_i$ in WT β -cells increased rapidly to plateau levels. In contrast, the $[Ca^{2+}]_i$ rise was impaired in *Sid2*^{-/-} β -cells (Fig. 4A). The peak height of $[Ca^{2+}]_i$ in *Sid2*^{-/-} β -cells was decreased by 45.3% compared with WT (92.9 ± 9.5 AU vs 169.8 ± 16.4 AU, $P<0.01$, $n=20$ cells, Fig. 4B). Moreover, The time to the peak of $[Ca^{2+}]_i$ (T_{peak}) was longer in *Sid2*^{-/-} β -cells compared with WT (4.8 ± 0.9 min vs 1.1 ± 0.25 min,

Figure 2

Glucose-induced NAD(P)H changes in *Sid2*^{-/-} β -cells. (A) The representative curves for NAD(P)H autofluorescence. (B) Quantification of NAD(P)H fluorescence (β -cells from 6 mice per group). (C) NADPH responses to 20mM glucose compared with the basal levels at 3mM glucose with the enzymatic method ($n=300$ islets from 6 mice per group). (D) Quantification of the NADPH responses to 20mM glucose compared with the basal levels at 3mM glucose ($n=300$ islets from 6 mice per group). $\blacktriangle P>0.05$, $**P<0.01$.

Figure 3

Single β -cell K_{ATP} and K_v currents induced by 20 mM glucose. (A and B) Representative recordings of whole-cell K_{ATP} currents. Representative traces of K_v currents in (C) (WT) and (E) (*Sidd2*^{-/-}). The current-voltage relationship curves for the peak currents are plotted for WT and *Sidd2*^{-/-} in (D) and (F), respectively ($n=20$ cells for each group). * $P < 0.05$, ** $P < 0.01$.

$P < 0.01$, $n=20$ cells), and the maximal rate of $[Ca^{2+}]_i$ rise in *Sidd2*^{-/-} β -cells was smaller than WT (97.6 ± 11.7 AU/min vs 293.1 ± 8.6 AU/min, $P < 0.01$, $n=20$ cells).

Ca^{2+} response to 50 mM KCl was also impaired in cultured β -cells from the *Sidd2*^{-/-} mice (Fig. 4C). The Ca^{2+} peak height was 640.6 ± 10.9 AU vs 299.1 ± 14.6 AU in WT vs *Sidd2*^{-/-} β -cells, respectively ($P < 0.01$, $n=20$ cells).

Next we investigated where was the exact defective position of calcium signaling in *Sidd2*^{-/-} β -cells on glucose stimulation. As shown in Fig. 4D, $[Ca^{2+}]_i$ increased slowly to a low level in Ca^{2+} -free buffer (peak height, 53.7 ± 16.5 AU, $n=10$) upon stimulation of 20 mM glucose in WT β -cells. However, the rise in $[Ca^{2+}]_i$ was very low in *Sidd2*^{-/-} β -cells (peak height, 3.6 ± 1.8 AU, $n=10$) and was nearly abolished (WT vs *Sidd2*^{-/-}, $P < 0.01$), suggesting that the defect in $[Ca^{2+}]_i$ increase in *Sidd2*^{-/-} β -cells was caused by reduced Ca^{2+} mobilization from intracellular calcium stores.

We then treated the β -cells with inhibitors of intracellular Ca^{2+} channels. Both 100 μ M ryanodine (an inhibitor

of ER ryanodine receptors (RyRs)) and 50 μ M 2-APB (an inhibitor of ER inositol trisphosphate receptors (IP3Rs)) dramatically reduced the glucose-stimulated Ca^{2+} response in WT and *Sidd2*^{-/-} β -cells (Fig. 4E and Fig. 4F). There were no differences in the extent of $[Ca^{2+}]_i$ reduction by the ER blockers in both groups (ryanodine, $54.9 \pm 7.6\%$ vs $49.3 \pm 5.2\%$, $P > 0.05$; 2-APB, $53.8 \pm 6.3\%$ vs $47.5 \pm 5.4\%$, $P > 0.05$, $n=20$, WT vs *Sidd2*^{-/-}, Fig. 4G). These data indicate that there was no defect in Ca^{2+} trafficking from ER stores via RyRs or IP3R during glucose-induced CICR in *Sidd2*^{-/-} β -cells. Therefore, the release of Ca^{2+} from intracellular stores other than the ER might be impaired in *Sidd2*^{-/-} β -cells.

Treatment of cells with 3 μ M Baf-A1, an inhibitor of vacuolar H^+ -ATPase, induced a transient increase in $[Ca^{2+}]_i$ in both groups (peak height, 74.1 ± 2.1 AU in WT and 71 ± 8.3 AU in *Sidd2*^{-/-}, $P > 0.05$, $n=15$, Figs 4H and Fig. 4I). Subsequent stimulation with 20 mM glucose induced a similar $[Ca^{2+}]_i$ increase in both groups (peak height, 94.8 ± 8.5 AU vs 87.7 ± 7.2 AU, WT vs *Sidd2*^{-/-}, $P > 0.05$, $n=15$) (Fig. 4H and Fig. 4I). When Baf-A1

Figure 4

[Ca²⁺]_i in β -cells from *Sidd2*^{-/-} mice. (A) [Ca²⁺]_i responses of β -cells to 20 mM glucose. (B) Quantification of [Ca²⁺]_i peak height, $n=20$ cells for each group. (C) [Ca²⁺]_i response of β -cells to 50 mM KCl. (D) [Ca²⁺]_i responses in the absence of extracellular Ca²⁺. (E) [Ca²⁺]_i of β -cells pre-incubated with 100 μ M ryanodine. (F) [Ca²⁺]_i of β -cells pre-incubated with 50 μ M 2-APB. (G) Quantification of β -cell [Ca²⁺]_i decrements pre-incubated with ryanodine or 2-APB, $n=20$ cells. (H) [Ca²⁺]_i in β -cells pre-incubated with 3 μ M Baf-A1 and then stimulated with 20 mM glucose. (I) Summary of Baf-A1 effect on Ca²⁺ release from acidic compartments and [Ca²⁺]_i increase evoked by 20 mM glucose, $n=15$ cells. $\blacktriangle P > 0.05$. $**P < 0.01$.

A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0227>.

depleted the calcium stores in the acidic compartments, the glucose-induced Ca²⁺ release from the ER and other stores was comparable between *Sidd2*^{-/-} and WT β -cells. This suggests that the attenuated [Ca²⁺]_i increase in *Sidd2*^{-/-} β -cells was due to impaired Ca²⁺ release from acidic stores.

The pH of ISGs and lysosomes

The intracellular acidic organelles of β -cell, including ISGs and lysosomes, contain high levels of calcium, and their calcium contents are affected by pH (Christensen *et al.* 2002, Suckale and Solimena 2010). In 3 mM glucose, the granular pH-dependent fluorescence of

Figure 5

Measuring the pH in ISGs and lysosomes. (A) Live cell fluorescent images of pH using LysoSensor DND-189 in β -cells from *Sidd2*^{-/-} and WT mice. (B) The representative curves of LysoSensor-189 signals in β -cells from *Sidd2*^{-/-} and WT mice and qualification of fluorescence intensity ($n=20$ cells for each group). (C) Live cell fluorescent images of the lysosomal pH using LysoSensor DND-189 in fibroblasts from *Sidd2*^{-/-} and WT mouse. (D) Flow cytometric analysis of the fluorescence intensity of LysoSensor DND-189 in fibroblasts, $\blacktriangle P > 0.05$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0227>.

β -cells was stable for 3 min. The mean fluorescence intensity of WT and *Sidd2*^{-/-} ISGs was 103.85 ± 15.75 AU and 100.67 ± 13.43 AU, respectively ($n=20$ cells, $P > 0.05$). Stimulation with 20 mM glucose caused a rapid and monophasic increase of fluorescence intensity in both WT and *Sidd2*^{-/-} β -cells (52.2 ± 6.7 AU vs 54.2 ± 9.1 AU, respectively, $P > 0.05$, $n=20$, Figs 5A and 5B). These data indicate that SIDD2 deficiency had no effect on the acidification of ISGs.

Similarly, no difference was obtained in the lysosomal pH of *Sidd2*^{-/-} fibroblasts compared with WT, which was observed by confocal microscopy (Fig. 5C) and assessed by flow cytometer (785 ± 72.1 AU vs 798 ± 31.8 AU, WT vs *Sidd2*^{-/-}, $P > 0.05$, Fig. 5D).

Efficacy of NAADP treatment in *Sidd2*^{-/-} β -cells

It was reported that NAADP-induced calcium signals were not observed in extracellular calcium-free conditions (Kim *et al.* 2008), which is similar to our observation that the rise in $[Ca^{2+}]_i$ in response to glucose was nearly abolished in *Sidd2*^{-/-} β -cells without extracellular calcium. Therefore, we assessed the effect of NAADP on *Sidd2*^{-/-} β -cells. The peak height of $[Ca^{2+}]_i$ in *Sidd2*^{-/-} β -cells in the presence of 50 nM NAADP was comparable with WT β -cells without NAADP to the stimulus of 20 mM glucose (156.9 ± 12.1 AU vs 169.8 ± 16.4 AU, *Sidd2*^{-/-} vs WT, $n=10$ cells, $P > 0.05$); the respective T_{peak} was also comparable (1.0 ± 0.3 min vs 1.1 ± 0.3 min, $n=10$ cells, $P > 0.05$). Thus,

Figure 6

Efficacy of NAADP treatment in *Sidt2*^{-/-} β-cells and CD38 expression. (A) Representative trace of $[Ca^{2+}]_i$ in *Sidt2*^{-/-} β-cells upon stimulation with 20 mM glucose in the presence of 50 nM NAADP. (B) $[Ca^{2+}]_i$ responses to 50 nM NAADP in the presence of 12 mM glucose. (C) Western blotting of glucose-induced CD38 expression in *Sidt2*^{-/-} and WT islets. (D) Qualification of glucose-induced CD38 expression. ** $P < 0.01$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0227>.

bath-mediated application of NAADP rescued $[Ca^{2+}]_i$ response of *Sidt2*^{-/-} β-cells to glucose (Fig. 6A).

In the presence of 12 mM glucose, stimulation with 50 nM NAADP induced a comparable $[Ca^{2+}]_i$ response in both the WT and *Sidt2*^{-/-} groups (peak height, 112.9 ± 6.5 AU vs 111.5 ± 8.6 AU, WT vs *Sidt2*^{-/-}, $P > 0.05$, $n = 10$) (Fig. 6B).

CD38 expression in WT and *Sidt2*^{-/-} islets

Since the above data implied reduced $[Ca^{2+}]_i$ increase in *Sidt2*^{-/-} β-cells was related with NAADP signaling, we investigated whether NAADP generation was deficient in *Sidt2*^{-/-} β-cells. CD38 is located in endosomes, secretory granules, and lysosomes. It is an ADP-ribosyl cyclase responsible for endogenous NAADP generation in β-cells (Kim *et al.* 2008). As shown in Figs 6C and 6D, glucose-induced CD38 expression increased by $27 \pm 9.1\%$ in WT and $32.3 \pm 10.8\%$ in *Sidt2*^{-/-} β-cells ($P > 0.05$), suggesting that NAADP production was normal in *Sidt2*^{-/-} β-cells upon glucose stimulation.

Discussion

Previous proteomic studies identified some lysosomal-associated membrane proteins such as LAMP2, Syntaxin7, VAMP8, and Cathepsins L1, D, and F are expressed in ISGs (Brunner *et al.* 2007, Schwartz *et al.* 2012). The current study demonstrated that SIDT2, which was identified previously in mouse/rat liver lysosomes, was also endogenously expressed in mouse islet β-cells, and that it co-localized with ISGs.

The β-cells in rodent models have been estimated to contain ~10,000 ISGs, which corresponds to 12–20% of the total cell volume (PM 1973). ISGs contain the highest calcium concentrations among all intracellular compartments. Compared with the total calcium concentrations in the cytoplasm of β-cells, ISGs contain two-fold higher

levels of calcium (Norlund *et al.* 1987). Only a small number of lysosomes are present in the islet β-cells (Barg *et al.* 2001), and the lysosomal volume of mouse islet β-cells was only 3–5% of that of ISGs (Schnell Landstrom *et al.* 1991). Therefore, the impaired Ca^{2+} response in *Sidt2*^{-/-} β-cells likely results mainly from defects in calcium release from the ISG stores.

NAADP is the second messenger that mobilizes Ca^{2+} from acidic organelles. NAADP-mediated Ca^{2+} release plays a key role in triggering glucose-induced Ca^{2+} signaling events (Arredouani *et al.* 2010). However, the identity of the NAADP receptor channel in acidic organelles remains elusive. Recent data have indicated that neither TPCs nor the transient receptor potential channel mucolipin 1 (TRP-ML1) is the NAADP receptor (Lin-Moshier *et al.* 2012, Zhang *et al.* 2011). Bath-application of extracellular NAADP has been shown to induce intracellular calcium increase, at least partially through increase in intracellular NAADP (Djerada and Millart 2013, Singaravelu and Deitmer 2006). In the current study, the bath-application of NAADP corrected the impaired $[Ca^{2+}]_i$ increase in *Sidt2*^{-/-} β-cells, suggesting that SIDT2 exerts its function on the upstream of NAADP-mediated calcium release from acidic store. In other words, SIDT2 may regulate NAADP production in ISGs or function as a modulator to the NAADP-regulated Ca^{2+} channel.

In this study, the equal glucose-induced CD38 expression in WT and *Sidt2*^{-/-} islets indicates that the generation of NAADP was not different between the two groups. Based on the localization of SIDT2 on the membranes of intracellular acidic compartments and its nine transmembrane domain structures (Jialin *et al.* 2010), and taken together the results of this study, we propose that SIDT2 may be required to assembly with TPCs to form a functional Ca^{2+} -release channel, or function as a modulator to the NAADP-regulated Ca^{2+} channel in ISGs.

In conclusion, this study demonstrated that SIDT2 was expressed in mouse pancreatic β-cells, and co-localized

predominantly with ISGs. It plays an important role in regulating NAADP-mediated ISG calcium release and glucose-induced insulin secretion.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

All authors contributed to the study conception and design. G C and R Y researched the data. Y C and A N helped the electrophysiological recording of cells. G C and H Z wrote the paper. H Z and X G designed the project.

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