Disruption of the WFS1 gene in mice causes progressive β-cell loss and impaired stimulus–secretion coupling in insulin secretion

Hisamitsu Ishihara1, Satoshi Takeda4, Akira Tamura1, Rui Takahashi1, Suguru Yamaguchi1, Daisuke Takei1, Takahiro Yamada1, Hiroshi Inoue5, Hiroyuki Soga2, Hideki Katagiri3, Yukio Tanizawa6 and Yoshitomo Oka1,*

1Division of Molecular Metabolism and Diabetes, 2Division of Immunology and Embryology, and 3Division of Advanced Therapeutics for Metabolic Diseases, Tohoku University Graduate School of Medicine, Sendai, Japan, 4Otsuka GEN Research Institute, Otsuka Pharmaceutical Co., Tokushima, Japan, 5Division of Diabetes and Endocrinology, Department of Medicine, Kawasaki Medical School, Kurashiki, Japan and 6Division of Molecular Analysis of Human Disorders, Department of Bio-Signal Analysis, Yamaguchi University Graduate School of Medicine, Ube, Japan

Received February 8, 2004; Revised and Accepted March 26, 2004

Wolfram syndrome, an autosomal recessive disorder characterized by juvenile-onset diabetes mellitus and optic atrophy, is caused by mutations in the WFS1 gene. In order to gain insight into the pathophysiology of this disease, we disrupted the wfs1 gene in mice. The mutant mice developed glucose intolerance or overt diabetes due to insufficient insulin secretion in vivo. Islets isolated from mutant mice exhibited a decrease in insulin secretion in response to glucose. The defective insulin secretion was accompanied by reduced cellular calcium responses to the secretagogue. Immunohistochemical analyses with morphometry and measurement of whole-pancreas insulin content demonstrated progressive β-cell loss in mutant mice, while the α-cell, which barely expresses WFS1 protein, was preserved. Furthermore, isolated islets from mutant mice exhibited increased apoptosis, as assessed by DNA fragment formation, at high concentration of glucose or with exposure to endoplasmic reticulum-stress inducers. These results strongly suggest that WFS1 protein plays an important role in both stimulus–secretion coupling for insulin exocytosis and maintenance of β-cell mass, deterioration of which leads to impaired glucose homeostasis. These WFS1 mutant mice provide a valuable tool for understanding better the pathophysiology of Wolfram syndrome as well as WFS1 function.

INTRODUCTION

Wolfram syndrome (OMIM 222300) is a rare autosomal recessive disorder characterized by juvenile-onset diabetes mellitus, optic atrophy, sensorineural deafness and diabetes insipidus (1). In addition, psychiatric illnesses such as depression and impulsive behavior are frequently observed in affected individuals (2). The nuclear gene responsible for this syndrome was identified by us (3) and others (4), and designated WFS1 (3). More than 100 mutations of the WFS1 gene have been identified to date in Wolfram syndrome patients. Most are inactivating mutations, suggesting loss of function to be responsible for the disease phenotype (5). WFS1 mutations underlie not only autosomal recessive Wolfram syndrome but also autosomal dominant low-frequency sensorineural hearing loss (LFSNHL). Heterozygous, non-inactivating WFS1 mutations were recently found in families with LFSNHL linked to chromosome 4p16 (DFNA6/14/38) (OMIM 600965) (6,7). The observation that the first-degree relatives of Wolfram syndrome patients have increased frequencies of diabetes mellitus and certain psychiatric disorders suggests sequence variants of the WFS1 gene predispose these individuals to such conditions (2,8). Indeed, several WFS1 sequence variants have been shown to be significantly associated with more common forms of diabetes mellitus (9,10) as well as with suicidal and impulsive behavior (11).
The WFS1 protein, also called wolframin (4), consists of 890 amino acids and was predicted to have nine or ten membrane spanning domains (3,4). Proteins with sequence similarity are now found in public databases of other organisms, *Drosophila melanogaster* (CG4917), *Anopheles gambiae* (EBI53764) and *Fugu rubripes* (SINFRUP82345), but little is known about their functions, suggesting WFS1 protein to belong to a novel family. The WFS1 protein is expressed in various tissues but at higher levels in the brain, heart, lung and pancreas (3,4). We showed the WFS1 protein to be localized predominantly in the endoplasmic reticulum (ER) and suggested a possible role of this protein in membrane trafficking, protein processing and/or regulation of cellular calcium homeostasis (12). A recent study showed this protein to contain nine transmembrane domains and to be embedded in the ER membrane with the amino-terminus in the cytosol and the carboxy-terminus in the ER lumen (13). ER dysfunction is known to cause apoptosis, which underlies a number of genetic disorders (14,15), possibly including a subset of diabetes (15). Since severe atrophic changes have been reported in the brain and in pancreatic islets of subjects with diabetes (15), it is reasonable to speculate that WFS1 protein plays an essential role in the survival of neuronal and islet β-cells.

In this study, to gain insight into the pathophysiology of Wolfram syndrome, we disrupted the *wfs1* gene in mice. The mice developed glucose intolerance or overt diabetes, depending on their genetic background. Our results demonstrate that the impaired glucose homeostasis in these mouse results from insufficient insulin secretion due to defects in both stimulus–secretion coupling and maintenance of β-cell mass.

**RESULTS**

**Targeted disruption of the WFS1 gene**

We first studied wfs1 protein expression in the pancreas, as this was essential to understand the diabetic phenotype in mice with a disrupted *wfs1* gene. Mouse pancreas sections were stained using an antibody raised against the 290 amino acid amino-terminus peptide of murine WFS1 (α-mWFS1-N) and those against islet hormones (Fig. 1A–L). Importantly, the WFS1 protein is strongly expressed in β-cells, and the majority of α, δ and F-cells are essentially devoid of wfs1 protein immunoreactivity. Double-staining of dispersed islet cells with these antibodies showed >80% of insulin-positive cells to be stained with anti-WFS1 antibody, while few cells express both WFS1 protein and one of the following: glucagon, somatostatin or pancreatic polypeptide (Fig. 1M–P).

In order to study the pathophysiology of Wolfram syndrome, we sought to inactive the *wfs1* gene by inserting a neomycin-resistance gene into the second exon of the *wfs1* gene which contains the initial ATG codon (Fig. 2A and B). When analyzed using an antibody against α-mWFS1-N, WFS1 protein bands of 95 kDa were abolished in whole-brain lysates from mutant mice (Fig. 2C). In addition, WFS1 protein staining was detected in neither pancreatic islets (Fig. 2D and E) nor the hippocampus (Fig. 2F and G) in mutant animals. It was subsequently recognized that our disruption strategy resulted in altered splicing transcripts in mutant animals. Reverse transcription–polymerase chain reaction on brain, heart and islet mRNA revealed existence of a wfs1 mRNA that lacks exon 2 in mutant animals (data not shown). Such an altered mRNA was not detected in wild-type tissues. The mutant transcript could generate amino-terminus-truncated WFS1 protein resulting from initiation of translation from one of the internal methionines. There exist methionine residues at 81, 184, 230 and 299, as well as further downstream, in murine WFS1 protein. We constructed a cDNA encoding WFS1 protein lacking the first 80 amino acids (WFS1-del80) and expressed it in COS7 cells. The WFS1-del80 protein was recognized by the antibody α-mWFS1-N (data not shown), while no bands were detected in brain lysates from mutant animals (Fig. 2C), indicating that WFS1-del80 is not expressed in mutant mice and that mutant proteins, if present, would be WFS1 protein lacking the first 183 amino acids or with larger truncations. We speculate that such truncated WFS1 proteins do not have normal functions since human substitution mutations at alanine 126, alanine 133 or glutamate 169 and a deletion mutation that lacks both lysine 178 and alanine 179 residues cause Wolfram syndrome (5). Therefore, we conclude that WFS1 function is lost, or at least severely impaired, in mice with a disrupted *wfs1* gene.

Mice homozygous for the mutated *wfs1* gene constitute the expected 25% of offspring born to heterozygous mutant parents, and are normal in appearance, growth and fertility. We did not see ataxic posture or gait disturbance. In addition, there were no differences in urine osmolality between wild-type and mutant mice. In the following experiments only male mice were used because an earlier study indicated females to have a milder phenotype. Since juvenile-onset diabetes mellitus is the most prominent feature of Wolfram syndrome, we have focused on this issue herein. Detailed studies on other aspects of this syndrome, including optic atrophy, hearing disorders, diabetes insipidus or psychiatric illness, are currently underway.

**Impaired glucose homeostasis in mutant mice**

Blood glucose levels in these mice were studied in non-fasted states. Initially, using mice on the [(129Sv × B6) × B6]F2 hybrid background, we found that blood glucose levels of mutant mice started to rise at around 16 weeks of age and >60% of mice (8 out of 13) had overt diabetes by 36 weeks (Fig. 3A). Since the heterogeneous contribution of B6 and 129Sv strains in the mixed background mice could cause a large variance in data, making interpretation difficult, we sought to generate mutant animals on a nearly homogenous genetic background. For this purpose, male mice with a disrupted *wfs1* gene were backcrossed for five successive generations with female mice of the B6 strain, which is frequently used for diabetes and obesity research. On the B6 background, no apparent increase in blood glucose levels was observed even at 36 weeks in mice homozygous for disrupted *wfs1* alleles (Fig. 3B). However, impaired glucose homeostasis was evident in mice on the B6 background when they were subjected to oral glucose tolerance test (Fig. 3C). Blood glucose levels at 15 and 30 min were significantly higher in mutant than in wild-type mice at 17 weeks of
These data indicated that disruption of the \( \text{wfs1} \) locus induced impaired glucose homeostasis in mice, as is seen in human Wolfram syndrome.

In order to investigate the pathophysiology of impaired glucose homeostasis in mutant mice, plasma immunoreactive insulin (IRI) levels in response to a glucose load were evaluated. Although plasma insulin levels after a 6 h fast were comparable between wild-type and mutant animals at 17 weeks of age (Fig. 3D), hormone responses were markedly blunted in \( \text{WFS1} \)-deficient mice. We also studied non-fasting plasma insulin levels in these mice. Plasma insulin levels in mutant mice were similar to that in wild-type mice at 24 weeks but had decreased to half the wild-type level at 36 weeks (Fig. 3E). Intraperitoneal insulin injection tests did not show insulin resistance in mutant mice at 14 (data not shown) and 19 weeks (Fig. 3F). In fact, \( \text{WFS1} \)-deficient mice were somewhat more insulin sensitive. Taken together, these data indicate impaired glucose homeostasis in mice with a disrupted \( \text{wfs1} \) gene to be due to insulin secretory defects rather than insulin resistance.

**Impaired stimulus–secretion coupling in \( \beta \)-cells from mutant mice**

Since defects in both stimulus–secretion coupling and insulin production could be the cause of insulin secretory defects in vivo, insulin secretory responses were studied using isolated islets. When we isolated islets from these mice, we noticed that

---

**Figure 1.** \( \beta \)-Cell specific expression of \( \text{WFS1} \) protein in the pancreas. (A–L) Paraffin embedded mouse pancreatic sections were immunostained with antibodies against \( \text{WFS1} \) protein (green) (A–D) and islet hormones (red): insulin (E), glucagon (F), somatostatin (G), or pancreatic polypeptide (H). A and E are the same section, and the two are merged in I. Similarly, J, K, L are merged versions of B and F, C and G, D and H, respectively. Bars = 10 \( \mu \)m. Ins, insulin; Glu, glucagon; Sms, somatostatin; PP, pancreatic polypeptide. (M–P) Dispersed islet cells were stained with anti-\( \text{WFS1} \) antibody (green) together with those against islet hormones (red): insulin (M), glucagon (N), somatostatin (O) or pancreatic polypeptide (P). Bars = 10 \( \mu \)m.
it was possible to obtain only 100 islets or even less from a mutant mouse, while around 200 islets can normally be isolated from a wild-type mouse. Insulin content in the WFS1-deficient islets was slightly (16%) but significantly less than that in islets of wild-type mice \( [61.8 \pm 2.3 \text{ ng/islet} (n = 10 \text{ experiments}) \] versus \( 73.4 \pm 3.3 \text{ ng/islet} (n = 10 \text{ experiments}) \), \( P = 0.039 \), mutant and wild-type islets, respectively.\) We used these islets infected with either AdCAGlacz (as a control) or AdCAGmWFS1 (Fig. 4A), because we also wanted to examine effects of WFS1 re-expression in WFS1-deficient islets and of its overexpression in wild-type islets. Glucose \( (15 \text{ mM}) \)-stimulated insulin secretion, after normalization with insulin content, was reduced by 23% in islets from mutant mice (Fig. 4B). Carbachol \( (1.0 \text{ mM}) \)-stimulated insulin secretion, which is thought to be evoked by \( \text{Ca}^{2+} \) release from the ER and \( \text{Ca}^{2+} \) entry through the \( \text{Ca}^{2+} \) release-activated channel, was also reduced by 26% (Fig. 4C). When WFS1 protein was re-expressed in islets from mutant animals via a recombinant adenovirus,
Figure 3. Impaired glucose homeostasis in WFS1-deficient mice. (A) Non-fasted blood glucose levels in male mice on the [(129Sv × B6) × B6]F2 hybrid background at indicated ages (n = 8–13). (B) Non-fasted blood glucose levels in male mice on the B6 background (n = 9–16). (C, D) Oral glucose (2 mg/g body weight) tolerance test in 17-week-old mice on the B6 background (n = 6). Blood glucose levels (C) at indicated points and plasma IRI levels (D) before and 30 min after the glucose load are shown. Glucose tolerance tests were performed on two other occasions using different animals with essentially same results. (E) Plasma IRI levels at 24 and 36 weeks of age (n = 6–8). (F) Insulin (0.75 units/kg body weight) tolerance test at 19 weeks (n = 5). Insulin tolerance tests were performed on two other occasions with essentially same results. White circles and bars, wild-type mice; black circles and bars, mutant mice. *P < 0.05, **P < 0.01.
Figure 4. Impaired stimulus–secretion coupling in WFS1-deficient β-cells. (A) Islets from wild-type and mutant mice were infected with either AdCAGlacZ or AdCAGmWFS1. After 36 h, islets were subjected to western blot analyses using anti-WFS1 antibody. Lane 1, wild-type islets infected with AdCAGlacZ; lane 2, mutant islets infected with AdCAGlacZ; lane 3, wild-type islets infected with AdCAGmWFS1; lane 4, mutant islets infected with AdCAGmWFS1. Western blot experiments were performed twice with similar results and one of them is shown. (B, C) Islets were challenged with 15 mM glucose (B) or 1 mM carbachol in the presence of 2.5 mM glucose (C) for 1 h. Absolute insulin secretion in response to glucose was $3.11 \pm 0.34$ and $2.03 \pm 0.26$ ng/islet/h, respectively, for wild-type and mutant islets infected with the control virus (AdCAGlacZ). Data are means ± SEM, $n = 5$ experiments. White bars, wild-type islets infected with AdCAGlacZ; black bars, mutant islets with AdCAGlacZ; hatched bars, wild-type islets with AdCAGmWFS1; dotted bars, mutant islets with AdCAGmWFS1. (D, E) Intracellular Ca$^{2+}$ responses to 15 mM glucose in wild-type (gray line and white bar) and WFS1-deficient (black line and black bar) β-cells. Representative traces out of 21 wild-type and 24 WFS1-deficient β-cells from one experiment were shown in D. Areas under the curve during a 5 min period after the onset of Ca$^{2+}$ rises to glucose were summarized in E. Similar significant differences were observed in the other four experiments. *$P < 0.05$, **$P < 0.01$. 
glucose- and carbachol-stimulated insulin secretion was restored (Fig. 4B and C), indicating reduced insulin secretion in islets from mutant mice to be a direct consequence of absence of normal WFS1 function. Interestingly, glucose- and carbachol-stimulated insulin secretion from wild-type islets increased by 41 and 53%, respectively, with overexpression of the WFS1 protein, suggesting involvement of the WFS1 protein in stimulus–secretion coupling for insulin exocytosis (Fig. 4B and C).

To gain insight into the mechanisms of impaired insulin secretion in islets from mutant mice, intracellular calcium dynamics were then studied in single β-cells. The glucose-stimulated rise in the cytosolic Ca^{2+} response was reduced by 36% in WFS1-deficient β-cells when compared with that in wild-type β-cells (Fig. 4D and E).

**Progressive β-cell loss in mutant mice**

We then focused on aspects of insulin production, deterioration of which could be a cause of impaired glucose homeostasis in mice with disruption of the wfs1 gene. There were no differences in pancreatic weight between wild-type and mutant mice (data not shown). Whole-pancreas insulin content was already decreased at 2 weeks, the earliest age studied, and dropped further with age (Fig. 5A). Immunohistochemical studies (Fig. 5B–E) showed the number of insulin-positive cells to be reduced at 36 weeks in the mutant mouse pancreas (Fig. 5E). Morphometric analysis demonstrated a marked reduction in the insulin-positive area per pancreatic area in mutant mice when compared with wild-type mice (Fig. 5H), indicating the decrease in insulin content to be due to loss of islet β-cells. These features were more prominent in the mutant mouse pancreas on the [(129Sv × B6) × B6]F2 background, which was associated with overt diabetes (Fig. 5F and G). In contrast to the β-cell changes, glucagon-positive cells were increased and scattered throughout WFS1-deficient islets (Fig. 5E and G). Indeed, the pancreatic glucagon content in mutant mice at 36 weeks of age on the B6 background was 2.4-fold higher than that in wild-type mice [12.3 ± 1.8 ng/mg (n = 4) versus 5.2 ± 0.7 (n = 4), P = 0.0296].

**Increased susceptibility of WFS1-deficient islets to apoptosis**

To study whether the observed β-cell loss was due to increased apoptosis, we conducted an extensive search for apoptotic β-cells in pancreatic sections. However, TUNEL or activated-caspase 3-positive cells were sparse within islets in pancreatic sections from both mutant and wild-type animals (data not shown). Therefore, we turned to in vitro studies, and examined whether WFS1-deficient islet cells are more susceptible to apoptotic insults. For this purpose, apoptotic DNA fragmentation was studied in isolated islets by the ligation-mediated PCR (LM-PCR) method. When islets were cultured for 3 days in RPMI media with 5 or 25 mM glucose, ladder formation was increased at 25 mM glucose in both wild-type and mutant islets when compared with that at 5 mM glucose, indicating that apoptotic cell death may have been induced by glucose toxicity (Fig. 6A). Importantly, at 25 mM glucose, islets from mutant mice showed more DNA fragment formation than wild-type islets (1.7 ± 0.3-fold, n = 5), while no significant differences were observed at 5 mM glucose. Since recent studies have suggested so-called ER-stress to be an important mediator of apoptosis in β-cells (14,15), DNA fragmentation was studied after treatment with two different ER-stress inducers (18), tunicamycin (2 μg/ml) and thapsigargin (2 μM). DNA fragmentation at 5 mM glucose was significantly increased, by 2.2 ± 0.4-fold and 2.4 ± 0.4-fold after tunicamycin (Fig. 6B) and thapsigargin (Fig. 6C) treatments, respectively, in WFS1-deficient islets when compared with wild-type islets. In contrast, there were no differences in DNA fragmentation after combined tumor necrosis factor-α and interferon-γ treatment (Fig. 6D), which triggers apoptosis through a signaling pathway different from that originating in the ER.

**DISCUSSION**

We generated mice with a disrupted wfs1 gene. Although the diabetic phenotype was milder than that seen clinically in Wolfram syndrome (1), the progressive β-cell loss and impaired glucose homeostasis observed in these mice are essentially consistent with findings in patients (1,17). Thus, the mutant mice are indeed a model of Wolfram syndrome. The underlying anatomic condition of this syndrome has not been studied in great detail in humans, and the cellular basis for the diabetic phenotype and associated neuro-psychiatric disorders remains obscure. Creation of an animal model that reflects aspects of the disease is thus an important first step in understanding Wolfram syndrome.

The present data demonstrate that the pathophysiological basis of diabetes in Wolfram syndrome is insufficient insulin secretion due to progressive β-cell loss and impaired stimulus–secretion coupling in β-cells. Progressive β-cell loss has been expected from clinical observations of progressive deterioration of insulin-requiring states in affected patients as well as their postmortem findings, i.e. selective β-cell loss with an increase in α-cells and preservation of δ-cells (17). In contrast, impaired stimulus–secretion coupling in the β-cell, a quite unexpected result, was demonstrated for the first time in this study. In addition, we also showed for the first time that WFS1 protein is expressed selectively in β-cells, but very little in α, δ and F-cells, within the endocrine pancreas, suggesting that β-cell loss is a direct consequence of WFS1 deficiency.

The severity of the diabetic phenotype due to wfs1 gene disruption was dependent on the mouse genetic background: >60% of mice on the [(129Sv × B6) × B6]F2 background developed overt diabetes, while mutant mice on the B6 background had impaired glucose tolerance but not overt diabetes. Modifying effects of genetic background on glucose homeostasis have been reported previously in a number of mutant mice. An earlier pioneering study established that the B6 background confers more diabetes resistance to db/db and ob/ob mice (19). A diabetes-resistant phenotype has also been reported in insulin receptor substrate (IRS)-2 knockout mice on the B6 background (20), while anti-sense glucokinase mRNA expressing mice (21) and mice double heterozygous for deletion of the insulin receptor and IRS-1 (22), on the same B6 background, were reportedly diabetes prone. Therefore, the...
Figure 5. Progressive β-cell loss in mutant mice. (A) Insulin content extracted from whole pancreata of wild-type and mutant mice. Data represent percent of insulin content in wild-type littermates. Absolute insulin content in wild-type pancreata were 1367 ± 103 ng/mg pancreas at 2 weeks, 268 ± 18 (8 weeks), 329 ± 25 (24 weeks) and 372 ± 33 (36 weeks), n = 4–7. White bars, wild-type pancreata; black bars, WFS1-deficient pancreata. (B–G) Insulin (green) and glucagon (red) are stained in pancreatic sections from 8-week-old wild-type (B), mutant (C), 36-week-old wild-type (D) and mutant mice (E) on the B6 background, and 24-week-old wild-type (F) and mutant (G) mice on the [(129Sv × B6) × B6]F2 background. Bars = 10 μm. (H) Ratios of total insulin-positive area per whole pancreatic area in pancreas from wild-type and mutant mice on the B6 background. n = 4 animals for each group. *P < 0.05, **P < 0.01.
contribution of genetic background is apparently complex. In any case, progressive β-cell loss was observed in mutant mice in both [(129Sv x B6) x B6]F2 and B6 strains, independent of the mouse genetic background. It is not surprising that mutant mice on the B6 background did not develop overt diabetes. Overt diabetes was known to be induced when >90% of the pancreas was removed (23), while the insulin content of mutant mouse pancreas at 36 weeks was decreased by 73% on the B6 background in this study.

The present data provide an intriguing clue that may help to elucidate WFS1 protein function. WFS1-deficient islets exhibited impaired insulin secretion in response to glucose and carbachol, which was restored by re-expression of WFS1 protein. In addition, overexpression of WFS1 protein in wild-type islets resulted in an increase in glucose- and carbachol-induced insulin secretion. These data from islets with different WFS1 protein levels demonstrated this protein to be involved directly in the regulation of insulin secretion. Furthermore, impaired calcium responses to glucose suggested that WFS1 protein is involved in regulation of calcium homeostasis in the β-cell. This notion is supported by the recent report that expression of WFS1 protein in Xenopus oocytes confers a novel cation channel activity (24). The present data also provide insight into the mechanism of β-cell loss in mice with a mutant wfs1 gene. Although we rarely detected apoptotic cells in pancreatic sections from mutant mice, apoptosis cannot be excluded as a possible mechanism of β-cell loss, since our failure could presumably be due to slow progression of apoptosis in vivo

Figure 6. Increased apoptosis susceptibility in islets from mutant mice. (A) Islets from wild-type and mutant mice were cultured for 3 days in 5 and 25 mM glucose concentrations and DNA fragmentation was assessed by the LM-PCR method. (B–D) Islets from wild-type and mutant mice were treated with tunicamycin (Tm; 2 μg/ml) (B), thapsigargin (Tg; 2 μM) (C) for 24 h or with the combination of tumor necrosis factor-α (TNFα; 500 units/ml) and interferon-γ (IFNγ; 100 units/ml) (D) for 48 h and DNA fragmentation was assessed by the LM-PCR method; n = 4–6 experiments. *P < 0.05.
and rapid clearance of cells undergoing apoptosis, as was suggested recently in another animal model of diabetes (25).

Increased apoptosis susceptibility in response to high glucose and ER-stress inducers, demonstrated in isolated islets from mutant mice, is likely to contribute to β-cell loss. In contrast, the apoptosis induced by exposure to tumor necrosis factor-α and interferon-γ, in which the ER-stress response is not involved, did not differ between wild-type and WFS1-deficient islets. Although the mechanism whereby high concentrations of glucose induce apoptosis is not completely understood, it is possible that increased DNA fragmentation in WFS1-deficient islets at 25 mM glucose could also be attributable to increased susceptibility to ER-stress-induced apoptosis. However, it remains to be clarified why WFS1 deficiency renders β-cells more susceptible to apoptosis, especially to ER-stress-induced apoptosis.

Recent studies showing β-cell mass to be decreased in human type 2 diabetes, due to increased β-cell apoptosis (29), have attracted considerable attention to this potential pathogenic mechanism of type 2 diabetes development. Therefore, maintaining β-cell mass is an important strategy for preventing diabetes as well as halting disease progression. Since the WFS1 protein is likely to belong to a novel family, elucidating the WFS1 protein function could lead to establishment of new treatments not only for Wolfram syndrome but also for more common forms of diabetes mellitus.

**MATERIALS AND METHODS**

**Targeted disruption of the wfs1 gene**

The wfs1 gene was cloned from a 129Sv mouse genomic DNA library using its cDNA probe (3). A targeting vector was constructed by inserting a neomycin-resistance gene at the SmaI site in exon 2 of the wfs1 gene. The diphtheria toxin A chain expressing unit was inserted downstream (Fig. 2A). The wfs1 gene targeting vector was microinjected into 129Sv embryonic stem cells. Homologous recombination was successful in two independent embryonic stem cell lines (lines 133 and 190). Positive chimeric male mice were then crossed with female C57BL/6J (B6) mice to produce wfs1 heterozygous mice. Initial analyses demonstrated essentially the same phenotypes between the two lines, and therefore we have analyzed line 133 mice. In order to analyze animals with as homogenous a genetic background as possible, male wfs1 heterozygous mice were backcrossed with female B6 mice for five successive generations. We also analyzed wfs1 homozygous mice on the [(129Sv × B6) × B6]F2 hybrid background. The mice were kept in standard, specific pathogen-free conditions under a constant dark/light cycle. All animal experiments were approved by the local ethical committee for animal research at the Tohoku University.

**Physiological studies**

Control animals were age-matched siblings. Blood glucose levels in the non-fasting state were measured at 9:00–10:00 a.m. using a GluTest blood glucose monitor (Sanwa Chemicals, Tokyo, Japan). Serum insulin levels were determined by radioimmunoassay using a rat insulin RIA kit (Linco Research, St Charles, MO, USA). For oral glucose tolerance tests, animals after a 6 h fast were administered with 20% glucose solution (2 mg/g body weight) by gastric tubes. Whole-blood samples were collected from the tail tip at the indicated time points. Insulin tolerance tests were performed after a 6 h fast by an intraperitoneal injection of human regular insulin (0.75 units/kg body weight).

**Immunohistochemistry and morphometry**

For brain sections, animals were anesthetized by ethyl ether, and 4% formalin was perfused from the left ventricle. For pancreatic sections, the animals were killed by cervical dislocation. Dissected pancreas pieces were fixed in 4% formalin. Formalin-fixed paraffin-embedded sections of pancreas were de-paraffinized and re-hydrated. For insulin and glucagon staining, the sections were then incubated with a guinea pig anti-insulin IgG (DAKO Japan, Kyoto, Japan) diluted 1:1000 and a mouse anti-glucagon IgG (Sigma-Aldrich Japan, Tokyo, Japan) diluted 1:2000 for 1 h at room temperature. The anti-insulin and -glucagon primary antibodies were followed by a 45 min incubation with a fluorescein isothiocyanate (FITC)-conjugated anti-guinea pig IgG and a Texas Red-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA). The antibody raised against the 290 amino acid α-mWFS1-N was described previously (30). Pancreatic sections incubated with the anti-WFS1 antibody were then stained with an FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch). Immunohistochemical analyses were performed, sacrificing at least four different animals for each condition. For measurements of β-cell area, more than 10 pancreatic tissue sections per animal were randomly selected, stained with anti-insulin IgG and eosin. Pancreatic area and β-cell area were each estimated using the intensity thresholding function of the NIH Image software. Four animals were analyzed for each group.

**Pancreatic insulin and glucagon content**

Pancreases were suspended in cold acid ethanol and minced by scissors, and left at −20°C for 48 h, with sonication every 24 h. Insulin content in the acid ethanol supernatant was determined with a rat insulin RIA Kit (Linco Research). Glucagon content in the same extract was measured by a glucagon RIA kit (Linco Research).

**Islet studies**

**Construction of a recombinant adenovirus expressing murine WFS1 protein.** A recombinant adenovirus AdCAmWFS1, bearing an EcoR1 fragment of murine WFS1 cDNA, was constructed by the method described previously (31,32). AdCAmGlacZ expressing β-galactosidase was used as a control adenovirus. Isolated islets were infected with the recombinant adenoviruses at 1.2 × 10⁵ particles per islet in 1.0 ml medium for 60 min.
Isolation and static incubation of islets. Islets isolated from age-matched wild-type and mutant siblings at 14–17 weeks were isolated by retrograde injection of collagenase (Serva, Heidelberg, Germany) into the pancreatic duct according to standard procedures. For secretion studies, batches of 10 islets (triplicates for each condition) were kept in Krebs–Ringer-bicarbonate-HEPES buffer [KRBH; 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 1.5 mM CaCl2, 2 mM NaHCO3, 10 mM HEPES (pH 7.4)] containing 0.1% BSA and stimulators indicated. Islet insulin content was measured following extraction by acid ethanol. Insulin was detected by radioimmunoassay.

Single cell Ca2+ measurement. Islets isolated from mice at 12–16 weeks were dispersed, plated on glass-bottomed dishes and cultured for 3 days before measurement. β-Cells were identified by adenosine-5′-monophosphate expression of green fluorescent protein driven by the insulin promoter (33). We performed experiments without adenosine-5′-monophosphate expression of green fluorescent protein, identifying β-cells with immunostaining after perfusion, and observed similar results (data not shown). Cells were incubated with 1 μM Fura 2-AM (Dojindo, Kumamoto, Japan) for 30 min, perfused with KRBH and excited at 340 and 380 nm. A cooled CCD camera (Hamamatsu Photonics, Shizuoka, Japan) mounted on a microscope (Leica Microsystems, Heerbrugg, Switzerland) was used to capture fluorescence images. Ca2+ rises were compared by calculating areas between Ca2+ curves and baselines for the 300 s after the onset of Ca2+ rises.

LM-PCR amplification of DNA fragments. Groups of 50 islets isolated from mice at 15–17 weeks of age were cultured for 3 days in RPMI with different glucose concentrations. In another series of experiments, groups of 50 islets were treated with 2 μg/ml tunicamycin (Sigma-Aldrich Japan), 2 μM thapsigargin (Alamone Labs, Jerusalem, Israel) or a combination of interferon-γ (100 units/ml; PeproTech, London, UK) and tumor necrosis factor-α (500 units/ml; PeproTech). Genomic DNA was isolated from treated islets using the DNeasy kit (Qiagen-Japan, Tokyo, Japan). The PicoGreen® dsDNA quantitation kit (Molecular Probes, Eugene, OR, USA) was used to determine the DNA concentrations. 200 ng of the genomic DNA was ligated with an adaptor, which has been generated by annealing two synthetic oligonucleotides 5′-AGCAGTCTGAGGCCCTTACCCGCA-3′ and 5′-TGCGGTTAGGGA-3′. A portion of ligation mixture (30%) was used for the PCR amplification with a primer 5′-AGCAGTCTGAGGCCCTTACCCGCA-3′ and 5′-TGCGGTTAGGGA-3′. The resulting PCR products were run on 1.2% agarose gels. Intensities of ladders between 500 and 1000 bp were analyzed using the Scion Image software. In order to compare data from separate gels, band intensity was normalized to the average laddering of 1 kb DNA standards.

Statistical analyses

Data are presented as mean ± SE, unless otherwise noted. Differences between wild-type and mutant animals were assessed by Student’s t-test.

ACKNOWLEDGEMENTS

We thank Professor H. Takeshima, Dr Y. Ohwada and Professor T. Itoh, Tohoku University, for their help in Ca2+ imaging and immunohistochemical analyses. We are also grateful to N. Nishino, T. Wadatsu and N. Miyazawa, Otsuka GEN Research Institute, for their help in generation of WFS1-deficient mice. Y. Takahashi is gratefully acknowledged for her excellent technical assistance. This study was supported by Grants in Aid for Scientific Research (15204062) to Y.O. from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES


