
DOI: http://dx.doi.org/10.1210/en.2011-2149

© 2012 The Endocrine Society. Paper reproduced here with permission from the publisher.
Increased Expression of the Glucose-Responsive Gene, RCAN1, Causes Hypoinsulinemia, \(\beta\)-Cell Dysfunction, and Diabetes

Heshan Peiris, Ravinarayan Ragupathi, Claire F. Jessup, Mark P. Zanin, Daisy Mohanasundaram, Kimberly D. Mackenzie, Tim Chataway, Jennifer N. Clarke, John Brealey, P. Toby Coates, Melanie A. Pritchard, and Damien J. Keating

Flinders Medical Science and Technology and Centre for Neuroscience (H.P., R.R., C.F.J., M.P.Z., K.D.M., T.C., J.N.C., D.J.K.), Flinders University, Adelaide, Bedford Park SA 5042, Australia; School of Medicine (C.F.J., D.M., P.T.C.), University of Adelaide, Adelaide SA 5005, Australia; Australia Islet Consortium (D.M., P.T.C.), Institute of Medical and Veterinary Sciences (J.B.), Adelaide SA 5000, Australia; and Department of Biochemistry and Molecular Biology (M.A.P.), Monash University, Victoria 3842, Australia

\(RCAN1\) is a chromosome 21 gene that controls secretion in endocrine cells, regulates mitochondrial function, and is sensitive to oxidative stress. Regulator of calcineurin 1 (\(RCAN1\)) is also an endogenous inhibitor of the protein phosphatase calcineurin, the inhibition of which leads to hypoinsulinemia and diabetes in humans and mice. However, the presence or the role of \(RCAN1\) in insulin-secreting \(\beta\)-cells and its potential role in the pathogenesis of diabetes is unknown. Hence, the aim of this study is to investigate the presence of \(RCAN1\) in \(\beta\)-cells and identify its role in \(\beta\)-cell function.

\(RCAN1\) is expressed in mouse islets and in the cytosol of pancreatic \(\beta\)-cells. We find \(RCAN1\) is a glucose-responsive gene with a 1.5-fold increase in expression observed in pancreatic islets in response to chronic hyperglycemia. The overexpression of the human \(RCAN1.1\) isoform in mice under the regulation of its endogenous promoter causes diabetes, age-associated hyperglycemia, reduced glucose tolerance, hypoinsulinemia, loss of \(\beta\)-cells, reduced \(\beta\)-cell insulin secretion, aberrant mitochondrial reactive oxygen species production, and the down-regulation of key \(\beta\)-cell genes. Our data therefore identifies a novel molecular link between the overexpression of \(RCAN1\) and \(\beta\)-cell dysfunction. The glucose-responsive nature of \(RCAN1\) provides a potential mechanism of action associated with the \(\beta\)-cell dysfunction observed in diabetes. (\textit{Endocrinology} 153: 5212–5221, 2012)

Diabetes is a metabolic disorder characterized by elevated fasting blood glucose, which arises when insulin-secreting \(\beta\)-cells cannot provide adequate insulin in the face of increasing insulin resistance. The pancreatic \(\beta\)-cell is the central regulator of glucose homeostasis and the primary source of endogenous insulin. Two major forms of diabetes exist, type 1 and type 2. Type 1 diabetes results from the autoimmune destruction of \(\beta\)-cells, whereas type 2 diabetes is associated with obesity, peripheral insulin resistance, and gradual insulin insufficiency. Increased levels of glucotoxicity in \(\beta\)-cells are thought to be central to the pathogenesis of \(\beta\)-cell dysfunction in type 2 diabetes (1), but the exact nature of the molecular mechanisms underlying \(\beta\)-cell dysfunction in the presence of insulin resistance and hyperglycemia is unknown. Candidate genes that might trigger \(\beta\)-cell dysfunction under such circumstances would need to have their expression induced by hyperglycemia and have potential roles in the regulation of \(\beta\)-cell function.

\(RCAN1\) is located on chromosome 21 within the q22.1–q22.2 region. The seven exons within the \(RCAN1\) gene give rise to two regulator of calcineurin 1 (\(RCAN1\))
isoforms, the longer and more abundant RCAN1.1 iso-
form and the shorter and lowly expressed RCAN1.4 iso-
form. Expression of RCAN1 is highest in brain, heart, and
skeletal muscle (2). RCAN1 expression is induced by a
number of stress-associated stimuli including Ca^{2+}, am-
loyd-β and oxidative stress (3). Oxidative stress is pre-
valent in β-cells exposed to hyperglycemia (4), and this is
due to the low expression levels of endogenous anti-
oxidant systems in these cells.

The most well-defined function of RCAN1 is as an in-
hibitor of the phosphatase calcineurin (5–9), the activity of
which is central to normal β-cell function and plasma in-
sulin levels (10). Increased RCAN1 negatively affects the
secretion of catecholamines from adrenal chromaffin cells
(11) and mitochondrial function in neurons (12) as well as
cell proliferation and tissue growth (13, 14). It is worth
comparing these functions with the compromised β-cell
insulin secretion, mitochondrial function, islet growth,
and β-cell proliferation that are all observed in type 2
diabetes (15). Hyperglycemia-induced oxidative stress is
thought to be central to β-cell dysfunction in type 2 dia-
betes (15), and oxidative stress induces RCAN1 expres-
sion (3). The combination of these facts suggests that ab-
errant RCAN1 function in β-cells may have implications
in the pathogenesis of β-cell failure in type 2 diabetes.
Based on its known roles in regulating secretion, mito-
ochondrial function, calcineurin activity, and cell prolif-
eration, we investigated the role of RCAN1 in β-cell func-
tion. Our findings demonstrate that the overexpression of
RCAN1 in mice causes diabetes characterized by hypoin-
sulinemia and β-cell dysfunction via multiple pathways.

Materials and Methods

Animals
RCAN1 transgenic (RCAN1<sup>ox</sup>) mice were generated using
human RCAN1 cDNA encoding the exon 1 splice variant as
described (11). The minigene transgene construct consisted of a
4-kb region up-stream of exon 1 and also contained exons 5–7
and their flanking introns. The minigene DNA was prepared for
microinjection by a double digestion with
and their flanking introns. The minigene DNA was prepared for
4-kb region up-stream of exon 1 and also contained exons 5–7
human RCAN1 cDNA encoding the exon 1 splice variant as
all studies.

Fasting blood glucose measurement
Fasting blood glucose levels were measured at the ages in-
dicated. Before measurement, mice were transferred to a fresh cage
without food for 16 h. All mice had access to water ad libitum
during this period. Samples were obtained at the ages indicated.
Blood was sampled from the tail, and fasting blood glucose levels
were measured using an ACCU-CHEK Performa glucometer
(Roche Diagnostics, Castle Hill, Australia).

Isolation of mouse pancreatic islets
Mice were killed by an anesthetic overdose of isoflurane and
islets isolated as previously described (16).

Quantitative real-time PCR
RNA was extracted from mouse islets using the RNeasy Mini
Kit (QIAGEN, Doncaster, Australia). Real-time PCR was car-
ried out using the QIAGEN Quantitect SYBR Green PCR kit
(QIAGEN, Australia) and RotorGene 3000 thermocycler (Cor-
bett Life Science, Sydney, Australia). All real-time experiments
were carried out using 3 μl of diluted cDNA. The 18s rRNA
primer sets were used as the housekeeping gene to normalize
samples and evaluate changes in expression. A sample with a
no-cDNA template was the negative control. All samples were
run in triplicate. Primer sequences and other information for all
target genes are provided in Supplemental Table 1 (published on
The Endocrine Society’s Journals Online web site at

Immunoblot analysis of RCAN1
Immunoblot analysis of RCAN1 was performed using anti-
RCAN1 antibodies at a dilution of 1:200 (Sigma-Aldrich, St.
Louis, MO). Proteins were extracted in RIPA buffer containing
0.1% SDS using a tissue-lyser (QIAGEN, Australia). Protein
concentration was estimated using the EZQ assay (Invitrogen,
Australia). 25 μg of each sample was separated on a Criterion
TGX stain-free gel (Bio-Rad, Australia) and transferred onto
0.2-μm polyvinylidene fluoride membrane (Roche Applied Sci-
ence, Australia). Blots were probed using a donkey antirabbit-
horseradish peroxidase-conjugated secondary antibody at a di-
lution of 1:100 (Thermo Scientific) and West Pico (Pierce) as
the substrate for chemiluminescent detection. Blots were visualized
on a Fujifilm LAS4000 and Gel Doc Ez-Imagers (Bio-Rad, Aus-
tralia). Densitometry was performed with Carestream Mole-
cular Imaging Software (Carestream Health).

Insulin measurements
Blood insulin levels were measured after 16 h fasting using an
ultrasensitive mouse insulin ELISA kit (Crystal Chem Downers
Grove, IL) according to the manufacturer’s instructions for a
low-range assay (17). Absorbance was measured at 450 and 620
nm using a BIOMEK-3000 microplate reader (Beckman-
Coulter Brea, CA) and MultiMode detection software. For in
vitro glucose-stimulated insulin secretion (GIS), 10 islets were
incubated in 50 μl of Krebs buffer (supplemented with 3 mM
glucose) at 37 C for 1 h (basal secretion). Islets were exposed to
Krebs buffer with 20 mM glucose for 10 min (first-phase GIS)
and 50 min (second-phase GIS). Islets were allowed to recover
for 1 h at 3 mM glucose before being exposed to a high-K⁺ (70
mM) Krebs solution for 20 min. Samples were centrifuged at
250 × g for 1 min, the supernatant removed, and insulin content
measured as described previously. To measure islet insulin content, 10 islets were subjected to 12 freeze-thaw cycles in RIPA buffer and centrifuged at 250 × g for 1 min. The supernatant was diluted 1:1000 in PBS and insulin content measured by ELISA.

**Glucose tolerance test**

Glucose tolerance tests were carried out on mice fasted for 16 h. Glucose (2 mg/g body weight) (PharmLab, Australia) was injected (ip) into mice and blood glucose measured at 15-min intervals from 0–240 min via a tail bleed using an ACCU-CHEK Performa glucometer (Roche Diagnostics, Australia).

**Immunohistochemical labeling in pancreatic sections and MIN6 cells**

Whole pancreatic tissue was fixed in 10% buffered formalin for 24 h after excision from the animal. Tissue was then washed in PBS (pH 7.2) supplemented with sodium azide for 2 h and then washed in running deionized water for 2 h. The tissue was subsequently dehydrated in ethanol (80%, 90%, and 100% for 2 h in each solution) and then transferred to chloroform overnight. The tissue was embedded in Paraplast wax for 1 h (62°C) and fresh wax for 1.5 h and finally embedded in fresh wax. The 5-μm sections were allowed to dry overnight in a 37°C oven. Guinea pig polyclonal antibody to insulin (AbCam, Cambridge, MA) was used diluted 1:3200 in antibody diluent [NaCl, Na2HPO4, polyclonal antibody to insulin (AbCam, Cambridge, MA) was diluted in PBS, the coverslips were mounted onto glass slides in buffered DAPI as described in the previous paragraph. After washing with key antirabbit IgG-Cy5 (Jackson Immunoresearch) (final dilution 1:3200). The cells were washed with PBS and incubated in donkey anti-rabbit IgG-Cy5 (Jackson Laboratories) (final dilution 1:10) and guinea pig 10% normal donkey serum, placed for 24 h at room temperature times for 5 min each). Fixed cells were incubated for 30 min in dimethylsulfoxide (three times for 5 min each), and PBS (four times for 5 min each), 100% ethanol (twice for 5 min each), 80% ethanol, 90% ethanol, and 100% ethanol for 2 h. The tissue was subsequently dehydrated in ethanol (80%, 90%, and 100% for 2 h in each solution) and then transferred to chloroform overnight. The tissue was embedded in ParaPlast wax for 1 h (62°C) and fresh wax for 1.5 h and finally embedded in fresh wax. The 5-μm sections were allowed to dry overnight in a 37°C oven. Guinea pig polyclonal antibody to insulin (AbCam, Cambridge, MA) was used diluted 1:3200 in antibody diluent [NaCl, Na2HPO4, NaHPO4 2H2O, 10% NaN3 in distilled water (pH 7.1)]. Biotin-streptavidin (Jackson Laboratories), and 3 μM 4',6-diamino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen, Australia) was used as a nuclear marker.

MIN6 ß-cells were grown for 24 h on glass coverslips coated with 10 μg/ml poly-D-lysine and 10 μg/ml laminin (Sigma-Aldrich) and in DMEM containing 10% FBS, 1% penicillin-streptomycin (1 mg/ml), 1% l-glutamine, and 3 μl/liter β-mercaptoethanol (Sigma-Aldrich). Cells were fixed for 18–20 h in Zamboni’s fixative at 4°C followed by washes in 80% ethanol (four times for 5 min each), 100% ethanol (twice for 5 min each), dimethylsulfoxide (three times for 5 min each), and PBS (four times for 5 min each). Fixed cells were incubated for 30 min in 10% normal donkey serum, placed for 24 h at room temperature in a humidifier with rabbit monoclonal antibody against RCAN1 (Sigma Aldrich) (final dilution 1:10) and guinea pig monoclonal antibody against insulin (Abcam) (final dilution 1:3200). The cells were washed with PBS and incubated in donkey antirabbit IgG-Cy5 (Jackson Immunoresearch) (final dilution 1:400) and donkey anti-guinea pig IgG tagged with Cy3 and DAPI as described in the previous paragraph. After washing with PBS, the coverslips were mounted onto glass slides in buffered glycerol and fluorescence visualized on a Leica TCS SP5 Spectral confocal microscope.

**Measurement of islet size and ß-cell density**

Islet size was determined by measuring the insulin-positive area of individual islets in paraffin embedded pancreatic sections. ß-cell density of an islet was measured by counting the number of DAPI-positive cells per islet area. Analysis was carried out on ImageJ image analysis software. 8–10 islets were measured from each animal.

**Insulin tolerance test**

Insulin tolerance tests were carried on mice fasted for 1 h, and 0.75 or 1 U/kg body weight insulin (Novo Nordisk, Australia) was injected (ip) into 40- and 120-d-old mice, respectively. Blood glucose was measured via a tail bleed from 0–120 min at 15-min intervals using an ACCU-CHEK Performa glucometer (Roche Diagnostics, Australia).

**Islet reactive oxygen species (ROS) production**

After isolation, islets were allowed to recover overnight in phenol red-free RPMI (10% heat inactivated fetal calf serum). Islets were transferred into media supplemented with 20 or 5 mM glucose (control) for 24 h. Islets were incubated with 5 μM Mitofix Red (Invitrogen, Australia) for 10 min, washed twice in Krebs solution, and visualized on an IX-71 fluorescent microscope (Olympus, Japan). Mean fluorescence intensity of individual islets was calculated using ImageJ image analysis software. Seven to 10 islets were measured from each animal.

**Electron microscopy studies**

The pancreas was cut into cubes of approximately 0.5 mm3 and processed for electron microscopy as reported (18). Sections were examined using a Hitachi H-600 transmission electron microscope (Tokyo, Japan). ß-Cells were distinguished from α- and δ-cells by the presence of their characteristic granules as described in previous studies (18). Insulin secretory vesicle density was calculated by counting the number of large dense-core vesicles (LDCVs) per cell and normalized to cell area. To measure vesicle localization, 200-nm-wide concentric zones starting from the cell membrane were defined within each ß-cell and the number of dense core vesicles within each region counted and presented as a percentage of the total number of vesicles within each cell. Filled secretory vesicles containing insulin were clearly identified by the presence of a dense core within the vesicle. Empty secretory vesicles were identified as being a membrane-bound vesicle in the range of 100–300 nm in diameter, the same size range as that found for filled vesicles. All analysis was carried out on ×4000 electron micrographs using ImageJ image analysis software.

**Statistical analysis**

Parametrically distributed data were analyzed using an unpaired Student’s t tests and for nonparametric data sets a Mann-Whitney U test was used. Statistical significance was P < 0.05. All data are shown as mean ± SEM.

**Results**

**RCAN1 is a glucose-responsive gene present in ß-cells that regulates the expression of islet gene products**

To study the role of RCAN1 in ß-cell function, we confirmed the presence of the RCAN1 gene in mouse pancreatic islets and MIN6 ß-cells (Fig. 1A). Using immunohistochemistry, we also observed a largely cytoplasmic expression of RCAN1 protein in MIN6 ß-cells (Fig. 1B). Chronic high glucose is thought to induce oxidative stress
in β-cells, leading to β-cell dysfunction and β-cell loss in type 2 diabetes. Because oxidative stress induces RCAN1 expression, we hypothesized that chronic high glucose exposure would induce β-cell RCAN1 expression. Using real-time PCR (Fig. 1C) and Western blotting (Fig. 1D), we found that in vitro islet RCAN1 expression is induced by chronic (6 d) hyperglycemia. To study the effect of RCAN1 overexpression on glucose homeostasis and β-cell function, we used mice in which human RCAN1.1 is endogenously expressed in mouse MIN6 β-cells; from left to right, bright-field image of MIN6 cells, insulin (red), RCAN1 (green), and merged image of insulin, RCAN1, and DAPI (blue). C and D, Real-time RT-PCR (C) and Western blot analysis (D) in WT islets illustrates that chronic (6 d) hyperglycemia induces islet RCAN1 expression. E, Real-time RT-PCR was performed on isolated islets from 120-d-old WT and RCAN1ox mice. F, Western blot analysis illustrates RCAN1 expression in WT islets and RCAN1 overexpression in RCAN1ox islets at 40 d (lane 1 and 2) and 120 d (lane 3 and 4). G, Real-time RT-PCR was performed on isolated islets from WT and RCAN1ox mice for β-cell gene products. White bars, WT; black bars, RCAN1ox (n = 5 animals per genotype). *, P < 0.05.
both reduced in RCAN1ox islets. The expression of genes encoding the glucose transporter 2 and the insulin receptor are also reduced. Ins-1 expression is not altered, in agreement with the lack of change in total insulin content we observe in RCAN1ox islets (see Fig. 4B). We also observe an increased expression of the apoptotic markers caspase-3 and caspase-9 in RCAN1ox islets (Fig. 1G).

**RCAN1ox mice develop diabetes**

RCAN1ox mice develop increasingly severe hyperglycemia and overt diabetes from 60 d of age compared with age-matched WT controls (Fig. 2A). This is not due to increased body weight in RCAN1ox mice (Fig. 2B). Glucose tolerance was assessed by ip glucose tolerance tests at two different ages, before and after the onset of hyperglycemia in RCAN1ox mice. Glucose tolerance was unchanged at 40 d (Fig. 2C), but RCAN1ox mice demonstrate impaired glucose tolerance at 120 d (Fig. 2D). The hyperglycemia observed in RCAN1ox mice is likely caused either by increased insulin resistance or by decreased plasma insulin levels. To identify whether such changes occur, we measured insulin resistance in RCAN1ox mice. No significant difference in blood glucose between WT and RCAN1ox mice was observed at any of the time points in response to the injected insulin at both 40 d (data not shown) and 120 d of age (Fig. 2E). However, when we transform these results into relative changes in insulin-induced blood glucose (Supplemental Fig. 1), we see that RCAN1ox mice have increased sensitivity to an exogenous insulin load at 120 d. Thus, RCAN1ox mice may develop an increased response to a given amount of insulin as they age, and RCAN1ox mice clearly do not develop any insulin resistance. The absence of insulin resistance in RCAN1ox mice suggests that hypoinsulinemia may underlie the age-associated diabetes observed in RCAN1ox mice. Consistent with this, circulating insulin levels were significantly lower at 120 d in RCAN1ox mice (Fig. 2F).

**RCAN1 regulates islet morphology**

To identify whether this reduction in circulating insulin could be caused by a loss of β-cells, we stained pancreatic sections for insulin (Fig. 3A) and measured total β-cell mass per islet. Islet size was significantly smaller in RCAN1ox mice at 100 d (P < 0.001) but not at 40 d (Fig. 3B). Islet shrinkage was due to a reduction in total β-cell number per islet (Fig. 3C) rather than decreased β-cell size (Fig. 3D).

**RCAN1 regulates insulin secretion and insulin loading in β-cells**

Given the reductions in circulating insulin levels and β-cell number in RCAN1ox mice, we next wished to identify whether elevated RCAN1 expression affects insulin secretion. Static in vitro islet insulin secretion (normalized to total islet protein content to compensate for variations in islet size) was measured to gauge β-cell secretory capacity. Basal (P < 0.05) and first-phase (10 min, P < 0.05) GSIS were both reduced in RCAN1ox islets, whereas second-phase secretion (10–60 min after glucose stimulation) was not altered (Fig. 4A). The reduction
in secretory capacity was not due to decreased insulin content within pancreatic islets (Fig. 4B). Electron microscopic analysis of β-cells was carried out to investigate potential mechanisms underlying the insulin secretion defect. Electron micrographs of WT and RCAN1ox β-cells clearly identified both filled and empty large vesicles (Fig. 4C). Analysis of these images reveals that the number of empty vesicles (Fig. 4D), but not insulin-containing LDCVs (Fig. 4E), was significantly higher in RCAN1ox β-cells. The proximity of insulin-containing LDCVs to the plasma membrane was unchanged in RCAN1ox β-cells (Fig. 4F). The number of docked vesicles (filled vesicles in contact with the plasma membrane) was also unchanged in RCAN1ox β-cells (Fig. 4G).

Because RCAN1 overexpression caused an increase in empty vesicles, we hypothesized that the filling of LDCVs might be negatively affected in RCAN1ox β-cells. We detected a small (~8%) but significant reduction in the diameter of the dense core (containing insulin) within LDCVs in RCAN1ox β-cells (111.0 ± 1.6 nm) compared with WT β-cells (120.8 ± 1.8 nm, P < 0.01) (data not shown), indicating reduced insulin loading into RCAN1ox LDCVs. This is not due to a concomitant reduction in total LDCV diameter in RCAN1ox β-cells (data not shown).

**RCAN1 affects mitochondrial morphology and function**

Mitochondrial changes and increased ROS production are observed in β-cells from type 2 diabetes patients (15) and upon overexpression in neurons of the *Drosophila melanogaster* RCAN1 ortholog nebula (12). The ultrastructural appearance of mitochondria is unchanged in RCAN1ox β-cells (Fig. 5A). Although the same number of mitochondria were seen in each group (Fig. 5B), mitochondrial size was reduced in RCAN1ox β-cells (P < 0.05, Fig. 5C). Hyperglycemia-induced oxidative stress reduces mitochondrial size (20). We therefore measured mitochondrial ROS production in WT and RCAN1ox islets using the mitochondrial ROS marker MitoSox (Fig. 5D). Basal (3 mM glucose) mitochondrial ROS production was not altered between groups (Fig. 5E). However, we observed a significant increase in mitochondrial ROS production in RCAN1ox β-cells (P < 0.05, Fig. 5F).

**FIG. 3.** RCAN1 regulates islet morphology and β-cell number. A, Pancreatic sections were labeled with an anti-insulin antibody (red) and nuclear marker DAPI (blue) to measure islet size. Scale bar, 50 μm. B–D, Islet size was similar between groups at 40 d but significantly smaller in RCAN1lox mice at 100 d (B) because there are fewer β-cells per islet in RCAN1lox mice (C) and not due to any change in β-cell size at 120 d as measured from electron microscopy images (D). *, P < 0.05; ***, P < 0.001; n = 5–9 animals per genotype. Black bars, RCAN1lox mice; white bars, WT mice.

**FIG. 4.** RCAN1 regulates insulin secretion and vesicle loading in β-cells. A, Insulin secretion is compromised in RCAN1ox β-cells. Basal (3 mM glucose for 1 h) and first-phase (time = 0–10 min in 20 mM glucose) insulin secretion are significantly reduced in RCAN1ox islets, whereas second-phase (time = 10–60 min in 20 mM glucose) secretion is not altered (n = 5 mice sampled in triplicate per group). B, Total insulin content of islets is unaltered (n = 13–15). C, Electron micrographs of a WT (left) and RCAN1ox (right) β-cell demonstrate the presence of both filled and empty (arrows) secretory vesicles within the cytosol of β-cells. D and E, The number of empty secretory vesicles (D) but not filled vesicles (E) is increased in RCAN1ox β-cells. F and G, We did not observe differences in the proximity of insulin-containing vesicles to the plasma membrane (F) or the number of docked vesicles (G) (n = 3–5 animals and 6–8 β-cells per genotype). *, P < 0.05. White bars, WT; black bars, RCAN1ox. Scale bar, 500 nm (C).
ROS production in islets was similar between both groups (Fig. 5E). After exposure to 20 mM glucose, WT islets displayed moderately increased mitochondrial ROS production (\(P < 0.05\)), consistent with previous reports (4). However, glucose-induced increase in mitochondrial ROS production was greater in RCAN1ox islets (\(P < 0.001\), Fig. 5E), illustrating RCAN1-induced mitochondrial dysfunction.

**Discussion**

Normal \(\beta\)-cell function and insulin secretion are essential for the regulation of circulating plasma glucose levels. This current study identifies the RCAN1 gene as being involved in the proper control of glucose homeostasis due to its effect on multiple facets of \(\beta\)-cell function. We further identify RCAN1 as a glucose-regulated gene that has increased expression in islets under glucotoxic conditions. We demonstrate that when RCAN1 expression is increased in vivo in mice, the result is the development of diabetes associated with \(\beta\)-cell failure. In RCAN1ox mice, age-dependent hyperglycemia, impaired glucose tolerance, hypoinsulinemia, reduced \(\beta\)-cell secretion, reduced \(\beta\)-cell number, decreased insulin granule filling, aberrant mitochondrial ROS production, and the altered expression of key \(\beta\)-cell genes occur. Thus, RCAN1 is an important \(\beta\)-cell gene, regulating multiple facets of \(\beta\)-cell function.

The experiments used mice in which RCAN1 is over-expressed in all cells that normally express RCAN1. Hence, the interpretations of our work cannot be solely confined to effects of RCAN1 directly on \(\beta\)-cells. Although studies have shown the presence of RCAN1 in the exocrine acinar cells of the pancreas (21), it is currently unknown whether RCAN1 is present in the endocrine \(\alpha\)- and \(\delta\)-cells of the pancreatic islet. Furthermore, we observed no overt changes in liver or kidney function or changes in blood lipids or in body weight. These results also demonstrate that RCAN1ox mice are not insulin resistant but rather show an increased response to insulin at 100 d. What underlies this increase in insulin sensitivity is unknown. It may be due to an undescribed role of RCAN1 in regulating insulin sensitivity in peripheral tissues in which it is expressed, such as skeletal muscle or liver (2). Alternatively, this change in insulin sensitivity may reflect a physiological adjustment by RCAN1ox mice in response to the lower levels of circulating insulin to maintain glucose homeostasis and minimize hyperglycemia. Many of our in vitro studies were also performed using isolated islets to alleviate concerns regarding non-\(\beta\)-cell effects of elevated RCAN1 expression. These islets were maintained in culture for several days before experimentation, potentially removing any indirect in vivo effects that could have occurred. This tissue was obtained at an age in which these mice are hyperglycemic, and there is the additional possibility that hyperglycemia may be driving some of the \(\beta\)-cell changes we observed, such as aberrant ROS production, gene expression changes, and reduced insulin secretion. If in vivo hyperglycemia was still affecting in vitro function, however, higher resting ROS levels would have been observed in RCAN1ox islets. Because we do not observe such changes, we are confident that the 72- to 96-h culture period used in our in vitro
experiments is sufficient to remove potential confounding effects of hyperglycemia in RCAN1\textsuperscript{ox} islets.

The diabetes associated with RCAN1 overexpression is due to hypoinsulinemia caused by β-cell loss and dysfunction. One mechanism associated with this β-cell loss may be increased apoptosis, as indicated by the elevated expression of the apoptosis gene markers caspase-3 and caspase-9 in RCAN1\textsuperscript{ox} islets. Overexpression of RCAN1.1 in primary neurons activates both caspase-9 and caspase-3, which induces neuronal apoptosis (22). This neurotoxic effect of RCAN1.1 is inhibited in caspase-3\textsuperscript{−/−} neurons, indicating the potential for RCAN1 to induce β-cell apoptosis via this enzyme (23). The large in vitro increase in ROS production under hyperglycemic conditions in RCAN1\textsuperscript{ox} islets illustrates that in vivo oxidative stress is likely higher in RCAN1\textsuperscript{ox} islets once RCAN1\textsuperscript{ox} mice become diabetic. Oxidative stress induces apoptosis in β-cells and reduces β-cell proliferation (24). Additionally, loss of calcineurin function reduces β-cell proliferation (10) and loss of NeuroD function, which is down-regulated in RCAN1\textsuperscript{ox} islets, reduces β-cell generation, and increases apoptosis (25). The loss of β-cells in RCAN1\textsuperscript{ox} mice may also be indirectly due to the effects of hyperglycemia itself. Chronic hyperglycemia has a clear link to increased β-cell apoptosis and reduced proliferation (26). Therefore, several mechanisms may explain how RCAN1 overexpression results in loss of β-cells.

Not only are β-cells lost as RCAN1\textsuperscript{ox} mice age but the secretory capacity of remaining RCAN1\textsuperscript{ox} β-cells is also reduced. The negative effect of RCAN1 on insulin release is not due to reduced insulin synthesis or altered localization of insulin granules or vesicle docking at the plasma membrane. The smaller size of the insulin granules in RCAN1\textsuperscript{ox} β-cell LDCVs and increase in the number of empty vesicles indicates problems with vesicle loading. The ZnT-8 vesicle zinc transporter controls insulin loading into vesicles and is inhibited by calcineurin inactivity and RCAN1 overexpression (27). Although this could explain the smaller insulin granule size and higher number of empty vesicles in RCAN1\textsuperscript{ox} β-cells, these effects are relatively minor.

Basal insulin release and first-phase GSIS are each reduced in RCAN1\textsuperscript{ox} β-cells, similar to secretory defects occurring in islets isolated from type 2 diabetes patients (28). RCAN1 may affect distal steps in the exocytosis pathway as occurs in RCAN1\textsuperscript{ox} adrenal chromaffin cells in which the number of exocytotic events and the amount released per vesicle are reduced (11). However, this would not explain the specific decrease in first-phase, but not second-phase, GSIS. $\text{K}_{\text{ATP}}$ channel-dependent mechanisms drive first-phase GSIS (29), whereas $\text{K}_{\text{ATP}}$ channels are required only as an initiating event in the sustained second-phase insulin response (30). The reduced Glut2 and glucokinase expression in RCAN1\textsuperscript{ox} islets may limit glucose 6-phosphate availability for oxidative phosphorylation and glucose-regulated $\text{K}_{\text{ATP}}$ channel closure and potentially explain reduced first-phase GSIS. Whether the overexpression of RCAN1 affects oxidative phosphorylation and $\text{K}_{\text{ATP}}$ channel function resulting in altered first-phase but normal second-phase insulin secretion is unresolved and will be a matter of future study.

RCAN1\textsuperscript{ox} β-cell mitochondria are smaller and produce more ROS \textit{in vitro} when exposed to high glucose. Increased RCAN1 expression in \textit{Drosophila} neurons promotes mitochondrial fission and ROS production (12), and hyperglycemia increases mitochondrial fission through increased expression of dynamin-related protein 1 (31). Under conditions of chronic high glucose or free fatty acid exposure, β-cell mitochondria lose their ability to undergo fusion that would otherwise prevent β-cell apoptosis under such conditions (32). From the present study, we cannot conclude whether elevated RCAN1 expression directly reduced mitochondrial size or whether the aberrantly high hyperglycemia-induced ROS levels present in RCAN1\textsuperscript{ox} β-cells is the cause. Our data raise the interesting possibility that RCAN1 may act as a link between hyperglycemia, mitochondrial fission, ROS production, and β-cell apoptosis. Greater glucose-stimulated ROS production in RCAN1\textsuperscript{ox} islets than in WT islets was also observed. Glucose normally enters the glycolytic pathway and preferentially undergoes oxidative phosphorylation in β-cells. Under hyperglycemic conditions, however, glucose is metabolized via alternative pathways (33). These pathways include glyceraldehyde autopshorylation, PKC activation, dicarbonyl formation, sorbitol metabolism, and hexosamine metabolism, which produce more ROS than oxidative phosphorylation (34, 35). Changes in glucose metabolism via these pathways in RCAN1\textsuperscript{ox} β-cells might therefore underlie the increased ROS production we observed in RCAN1\textsuperscript{ox} islets.

The age of onset of fasting hyperglycemia, glucose intolerance, hypoinsulinemia, islet shrinkage, and the reduced expression of multiple key β-cell genes in RCAN1\textsuperscript{ox} mice is similar to changes reported in mice displaying a loss of β-cell calcineurin activity (10). This indicates that these changes in RCAN1\textsuperscript{ox} mice may be due to reduced calcineurin activity caused by higher RCAN1 expression. However, some of the observed differences in RCAN1\textsuperscript{ox} β-cells in mitochondrial size, ROS production, GSIS, and vesicle filling have not been previously associated directly with calcineurin activity. Elucidating the pathways underlying RCAN1 regulation of β-cell function and identifying those that are dependent and independent of effects on
calcineurin activity will be an important area of future research.

Loss of β-cells and ensuing hypoinsulinemia is the ultimate cause of both type 1 and type 2 diabetes. In type 1 diabetes, β-cell death is caused by sudden autoimmune destruction. We do not feel this mechanism underlies the diabetes in our RCAN1lox mouse model because these mice lose β-cells after 40–60 d and gradually display worsening hyperglycemia. In type 2 diabetes, insulin resistance causes an increase in β-cell apoptosis, a relatively inadequate of insulin, poorly controlled blood glucose, and ultimately type 2 diabetes. The finding that chronic, but not acute, hyperglycemia induces RCAN1 expression raises the potential for RCAN1 to be involved in the progressive β-cell dysfunction occurring in type 2 diabetes. By contrast, in primary neuronal cells, the short-term induction of RCAN1 expression is thought to have a protective effect, whereas prolonged elevated expression has a detrimental effect on cell survival (36). Our in vivo data illustrate that a chronic increase in RCAN1 expression leads to β-cell loss, hypoinsulinemia, and diabetes. Although 6 d of high glucose in WT mice causes a 1.5-fold induction of RCAN1 expression, the effect of more prolonged hyperglycemia on the level of RCAN1 expression is unknown. Our RCAN1lox mouse model has a 3.5- to 5-fold increase in islet RCAN1 expression yet develops hyperglycemia only at 60–70 d of age. We hypothesize that when hyperglycemia occurs over time, as occurs during the progression of both type 1 and type 2 diabetes, RCAN1 expression will increase, resulting in β-cell dysfunction, β-cell loss, and hypoinsulinemia. Our findings, therefore, not only link RCAN1 to the control of β-cell number, secretion, gene expression, and mitochondrial function but also identify that RCAN1 is regulated by chronic hyperglycemia, and as such, increased RCAN1 expression has the potential to underlie β-cell dysfunction and hypoinsulinemia in diabetes.

Acknowledgments

Address all correspondence and requests for reprints to: Dr. Damien Keating, Department of Human Physiology and Centre for Neuroscience, Flinders University, Adelaide, Bedford Park SA 5042, Australia. E-mail: damien.keating@flinders.edu.au.

Disclosure Summary: The authors have nothing to disclose.

This work was supported by a BioInnovation SA Fellowship, Australian Research Council Future Fellowship, National Health and Medical Research Council Project Grant, and Diabetes Australia Research Trust Grant.

References


