Heterozygous Inactivation of the Na/Ca Exchanger Increases Glucose-Induced Insulin Release, β-Cell Proliferation, and Mass

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OBJECTIVE—We have previously shown that overexpression of the Na-Ca exchanger (NCX1), a protein responsible for Ca2+ extrusion from cells, increases β-cell programmed cell death (apoptosis) and reduces β-cell proliferation. To further characterize the role of NCX1 in β-cells under in vivo conditions, we developed and characterized mice deficient for NCX1.

RESEARCH DESIGN AND METHODS—Biologic and morphologic methods (Ca2+ imaging, Ca2+ uptake, glucose metabolism, insulin release, and point counting morphometry) were used to assess β-cell function in vitro. Blood glucose and insulin levels were measured to assess glucose metabolism and insulin sensitivity in vivo. Islets were transplanted under the kidney capsule to assess their performance to revert diabetes in allooxan-diabetic mice.

RESULTS—Heterozygous inactivation of Ncx1 in mice induced an increase in glucose-induced insulin release, with a major enhancement of its first and second phase. This was paralleled by an increase in β-cell proliferation and mass. The mutation also increased β-cell insulin content, proinsulin immunostaining, glucose-induced Ca2+ uptake, and β-cell resistance to hypoxia. In addition, Ncx1+/− islets showed a two- to four-times higher rate of diabetes cure than Ncx1+/− islets when transplanted into diabetic animals.

CONCLUSIONS—Downregulation of the Na/Ca exchanger leads to an increase in β-cell function, proliferation, mass, and resistance to physiologic stress, namely to various changes in β-cell function that are opposite to the major abnormalities seen in type 2 diabetes. This provides a unique model for the prevention and treatment of β-cell dysfunction in type 2 diabetes and after islet transplantation.

The prevalence of type 2 diabetes is progressing in an alarming way in most regions of the world (1,2). Type 2 diabetes is a complex disease characterized by insulin resistance and β-cell dysfunction. One of the earliest abnormalities occurring in this disease is the alteration in pulsatile insulin release with the suppression of the first phase of insulin response to glucose (3). The second phase of insulin release is also diminished and a number of abnormalities of continuous insulin release have been observed (4,5). In addition to a defect in β-cell function, a reduction in islet and β-cell mass has been observed (6,7). This reduction could be related to increased programmed cell death (apoptosis), to decreased β-cell replication, or both (8).

In a previous work, we observed that overexpression of the Na/Ca exchanger (isoform 1: Na-Ca exchanger [NCX1]), a protein responsible for Ca2+ extrusion from cells (9,10), increased β-cell apoptosis and reduced β-cell proliferation (11). The increase in apoptosis resulted from endoplasmic reticulum (ER) Ca2+ depletion with resulting ER stress (11). The increase in apoptosis resulted from endoplasmic reticulum (ER) Ca2+ depletion with resulting ER stress (11).

If it is possible to increase apoptosis and to decrease β-cell proliferation by increasing the activity of NCX1, it may be possible to obtain the opposite effects by down-regulating such a mechanism. To test this hypothesis, we generated Ncx1 heterozygous deficient mice (Ncx1+/−). Our data show that Ncx1+/− heterozygous inactivation induces several β-cell modifications, including an increase in glucose-induced insulin release and in β-cell proliferation and mass. Ncx1+/− islets also displayed an increased resistance to hypoxia, and when transplanted in diabetic animals, showed a two- to four-times higher rate of diabetes cure than Ncx1+/− islets.

RESEARCH DESIGN AND METHODS

Generation of Ncx1+/− mice. Exon 11 of the murine Ncx1 gene (GenBank, accession number AF109080) was cloned from a 129/Sv genomic phage library. The first 206-bp were amplified by PCR and a BanHI site was introduced for cloning. A targeting vector was constructed by inserting the neomycin resistance cassette into the targeted gene. The targeting vector was linearized with BstXI and injected into the 129/Sv strain. Embryonic stem cells were transfected, selected, and analyzed for homologous recombination. The targeted allele was confirmed by PCR and Southern blot analysis. Homozygous Ncx1−/− mice were bred with wild-type mice to generate the heterozygous Ncx1+/− mice for all subsequent studies.

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Received 1 July 2010 and accepted 19 April 2011.

Diabetes 2011;60:1–8. doi:10.2337/db10-0924

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-0924/-/DC1.

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resistance cassette (neo) into the BamHI restriction site of that amplified exon. Standard procedures were used to generate Ncx1HETEROZYGOUS INACTIVATION IN β-CELLS

The ImageJ (National Institutes of Health, Bethesda, MD) image analysis program. described (24). Individual etry of insulin-peroxidase immunostained pancreatic sections, as previously described (25). The duration of hypoxia was 6 h. Viability of -cell apoptosis, the terminal deoxynucleotidyltransferase mediated dUTP nick-end labeling (TUNEL) method was used, with the In Situ Cell Death Detection Kit (Roche Diagnostics, Vilvoorde, Belgium). The method for -cell labeling and counting was similar to that for -cell proliferation, apoptosis, mass, and size. To measure -cell proliferation, mice were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO; 100 mg/kg body wt) 6 h before euthanasia, as previously described (21). An alternative method to measure -cell replication was the KI67 labeling method (rabbit monoclonal anti-KI67, Vector Laboratories, Brussels, Belgium) with a method similar to the BrdU immunofluorescence labeling (22).

To measure -cell apoptosis, the terminal deoxynucleotidyltransferase mediated dUTP nick-end labeling (TUNEL) method was used, with the In Situ Cell Death Detection Kit (Roche Diagnostics, Vilvoorde, Belgium). The method for -cell labeling and counting was similar to that for -cell proliferation.

Cell viability in vitro was measured using Hoechst 33342 (Ho3342) and propidium iodide (PI) (23). The percent viability in Ncx1–/– single -cells and islets (not exposed to thapsigargin or cyclosporaxid) was 65% to 70% and 85% to 95%, respectively. In some experiments, cytokines were used at the following concentrations: human IL-1β: 50 units/mL (R&D Systems, Oxon, UK); mouse interferon-γ: 1000 units/mL (tebu-bio, Boechout, Belgium).

Quantification of -cell mass was performed by point-counting morphometry of insulin-peroxidase immunostained pancreatic sections, as previously described (24). Individual -cell size was measured using the calibrated ImageJ (National Institutes of Health, Bethesda, MD) image analysis program. The -cell area of the pancreatic section was divided by the number of -cell nuclei identified in the area.

In vitro hypoxia studies. In vitro hypoxia studies were as previously described (25). The duration of hypoxia was 6 h. Viability of -cells was measured as described above.

Glucose metabolism. Glucose metabolism and insulin sensitivity, serum glucagon, growth hormone, and glucagon-like peptide 1 measurement in vivo. The measurement of glucose metabolism and insulin sensitivity in vivo were done as previously described (26,27). Serum glucagon, growth hormone, and glucagon-like peptide 1 (GLP-1) were measured using Glucagon Human/Mouse/Rat ELISA Kit (Alpeco, Salem, NH), Rat/Mouse Growth Hormone ELISA Kit (Millipore, St. Charles, MO), and Mouse GLP-1 ELISA kit (Antibodies-online.com, Aachen, Germany).

Diabetes induction and islets transplantation. Diabetes was induced in 10- to 12-week-old C57BL6N mice using a single intravenous injection of alloxan (90 mg/kg; Sigma) (25,26). Grafts of 50 to 400 islets from Ncx1+/+ or Ncx1–/– mice were transplanted under the kidney capsule in diabetic mice. Thereafter, the nonfasting blood glucose levels were measured in each animal up to 100 days, using a Glucometer (Abbott, Diegem, Belgium). Islet grafts were considered functional when the nonfasting blood glucose returned to normoglycemic levels (<220 mg/dL). Some animals, the graft-bearing kidney was removed to confirm islet graft function.

Statistics. The results are expressed as means ± SEM. The statistical significance of differences between data was assessed by using ANOVA, followed by the Tukey post-test.

RESULTS

Generation of Ncx1–/– mice. A mutant allele was constructed as described in RESEARCH DESIGN AND METHODS (Fig. IA). After electroporation with the targeting vector, the recombinant embryonic stem cell clones were identified by DNA hybridization and used to produce chimeric mice (Fig. 1B). Transmission of the mutant allele produced Ncx1 heterozygous mice that were mated to generate Ncx1 null mutants (Ncx1–/–). The Ncx1–/– mice were not viable and died during embryogenesis as described (28). Heterozygous and WT mice genotyping from tail biopsy specimen DNA revealed an Ncx1 WT amplicon of ~250 bp and an Ncx1 recombinant allele of ~100 bp, which were simultaneously amplified by PCR (Fig. 1C). Ncx1 mRNA levels in batches of islets from Ncx1+/+ and Ncx1–/– mice, measured using RT-PCR, showed a clear decrease in Ncx1–/– mice (Fig. 1D). Ncx1–/– mice were viable, fertile, had a normal body weight, and showed no macro- or microscopic abnormalities at the level of the brain, heart, lung, liver, spleen, stomach, intestine, and thymus (Supplementary Fig. 1). Likewise, serum levels of glucagon, growth hormone (GH) in the fasting state and GLP-1 in the fed state were comparable in Ncx1+/+ and Ncx1–/– mice at 12 weeks (0.48 ± 0.04 vs. 0.41 ± 0.14 ng/mL, 2.4 ± 0.44 vs. 1.67 ± 0.44 ng/mL, and 3.20 ± 0.28 vs 2.86 ± 0.15 pg/mL, respectively; n = 4 in each case, except for GH where n = 9–11, respectively, P > 0.05).

Na/Ca exchange activity in Ncx1–/– islets. To evaluate the functional consequences of Ncx1 heterozygous inactivation, the activity of the Na/Ca exchanger was evaluated by measuring the effect of extracellular Na+ removal on 45Ca uptake and cytosolic free Ca2+ concentration ([Ca2+]cytosol), and of K+-induced membrane depolarization on [Ca2+]cytosol. Removal of extracellular Na+ increased 45Ca uptake in Ncx1+/+ and Ncx1–/– islets (Fig. 1E), the uptake in Ncx1–/– islets recorded in the absence of extracellular Na+ being half of that measured in Ncx1+/+ islets. Removal of extracellular Na+ induced a sustained increase in [Ca2+]cytosol (Fig. 1F), that again was reduced in Ncx1–/– compared with Ncx1+/+ islets. Thus, the increase in [Ca2+]cytosol induced by the absence of extracellular Na+, measured as the area under the curve over the baseline value during a 5- to 25-min period was reduced by 24% in Ncx1–/– compared with Ncx1+/+ islets (P < 0.05). K+ increased [Ca2+]cytosol in both types of islets (Fig. 1G), but [Ca2+]cytosol was always slightly higher in Ncx1–/– than in Ncx1+/+ mice. The difference in mean concentration was higher (30%) during the period of K+ exposure (min 2–12) and of K+ removal (min 12–24) than during the baseline period (min 0–2; P < 0.001), indicating that Ca2+ extrusion was mainly impaired during the period of K+ stimulation and recovery. Taken as a whole, these data indicate that partial Ncx1 heterozygous inactivation reduces Na/Ca exchange activity in islet cells both in its forward and reverse mode. We then examined the effect of Ncx1 heterozygous inactivation on islet function.

Islet function in Ncx1+/– islets

Insulin release. Figure 2A and B shows the effect of an increase in glucose concentration from 2.8 to 11.1 mmol/L on insulin release from perfused islets (A: representative experiment, B: mean of 4–6 experiments). In Ncx1+/– islets, glucose induced an oscillatory increase in insulin...
Glucose-induced increase in \([\text{Ca}^{2+}]_i\). Oscillatory insulin release is associated with oscillatory \([\text{Ca}^{2+}]_i\) increases (29,30). Figure 2C and D shows the effect of 11.1 mmol/L glucose on \([\text{Ca}^{2+}]_i\) oscillations in intact islets. Ncx1+/− islets showed a rapid increase in \([\text{Ca}^{2+}]_i\), with an initial phase, followed by a second phase displaying regular oscillations. Ncx1+/− islets also showed a rapid increase in \([\text{Ca}^{2+}]_i\), but the initial phase was more distinct, separated from the second phase, which displayed disrupted \([\text{Ca}^{2+}]_i\) oscillations. Figure 2E and F shows at a larger scale the oscillations

release. In Ncx1+/− islets, glucose induced a marked first-phase insulin release, followed by a progressive increase with less clear oscillations. The amount of insulin released during the entire period of stimulation (16–60 min) was about 2.4-times higher in Ncx1+/− than in Ncx1+/+ islets (\(P < 0.02\)). A similar observation was made at 16.7 mmol/L glucose, where the fold-increase was 1.7 (\(P < 0.05\)), or by perifusing a single islet, where the fold-increase in response to 11.1 mmol/L glucose was 2.6 (\(P < 0.05\), Supplementary Figs. S2 and S3, respectively).
during the period 14–16 min. The number of oscillations is easily determined and counted in Ncx1+/- islets, at variance with Ncx1+/+ islets, where the oscillations are irregular and difficult to identify. PSD analysis of the oscillations showed that Ncx1+/- islets displayed a single peak with a very stable generator in frequency and magnitude, whereas Ncx1+/+ islets displayed an unstable dominant frequency and numerous peak intrusions above the frequency of 0.10 Hz.
illustrating the irregularity of the oscillations (Fig. 2G and H). Supplementary Figure S4A and B also shows that there was a significant difference both in the magnitude of the peak of power spectra (Max PSD) and in the crest factor (ratio Max/Integral) between Ncx1+/− and Ncx1+/− islets.  

**Glucose metabolism.** To stimulate insulin release, glucose must be metabolized by the pancreatic β-cell (31). In Ncx1+/− and Ncx1+/− islets, the utilization of d-[5-3H]glucose increased as a result of the rise in the glucose concentration from 2.8 to 16.7 mmol/L (Fig. 3A). At the low hexose concentration, \(^{1}H_{2}O\) generation from d-[5-3H]glucose was slightly higher in Ncx1+/− than in Ncx1+/− cells. Generation of \(^{13}CO_{2}\) from d-[U-14C]glucose was also increased in response to the rise in glucose concentration in both type of islets (Fig. 3B). However, although oxidation tended to be higher in Ncx1+/− islets at the low glucose concentration, glucose oxidation was significantly lower in Ncx1+/− than in Ncx1+/+ cells at the high glucose concentration. As a result of these differences, the paired ratio between d-[U-14C]glucose oxidation and d-[5-3H]glucose utilization increased with the rise in glucose concentration in Ncx1+/− cells (P < 0.05), but it did not change in Ncx1+/− cells. Compared with Ncx1+/− cells, ATP generation tended to be increased in Ncx1+/− islets at low (53%) but reduced at high glucose concentration (−20%, P < 0.05, Supplementary Table 1).  

**Uptake of \(^{45}Ca\) in response to glucose.** Glucose-induced insulin release is preceded by Ca\(^{2+}\) uptake in the β-cell (32). In Ncx1+/− and Ncx1+/− islets, 16.7 mmol/L glucose stimulated \(^{45}Ca\) uptake (Fig. 3C). Whereas at low glucose, there was no significant difference in \(^{45}Ca\) uptake between the two types of islet; the increase in uptake induced by 16.7 mmol/L glucose was twice as high in Ncx1+/− than in Ncx1+/− islets.  

**Insulin content.** In view of the major increase in insulin release in Ncx1+/− islets, the insulin content of the islets was measured and was about double that of Ncx1+/− islets (Fig. 3D).  

**Morphology.** There were no differences in islet morphology or in the position of β-, α-, and δ-cells between Ncx1+/− and Ncx1+/− islets (data not shown). Proinsulin staining, however, was increased in Ncx1+/− compared with Ncx1+/− islets (Fig. 4A) of adult mice (12–14 weeks), a finding in line with the increase in insulin content. Electron microscopic analysis revealed no difference in the features of pancreatic β-cells, including secretory granules, nucleus, mitochondria,
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A

NCX1+/+

NCX1−/−

B

NCX1+/+

NCX1−/−

C

NCX1+/+

NCX1−/−

FIG. 4. Effect of Ncx1 heterozygous inactivation on islet morphology. A: Immunohistochemistry: proinsulin labeling (immunoperoxidase in brown) is stronger in Ncx1−/− than in Ncx1+/+ islets. Representative images of six 12-week-old mice pancreas sections in each case. Inset: Same image at higher magnification (original magnification ×63). B: Transmission electron microscopy. Groups of 30 islets from Ncx1+/+ and Ncx1−/− mice were fixed, and three sections in each block demonstrating an islet were cut at randomly chosen depths. The characteristic ultrastructural features of pancreatic β-cells were comparable in both types of islets. Representative images are shown of β-cells from 12-week-old mice Ncx1+/+ (left panel) and Ncx1−/− islets (right panel). Scale bar = 2 μm. C: Representative images of pancreatic β-cells immunolabeled with immunoperoxidase (brown) using a polyclonal anti-insulin antibody, with hematoxylin used for the counterstain. The immunolabeled with immunoperoxidase (brown) using a polyclonal anti-insulin antibody, with hematoxylin used for the counterstain. Immature morphometry represent 12-week-old mice pancreatic sections of Ncx1+/+ (left panel) and Ncx1−/− (right panel). Scale bar = 0.2 μm. (A high-quality digital representation of this figure is available in the online issue.)

ER, and Golgi apparatus of Ncx1+/+ and Ncx1−/− adult mice (Fig. 4B). Quantitative analysis of relative volume of organelles demonstrated no significant difference between Ncx1+/+ and Ncx1−/− β-cells (data not shown).

As a whole, the data so far presented show that Ncx1 heterozygous inactivation strongly increases glucose-induced insulin production and release.

β-Cell mass, size, proliferation, and viability. β-Cell mass, size, and proliferation was measured in young (4 weeks) and adult (12 weeks) mice (Fig. 5A–C). As expected, β-cell mass was increased at 12 weeks in both types of islet, although the increase was of much larger magnitude in Ncx1−/− than in Ncx1+/+ islets (8.8- vs. 1.6-fold increase, respectively, P < 0.001; Fig. 4C). This increase was not due to β-cell or islet hypertrophy because no change in β-cell and islet size was observed between Ncx1+/+ and Ncx1−/− mice (Fig. 5B and data not shown); rather, it was due to an increase in the β-cell proliferation rate. As expected, β-cell proliferation, as measured using BrdU, was decreased at 12 weeks compared with 4 weeks (Fig. 5C), although the decrease was of a lower magnitude in Ncx1−/− than in Ncx1+/+ islets (−40 vs. −85%, P < 0.01). As a result, a 5.25-times higher proliferation rate was observed at 12 weeks in Ncx1−/− compared with Ncx1+/+ mice (Fig. 5C and Supplementary Fig. S5). We also observed an increase in proliferation rate of the exocrine pancreatic cells that was lower than in the endocrine pancreas (1.7- vs. 5.25-fold increase, respectively; P < 0.02, Supplementary Fig. S5). Ki67 staining showed an increased labeling in Ncx1−/− compared with Ncx1+/+ islets, with the number of positive nuclei averaging 2.75 ± 0.31 vs. 1.58 ± 0.28 per islet, respectively (P < 0.01).

Aptosis of β-cells was also measured, but no difference could be found between Ncx1−/− and Ncx1+/+ islets. Thus, whether using the TUNEL method at 4 and 12 weeks under basal conditions (Fig. 5D), or Ho342 and PI staining of islet cells (Fig. 5E–G) under basal or stimulated conditions created by the absence or presence of sarcoendoplasmic reticulum Ca2+ ATPase (SERCA) inhibitors (Fig. 5E), and 72-h exposure of intact islets to cytokines (Fig. 5F) or thapsigargin (Fig. 5G), no difference could be found between both types of islets.

Hypoxia studies. To test their resistance to physiologic stress, the islets were then subjected to hypoxia for 6 h, cell death being measured using Ho342 and PI staining of intact islets. Figure 5H shows that 71% of Ncx1+/+ islets showed a decrease in viability <60% compared with 45% of Ncx1−/− islets.

Transplantation. We then transplanted Ncx1−/− islets under the kidney capsule of alloxan-diabetic mice to examine their performances compared with Ncx1+/+ islets. First, we had to determine the minimum number of Ncx1+/+ islets to transplant to cure diabetes, which must be ~300 islets. Indeed, all transplantations with 400 islets were successful, with nephrectomy leading to a reincrease in glycemia (Supplementary Fig. S6A), whereas the rate of success of transplantation of 200 Ncx1−/− islets was only 2/5 (Supplementary Fig. S6B). In comparison, the rate of success of transplantation of 100 Ncx1−/− islets was 4/5 (Supplementary Fig. S6C), whereas the rate of success of transplantation of 50 Ncx1−/− islets was 2/3 (Supplementary Fig. S5D). This suggests that the Ncx1−/− islets are at least four- to seven-times more efficient to cure diabetes than the Ncx1+/+ islets. Finally, we performed a last series of parallel transplantations of 100 islets (Fig. 6A and B). The success rate was 4/5 and 2/5 for Ncx1+/+ and Ncx1−/− islets, indicating that the Ncx1+/+ islets are at least twice as efficient in curing diabetes than Ncx1−/− islets.

Morphometric analysis of the islet grafts after nephrectomy of mice which received a transplant of 100 islets showed that the relative volume of islet grafts was about seven-times higher in cured than in noncured animals (4.26 ± 1.09% vs. 0.61 ± 0.39%, respectively [n = 4–6]; P < 0.05), confirming that diabetes cure is attributable to islet transplantation.

Glucose metabolism and insulin sensitivity in vivo. Despite the major increase in insulin release seen in vitro, plasma glucose and insulin levels were comparable in Ncx1+/+ and Ncx1−/− mice both in the fasted and the fed state (Fig. 6C and E). However, the glucose-tolerance test showed an increased and earlier initial peak of insulin release (Fig. 6D and F), with a subsequent faster decrease in glucose levels during the ensuing 45 min (∼152 ± 20 compared with −81 ± 2 mg/dL in Ncx1+/+ and Ncx1−/− mice, respectively, P < 0.01). The intraperitoneal insulin sensitivity test showed no difference between the mice (Supplementary Fig. S7).
FIG. 5. Effect of Ncx1 heterozygous inactivation is shown on β-cell mass (A), size (B), proliferation rate (C), and apoptosis (D) between weeks 4 and 12 in Ncx1+/+ and Ncx1+/− mice. Mean ± SEM values are shown from five and six pancreas specimens, respectively. A: ***P < 0.001 vs. values at 4 weeks; #P < 0.001 vs Ncx1+/+ β-cells at 12 weeks. C: **P < 0.01; ***P < 0.001 vs respective value at 4 weeks; #P < 0.01 vs Ncx1+/+ islets at 12 weeks. Cell viability was measured in isolated islet cells (E) and in intact islets (F, G, H) using Hoe342 and PI staining in the absence or after 72-h exposure to thapsigargin (Thaps) or cyclopiazonic acid (CPA; E), cytokines (Cyt; F), and after 6-h exposure to hypoxia (H). Mean ± SEM values are shown from four individual experiments. E: *P < 0.05 vs. control in the absence of cytokines. F: *P < 0.05 vs. respective Ncx1 islets in the absence of Thaps; G: *P < 0.05 vs. Ncx1+/+ islets.
In a previous study we showed that NCX1 overexpression increased β-cell apoptosis and decreased β-cell proliferation by depleting ER Ca\(^{2+}\) stores (11). Therefore, we wondered whether the opposite changes seen in the current study in Ncx1\(^{+/2}\) mice were not due to an increase in ER Ca\(^{2+}\) stores. To test this hypothesis, the effect of the SERCA inhibitor thapsigargin on \([\text{Ca}^{2+}]_i\), in pancreatic islets was examined (Supplementary Fig. S8). In Ncx1\(^{+/+}\) islets, thapsigargin induced an important but transient increase in \([\text{Ca}^{2+}]_i\), a phenomenon that was increased by about 60% in Ncx1\(^{+/2}\) islets (\(P < 0.05\)), indicating that ER Ca\(^{2+}\) stores were increased in Ncx1\(^{+/2}\) compared with Ncx1\(^{+/+}\) islets.

**DISCUSSION**

We generated a mouse strain with heterozygous inactivation of the gene coding for NCX1, the isoform of the Na/Ca exchanger that is expressed in the pancreatic β-cell (33). RT-PCR studies confirmed heterozygous inactivation of the exchanger in Ncx1\(^{+/2}\) mice and the reduction of its expression in β-cells. The latter reduction led to a decrease in NCX1 activity as assessed at the physiologic...
level, with inhibition of both modes of the exchanger (forward and reverse). The reduction in Na/Ca exchange activity in Ncx1<sup>−/−</sup> β-cells had multiple consequences:

First, as expected from cells from which Ca<sup>2+</sup> extrusion is reduced, the rise in [Ca<sup>2+</sup>]<sub>i</sub> induced by membrane depolarization and the uptake of 45Ca induced by glucose were increased.

Second, the [Ca<sup>2+</sup>]<sub>i</sub> oscillations induced by the sugar were disrupted. This was not unexpected, because the exchanger generates an inward current when working in its forward mode that contributes to the duration of the bursts of electrical activity (34) that underlie [Ca<sup>2+</sup>]<sub>i</sub> oscillations (35). Hence, the reduction of the NCX1 inward current in Ncx1<sup>−/−</sup> cells led to the shortening and disorganization of the oscillations. However, in view of the decreased rate of ATP generation at high glucose in Ncx1<sup>−/−</sup>-compared with Ncx1<sup>+/+</sup>-islets, the disruption of the Ca<sup>2+</sup> oscillations could also be due to an attenuation of the metabolic oscillations that underlie the slow electrical and Ca<sup>2+</sup> oscillations via a relief of phosphofructokinase inhibition by ATP (37).

Third, glucose-induced insulin release was markedly increased. Both phases of insulin release were enhanced, which could also be related to the reduction in Ca<sup>2+</sup> extrusion with subsequent increase in cellular Ca<sup>2+</sup> content as objectified at the ER level. In view of this major increase in insulin release, we next examined insulin content, islet morphology, and glucose metabolism.

Insulin content was doubled in Ncx1<sup>−/−</sup>-compared with Ncx1<sup>+/+</sup>-β-cells, but no change in morphology was found between the cell types except for an increase in pro-insulin staining in Ncx1<sup>−/−</sup>-β-cells, a finding compatible with the increase in insulin content. In Ncx1<sup>−/−</sup>-β-cells, there was a tendency toward an increase in glucose utilization at low glucose concentration and a significant decrease in glucose oxidation at high glucose concentration, making it unlikely that the observed increase in glucose-induced insulin release in these islets is explained by an augmented mitochondrial glucose metabolism. The increase in the insulin secretory rate may result instead from increased mitochondrial glucose metabolism, making it unlikely that the observed increase in glucose content is the reason for the increased insulin release.

We next looked at β-cell mass, size, proliferation, and death. At 12 weeks (adult mice), β-cell mass had increased 100% in Ncx1<sup>−/−</sup>-compared with Ncx1<sup>+/+</sup>-mice, a phenomenon that was not due to an increase in β-cell size but rather to an increase in β-cell proliferation, the rate of which was five-times higher in Ncx1<sup>−/−</sup>-mice than in Ncx1<sup>+/+</sup>-mice. The view that a decrease in β-cell death could also contribute to this observation was examined, but the results obtained were negative except in the case of hypoxia. Indeed, although measurement by the TUNEL method showed no difference in the rate of apoptosis under basal conditions and no difference in viability between single cells or islets of Ncx1<sup>−/−</sup> and Ncx1<sup>−/−</sup>-mice under basal and stimulated conditions, Ncx1<sup>−/−</sup>-islets showed a 37% protection against hypoxia compared with Ncx1<sup>+/+</sup>-islets.

The increase in β-cell function, growth, and resistance to the physiologic stress of Ncx1<sup>−/−</sup>-islets was confirmed in transplantation studies, the Ncx1<sup>−/−</sup>-islets being at least twice as efficient in curing diabetes than the Ncx1<sup>+/+</sup>-islets. In this respect, it is important to know that islets transplantation represents a valuable approach in the treatment of diabetes. However, its applicability is limited by the need to transplant a high number of islets from two or more donors. In clinical islet transplantation, it has been estimated that up to 70% of the transplanted β-cell mass is destroyed in the early post-transplant period due to non-immune-mediated physiologic stress, namely prolonged hypoxia during the revascularization process (38).

The increase in insulin content, β-cell proliferation, and mass seen in Ncx1<sup>−/−</sup>-islets could result from the activation by raised cellular Ca<sup>2+</sup> of the calcineurin/nuclear factor of activated T-cell (NFAT) signaling pathway. Calcineurin is a calmodulin-dependent serine/threonine phosphatase that dephosphorylates the cytoplasmic sub-units of NFAT (NFATc) upon activation by Ca<sup>2+</sup>. Dephosphorylation of NFATc allows its rapid translocation to the nucleus, with subsequent activation of insulin transcription and promotion of β-cell proliferation by increasing the expression of cell cycle promoters such as cyclin D1, cyclin D2, and cyclin-dependent kinase 4 (CDK4) (39). β-Cell-specific calcineurin inactivation in knockout mice impaired insulin transcription and cyclins expression, leading to markedly decreased β-cell proliferation and mass and to diabetes onset (40). Interestingly, calcineurin inactivation did not increase β-cell apoptosis (39), a finding compatible with the absence of a decrease in β-cell apoptosis observed in this study.

Last, except for a higher initial peak of insulin release during the glucose tolerance test, no difference was found in vivo glucose metabolism or in insulin resistance between both mice.

In conclusion, heterozygous inactivation of a single gene (Ncx1) leads to an increase in insulin release, β-cell proliferation and mass, and to an increase in resistance to β-cell death, namely, to various changes in β-cell function that are opposite to the major abnormalities seen in type 2 diabetes. Moreover, it increased the success rate of islet transplantation in diabetic animals. Downregulation of the β-cell Na/Ca exchanger is thus a unique model providing a novel concept for the prevention and treatment of type 2 diabetes and to improve the applicability of islet transplantation.

**Note added in proof.** During the process of submitting the present work for publication, another study showed that KB-R7943, an NCX1 inhibitor, enhanced glucose-induced insulin release, a finding confirming part of our results (41).

**ACKNOWLEDGMENTS**

This work has been supported by grants from the Belgian Fund for Scientific Research (FRSM 3.4593.04, 3.4527.08), the European Foundation for the Study of Diabetes/Novo Nordisk Programme in Diabetes Research (2005/0), the Juvenile Diabetes Research Foundation International (1-2008-536), the Communauté Française de Belgique–Actions de Recherche Concertées (ARC), the European Union (Integrated Project Naitm [FP7] of the European Community), and the Belgium Program on Interuniversity Poles of Attraction initiated by the Belgium State (IUAP P6/40). CIBERDEM is an initiative of Instituto de Salud Carlos III (Spain). A.K.C. is a Research Associate, and J.M.V. is a Research Director of the Fonds National de la Recherche Scientifique. F.A. was supported by a fellowship from the “Crédit de Relations Internationales de l’ULB”. G.J. was supported by a grant from the Fundación Privada IDIBELL.

No potential conflicts of interest relevant to this article were reported.
E.N., S.S., S.B., and N.P. researched data and contributed to discussion. F.A., J.-M.V., and A.S. researched data, contributed to discussion, and reviewed and edited the manuscript. M.M. and M.D. researched data and contributed to discussion. J.M., G.J. researched data and contributed to discussion. E.M., J.R., A.K.C., and D.L.E. researched data, contributed to discussion, and reviewed and edited the manuscript. S.S.c. researched data and contributed to discussion. A.H. researched data, contributed to discussion, and wrote the manuscript.

The authors thank A. Van Praet, A. Iabkriman and M.P. Berghmans (Laboratory of Pharmacology, ULB) for excellent technical support.

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