EXPERIMENTAL STUDY

Endoplasmic reticulum stress induces *Wfs1* gene expression in pancreatic β -cells via transcriptional activation

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Abstract

Objective: The *WFS1* gene encodes an endoplasmic reticulum (ER) membrane-embedded protein. Homozygous *WFS1* gene mutations cause Wolfram syndrome, characterized by insulin-deficient diabetes mellitus and optic atrophy. Pancreatic β -cells are selectively lost from the patient's islets. ER localization suggests that *WFS1* protein has physiological functions in membrane trafficking, secretion, processing and/or regulation of ER calcium homeostasis. Disturbances or overloading of these functions induces ER stress responses, including apoptosis. We speculated that *WFS1* protein might be involved in these ER stress responses.

Design and methods: Islet expression of the *Wfs1* protein was analyzed immunohistochemically. Induction of *Wfs1* upon ER stress was examined by Northern and Western blot analyses using three different models: human skin fibroblasts, mouse pancreatic β -cell-derived MIN6 cells, and Akita mouse-derived *Ins2*^{96Y/Y} insulinoma cells. The human *WFS1* gene promoter-luciferase reporter analysis was also conducted.

Result: Islet β -cells were the major site of *Wfs1* expression. This expression was also found in δ -cells, but not in α -cells. *WFS1* expression was transcriptionally up-regulated by ER stress-inducing chemical insults. Treatment of fibroblasts and MIN6 cells with thapsigargin or tunicamycin increased *WFS1* mRNA. *WFS1* protein also increased in response to thapsigargin treatment in these cells. *WFS1* gene expression was also increased in *Ins2*^{96Y/Y} insulinoma cells. In these cells, ER stress was intrinsically induced by mutant insulin expression. The *WFS1* gene promoter-luciferase reporter system revealed that the human *WFS1* promoter was activated by chemically induced ER stress in MIN6 cells, and that the promoter was more active in *Ins2*^{96Y/Y} cells than *Ins2*^{wild/wild} cells.

Conclusion: *Wfs1* expression, which is localized to β - and δ -cells in pancreatic islets, increases in response to ER stress, suggesting a functional link between *Wfs1* and ER stress.

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Introduction

Wolfram syndrome is a rare recessively inherited genetic disorder, which is characteristically associated with juvenile onset diabetes mellitus and progressive optic atrophy (1). Sensorineural deafness, diabetes insipidus, ataxia, urinary-tract atony, peripheral neuropathy and psychiatric illness may also be present (2). We and another group succeeded in cloning the gene responsible for this disorder and designated it *WFS1* (3) or *wolframin* (4). Loss-of-function mutations in the *WFS1* gene have been linked to Wolfram syndrome. The *WFS1* gene consists of eight exons coding for a

putative 890 amino acid protein with an apparent molecular mass of ~ 100 kDa. *WFS1* protein (wolframin) is a hydrophobic protein with nine transmembrane segments and large hydrophilic regions at both termini. *WFS1* protein localizes primarily to the endoplasmic reticulum (ER) in a $N_{\text{cyt}}/C_{\text{lum}}$ membrane topology (5, 6). A recent report suggested that expression of *WFS1* protein in oocytes was associated with an increase in cytosolic Ca^{2+} and induced novel cation-selective channel activities in the ER membrane (7). However, its role in cellular functions and the mechanism by which mutations of this gene cause Wolfram syndrome remain largely unknown.

ER is a specialized organelle involved in a wide variety of cellular functions. Calcium regulation and post-translational modification, folding and trafficking of secreted and membrane integral proteins are well-defined ER functions (8). Various physiological and pathological conditions interfere with these functions, and overloading of these functions induces ER stress. Cells respond to such stress by activating several adaptive pathways including chaperone induction, protein translation attenuation, and occasionally apoptosis, collectively called the unfolded protein response (9). Characteristically, pancreatic β -cells have highly developed ER apparently due to the heavy demands of insulin biosynthesis and secretion. Beta-cells are highly susceptible to ER stress. Several studies have shown that β -cell mass is reduced in patients with type 2 diabetes, possibly due to apoptotic death of β -cells and to reduced cell proliferation (10). ER stress may be involved in this process (11). In the Akita mouse, an animal model of MODY (maturity onset diabetes of the young), which carries a conformation-altering missense mutation (Cys96Tyr) in the insulin-2 (*Ins2*) gene (12, 13), hyperglycemia and reduced β -cell mass are accompanied by ER stress-induced β -cell death (14). Based on the ER localization of WFS1 protein, it is reasonable to speculate that WFS1 protein may play an as yet undefined role in the ER stress-induced cell death of pancreatic β -cells. In fact, we showed islet cells lacking Wfs1 to be more susceptible to ER stress-induced apoptosis (15), and, more recently, Yamaguchi *et al.* reported that treatment with ER stress inducers increased Wfs1 protein expression in isolated mouse pancreatic islets (16).

In the present study, immunohistochemical staining confirmed β -cells to be the major site of *Wfs1* expression in the mouse pancreas. Furthermore, this expression was also evident in δ -cells but not in α -cells. The *WFS1* gene was clearly expressed in response to drug-induced ER stress in both fibroblasts and pancreatic β -cell-derived MIN6 cells. Under the same conditions, the human *WFS1* promoter luciferase reporter was activated suggesting transcriptional control of *WFS1* expression. Furthermore, *Wfs1* mRNA and protein levels were increased in Akita mouse-derived *Ins2*^{96Y/Y} insulinoma cells, in which the ER stress response had been triggered (17). Our results demonstrate that not only drug-induced but also intrinsic ER stress leads to *WFS1* expression in pancreatic β -cells, and this occurs, at least in part, via transcriptional activation of the *WFS1* promoter. These findings further suggest a functional link between *WFS1* and ER stress responses.

Research design and methods

Tissue preparation and immunohistochemical staining of the mouse pancreas

All experimental protocols for this study were approved by the committee on the Ethics of Animal

Experimentation at Yamaguchi University School of Medicine. The anti-Wfs1 antibodies were described previously (5, 15).

Double immunofluorescent staining was performed for co-localization studies. Sections were pre-incubated, bleached (18), and stained with a mixture of anti-Wfs1n (diluted 1:200) and mouse monoclonal anti-insulin (diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-glucagon (diluted 1:200; Sigma-Aldrich, St Louis, MO, USA), or anti-somatostatin (diluted 1:25; Biomed Corporation, Foster City, CA, USA) in 0.1 M sodium phosphate buffer containing 0.3% Triton X-100, 0.1% sodium azide, and 3% normal goat serum (PBT-NGS) for 24 h at 20 °C. Next, the sections were incubated with a mixture of two secondary antibodies in PBT-NGS for 24 h at 20 °C. The secondary antibodies used were Alexa Fluor 488 conjugated with goat anti-rabbit IgG (H + L), highly cross absorbed (Molecular Probes, Eugene, OR, USA) and diluted 1:100, and an Alexa Fluor 594 conjugated to goat anti-mouse IgG (H + L), F(ab')₂ fragment (Molecular Probes), diluted 1:100. The sections were coverslipped with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA). As a control, one of the two primary antibodies, for example either anti-Wfs1n or anti-insulin, was removed to check for cross-reactivity. In these control experiments, other procedures were the same as for Wfs1/insulin double staining. No cross-reactivity was observed in these experiments (data not shown).

In the case of double immunostaining for Wfs1 and pancreatic polypeptide (PP) detection, a mixture of anti-Wfs1n (diluted 1:200) and anti-PP (diluted 1:200; Linco Research, St Charles, MO, USA) was used for the primary antibody reaction. In the secondary antibody reaction step, sections were incubated in a mixture of Alexa Fluor 488 conjugated with donkey anti-rabbit IgG (H + L; Molecular Probes) diluted 1:100 and Alexa Fluor 594 conjugated to goat anti-guinea pig IgG (H + L), highly cross absorbed (Molecular Probes) and diluted 1:100 in PBT-NGS containing 3% normal donkey serum. Other procedures for Wfs1/PP double staining were the same as for Wfs1/insulin double staining.

Cell culture and reagents

The mouse insulinoma cell line, MIN6 (19), was a gift from Dr Junichi Miyazaki, Osaka University, Japan. Insulinoma cells derived from the Akita mouse and from normal littermates, *Ins2*^{96Y/Y} cells and *Ins2*^{WT/WT} cells respectively, were described previously (17). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 15% fetal calf serum in an atmosphere of 5% CO₂ at 37 °C. The genotype for the insulin-2 gene was confirmed by restriction fragment length polymorphism (RFLP), as previously described (12, 13). Human skin fibroblasts

(CCD-1059SK) were obtained from ATCC (Manassas, VA, USA). Thapsigargin, ionomycin, A23187, cyclopiazonic acid, 4-chloro-*m*-cresol, tunicamycin and brefeldin A were purchased from Sigma.

Northern blot analysis

Total RNA isolated using an ISOGEN kit (NIPPON GENE, Tokyo, Japan) was electrophoresed in 1% agarose formaldehyde gel and transferred to nylon filters (Hybond-N plus, Amersham Pharmacia Biotech). The filters were pre-hybridized and hybridized in a buffer containing 50% deionized formamide, 5 × sodium chloride-sodium phosphate-EDTA buffer (750 mmol/l NaCl, 43.25 mmol/l NaH₂PO₄, 6.25 mmol/l EDTA), 2 × Denhardt's solution (0.04% bovine serum albumin, 0.04% Ficoll, 0.04% polyvinylpyrrolidone), and 0.1% sodium dodecyl sulfate at 42 °C. The hybridization buffer contained a radio-labeled 3.0 kb fragment of mouse *Wfs1* cDNA (GeneBank Accession No. BC046988). After a stringent wash with 0.2 × sodium chloride-sodium citrate buffer (3.3 mmol/l Na-citrate, 3.3 mmol/l NaCl) and 0.1% SDS at 50 °C, autoradiographs were digitally scanned and quantified using FULA2000 (Fuji Film, Tokyo, Japan). The blots were stripped and re-probed with a 1122 bp fragment encompassing the entire coding region of the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The cDNA probes were labeled with a random primer DNA labeling kit (Ready-To-Go DNA Labeling Beads, Amersham Pharmacia Biotech) using α-[³²P]deoxy-CTP (Amersham Pharmacia Biotech).

Immunoblotting analysis

Cells were lysed in 20 mmol/l Tris-HCl (pH 7.6), 0.5% Nonidet P-40, 250 mmol/l sodium chloride, 3 mmol/l EDTA, 3 mmol/l EGTA, 1 mmol/l phenylmethylsulfonyl fluoride, 2 mmol/l sodium orthovanadate, 20 µg/ml aprotinin, 1 mmol/l dithiothreitol and 5 µg/ml leupeptin. Proteins in cell lysates were separated in 10% SDS-PAGE gel and then electrophoretically transferred onto a nitrocellulose membrane. All membranes were stained with Ponceau S to confirm equal protein loading. The membrane was blocked with 5% milk in TBS-T (50 mmol/l Tris-HCl, 300 mmol/l NaCl, pH 7.6, 0.1% Tween 20) for 1 h. SDS-PAGE and immunoblotting were carried out as described previously (20). Anti-Bip (GRP74), anti-Chop, anti-phosphorylated eIF2-α, and anti-poly(ADP-ribose) polymerase (PARP) antibodies were purchased from Santa Cruz Biotechnology. Detection was performed using the ECL system (Amersham Pharmacia Biotech).

Luciferase assay

To construct the *WFS1* promoter-luciferase reporter gene, the promoter region of the human *WFS1* gene (−3000 to +20, Genbank Accession No. AC004689)

was PCR-amplified from human genomic DNA. The fragment was inserted upstream from the luciferase cDNA in a pGL3-Basic vector (Promega, Madison, WI, USA). A plasmid, pCMVβ (Clontech, Palo Alto, CA, USA), containing the cytomegalovirus (CMV) promoter-driven β-galactosidase gene was used as an internal control for the normalization of transfection efficiency. One day before transfection, MIN6 cells or *Ins2*^{96Y/Y} cells were plated at 1 × 10⁵/well into 6-well tissue culture plates. The reporter plasmid (0.5 µg) and the pCMVβ (0.5 µg) were co-transfected into MIN6 cells or *Ins2*^{96Y/Y} cells in 6-well tissue culture plates using 10 µl LipofectAMINE 2000 (Invitrogen) in serum-free Opti-MEM medium (Invitrogen). Twenty-four hours after transfection, the medium was changed to DMEM containing 15% fetal calf serum and 20 mmol/l glucose, and cultured for a further 24 h. After this 24-h incubation, MIN6 cells were treated with thapsigargin or tunicamycin for an additional 6 h. Cell extracts were prepared, and luciferase and β-galactosidase activities were determined using a β-galactosidase enzyme assay system according to the manufacturer's protocol (Promega).

Results

Wfs1 expression in the mouse pancreatic islet

Using immunohistochemistry, it was demonstrated that mouse *Wfs1* protein was widely expressed in pancreatic islets except in some peripheral areas, while no signals for *Wfs1* protein were detected in exocrine acinar cells (Figs 1 and 2 and data not shown). Using double-immunofluorescent staining, the majority of *Wfs1*-immunoreactive cells were found to coincide with insulin-producing β-cells. Some minor part of the *Wfs1* immunoreactivity was, however, localized to non-β-cells seen in the islet periphery (Fig. 1A–F). Such *Wfs1*-immunoreactive non-β-cells were found to correspond to somatostatin-producing δ-cells (Fig. 1G–L). There was little difference in *Wfs1*-immunoreactive intensity between the two endocrine cell types (Fig. 1). *Wfs1*-immunoreactivity was not evident in glucagon-producing α-cells or in pancreatic polypeptide cells (PP-cells; Fig. 2).

ER stress induces WFS1 expression in fibroblasts

ER stress induces cellular responses, collectively termed the unfolded protein response, affecting diverse areas of cellular function such as gene expression, metabolism, cell signaling and apoptosis. Certain reagents are known to disturb ER calcium homeostasis or to inhibit post-translational processing or sorting, and thereby to cause ER stress (9). Chemical insults inducing ER stress, the calcium ionophor A23187 and ionomycin,

Figure 1 Mouse Wfs1 protein, insulin and somatostatin expression in mouse pancreatic islets. Double immunostaining for mouse Wfs1 (Wfs1: A, D, G, J; Alexa Fluor 488 label; green) and pancreatic hormones (insulin: B, E; somatostatin: H, K; Alexa Fluor 594 label; red) was performed. Panels C, F, I and L are overlaid images. All fluorescent photomicrographs were taken with a confocal microscope LSM 510 (Carl Zeiss Jena GmbH, Jena, Germany). The approximate positions of E and K are indicated by the rectangular frames in B and H respectively. Small solid arrows in C and F indicate non- β endocrine cells immunoreactive for Wfs1. Small solid arrows in J, K and L show somatostatin-producing δ -cells strongly immunoreactive for Wfs1. Note that insulin-producing β -cells and somatostatin-producing δ -cells display Wfs1 immunoreactivity. Scale bars = 50 μ m in C and I for A, B, and for G, H; 20 μ m in F and L for D, E, and for J, K.

Figure 2 Mouse Wfs1 protein, glucagon and pancreatic polypeptide expression in mouse pancreatic islets. Double immunostaining for mouse Wfs1 (Wfs1: A, D, G, J; Alexa Fluor 488 label; green) and pancreatic hormones (glucagon: B, E; pancreatic polypeptide: H, K; Alexa Fluor 594 label; red) was performed. Panels C, F, I and L are overlaid images. All fluorescent photomicrographs were taken with a confocal microscope LSM 510 (Carl Zeiss Jena GmbH). The approximate positions of E and K are indicated by the rectangular frames in B and H respectively. Large and small solid arrows in D, E and F indicate glucagon-producing α -cells negative for Wfs1 immunoreactivity and non- α endocrine cells positive for Wfs1 immunoreactivity respectively. Large solid arrows in J, K and L show pancreatic polypeptide cells (PP-cells) negative for Wfs1 immunoreactivity. Scale bars = 50 μ m in C and I for A, B, and for G, H; 20 μ m in F and L for D, E, and for J, K.

the ER Ca^{2+} -ATPase inhibitors (21) thapsigargin and cyclopiazonic acid, the ryanodine receptor activator 4-chloro-*m*-cresol, and the protein N-glycosylation inhibitor tunicamycin all induced WFS1 protein as shown in Fig. 3. Only brefeldin A had no effect. Ionomycin only weakly induced WFS1 protein. The differing effects of these chemicals, which have different mechanisms of action, may provide insights into the functions of Wfs1. The lack of WFS1 induction with brefeldin A, a Golgi apparatus disruptor, may be related to its instability in solution (22). Although we did not perform Northern blot analysis for each of these

reagents, A23187 induced WFS1 mRNA in fibroblasts (data not shown).

Effects of thapsigargin and tunicamycin on Wfs1 expression in MIN6 cells

We next examined the effects of thapsigargin and tunicamycin on the expression of Wfs1 mRNA in MIN6 cells. Thapsigargin and tunicamycin treatments are known to induce ER stress, and Chop/GADD153 is a transcription factor that plays a role in ER stress-induced apoptotic cell death (23, 24). Phosphorylation of the α -subunit of translation initiation factor-2 (eIF2- α) attenuates protein translation upon ER stress. Although the ER chaperone Bip/GRP78 expression did not change in MIN6 cells (Fig. 4B) probably due to its strong basal expression, thapsigargin and tunicamycin clearly generated ER stress as demonstrated by Chop induction and eIF2- α phosphorylation (Fig. 4A, B). Under these conditions, ER stress-induced caspase-3 activation, an event at the initiation of apoptosis (25), was evidenced by the cleavage of PARP (Fig. 4C). PARP is one of the substrates cleaved by caspase-3. Upon thapsigargin or tunicamycin treatment, the 113 kDa band decreased, and instead, the proteolytic PARP fragment (89 kDa) appeared (Fig. 4C). In association with ER stress induction and caspase-3 activation, Wfs1 mRNA expression increased (Fig. 4A,D). With thapsigargin, Wfs1 mRNA started to increase after 6 h and was maximal after 12 h. With tunicamycin, Wfs1 mRNA induction peaked at 6 h, and then declined. Wfs1 protein was also increased by thapsigargin treatment (Fig. 4B). In contrast, tunicamycin, despite the mRNA induction, did not increase the Wfs1 protein, but decreased it after 24 h (Fig. 4B). This is probably due to the instability of unglycosylated Wfs1 protein (6, 16).

Thapsigargin and tunicamycin enhance human WFS1 promoter activity in MIN6 cells

To determine the mechanism of WFS1 expression, we examined the effects of thapsigargin and tunicamycin on human WFS1 gene promoter activity by employing transient transfection assays in MIN6 cells. We used a WFS1 promoter-luciferase construct that contained a 3 kb DNA sequence upstream from the human WFS1 gene transcription initiation site. The human WFS1 gene promoter was active in MIN6 cells. Introduction of the WFS1 promoter-reporter plasmid produced a 20-fold increase in luciferase activity as compared with the promoterless pGL3-Basic vector. Treatment of the cells with thapsigargin or tunicamycin resulted in further 1.3- and 1.5-fold increases in luciferase activity respectively (Fig. 5). We conducted these experiments again using a 1 kb (−1000 to +20) WFS1 promoter-luciferase reporter gene. The results were essentially the same but the promoter activity was weaker than with the 3 kb construct (data not shown).

Figure 3 ER stress induces WFS1 protein in fibroblasts. Primary skin fibroblasts were cultured in the presence of (A) A23187 (A23, 1 $\mu\text{mol/l}$), thapsigargin (TG, 1 $\mu\text{mol/l}$), cyclopiazonic acid (CPA, 10 $\mu\text{mol/l}$) and 4-chloro-*m*-cresol (cres, 50 $\mu\text{mol/l}$) for 48 h. (B–D) Cells were treated for the indicated time periods with (B) tunicamycin (tunic), brefeldin A (brefA), (C) A23187, and (D) ionomycin. cont indicates control. Control samples included a vehicle (DMSO), also administered with all of the drugs. Cells were harvested after the incubation periods, and total cell lysates containing 20 μg protein were subjected to Western blot analysis using anti-WFS1c antibody.

Figure 4 Thapsigargin and tunicamycin increase *Wfs1* mRNA expression in MIN6 cells in association with ER stress and apoptosis induction. MIN6 cells were placed in culture dishes and serum-starved in serum-free DMEM for 12 h, and then treated with thapsigargin (1 μ mol/l) or tunicamycin (10 μ g/ml) for 6, 12 or 24 h. Dimethyl sulfoxide (DMSO) was used to dissolve thapsigargin and tunicamycin, and the same concentration of DMSO (final, 0.05%) was employed in all experiments, including controls. After incubation, cells were washed once with ice-cold phosphate-buffered saline, and harvested. (A) Ten micrograms RNA were subjected to Northern blot analysis. (B) Total cell lysates containing equal amounts of protein (50 μ g) were separated on 10% SDS-PAGE and analyzed by immunoblotting using anti-*Wfs1*n, anti-Bip (GRP74), anti-Chop or anti-phosphorylated eIF2- α . (C) Total cell lysates containing equal amounts of protein (50 μ g) were separated on 10% SDS-PAGE and analyzed by immunoblotting using the anti-PARP antibody. Activated caspase-3 cleaves the 113 kDa PARP, resulting in the appearance of the 89 kDa fragment. (D) Quantification of the *Wfs1* mRNA from the results obtained in (A), shown as means $\pm$ S.E. ($n = 4$). Statistical analysis, conducted using analysis of variance, indicated that the thapsigargin and tunicamycin treatments significantly increased *Wfs1* mRNA expression at 12 h and at 6 h and 12 h respectively (* $P < 0.05$, ** $P < 0.001$).

Figure 5 ER stress enhances *WFS1* promoter activity in MIN6 cells. MIN6 cells were transfected with a luciferase reporter plasmid containing a 3.0 kb human *WFS1* gene 5' flanking promoter region (from -3000 to +20) and were exposed to thapsigargin (1 μ mol/l) or tunicamycin (10 μ g/ml) for 6 h. Beta-galactosidase activity from the co-transfected expression vector pCMV β was used to calibrate for transfection efficiency. Basic represents luciferase activity from pGL3-Basic (promoterless) vector-transfected cells. Results are expressed as the fold increase as compared with basic (means $\pm$ S.E. of four independent experiments, each performed in triplicate). P values for comparison of results with versus without drug treatments are 0.034 (thapsigargin, *) and 0.005 (tunicamycin, **) (analysis of variance).

Wfs1 expression is transcriptionally upregulated in β -cells with intrinsic ER stress

In the Akita mouse, the C96Y mutation of the *ins2* gene disturbs intramolecular disulfide bond formation, resulting in progressive β -cell loss (12). ER stress and subsequent apoptosis are at least partially responsible for this progressive β -cell loss (14). To further examine the association between increased *Wfs1* expression and ER stress, we used mouse insulinoma cells derived from an Akita mouse homozygous for the *ins2* gene C96Y mutation (*Ins2*^{96Y/Y} cell) as a model. *Ins2*^{WT/WT} cells derived from normal littermates served as controls. Doubling of the ER chaperone Bip/GRP78 in *Ins2*^{96Y/Y} cells indicated persistent ER stress in these cells (Fig. 6A). In *Ins2*^{96Y/Y} cells, *Wfs1* protein increased sixfold as compared with that in *Ins2*^{WT/WT} cells (Fig. 6B). *Wfs1* mRNA expression was also increased twofold (data not shown). We next examined *WFS1* promoter activity in these cells. Introduction of the *WFS1* promoter-reporter plasmid into *Ins2*^{96Y/Y} cells approximately doubled luciferase activity as compared with that in wild type *Ins2*^{WT/WT} cells (Fig. 7). Luciferase activity after transfection of the SV40 promoter-reporter plasmid did not differ between *Ins2*^{96Y/Y} and *Ins2*^{WT/WT} cells.

Discussion

Herein, we have documented the localization of *Wfs1* expression in the mouse pancreatic islet. Insulin-producing β -cells are the major site of *Wfs1* expression, as shown in Ishihara *et al.* (15). *Wfs1* expression is also evident in somatostatin-producing δ -cells, but is absent from glucagon producing α -cells and PP-cells. No *Wfs1* expression is observed in pancreatic exocrine acinar

Figure 6 *WFS1* expression is increased in Akita mouse-derived *Ins2*^{96Y/Y} cells. Cell extracts of *Ins2*^{96Y/Y} cells containing equal amounts of protein (50 µg) were separated on 10% SDS-PAGE and analyzed by immunoblotting using (A) anti-Bip, and (B) anti-WFS1n and anti-GAPDH antibodies. Insulinoma cells derived from wild type littermates (wild) were used as the control. In (B), cell extracts were prepared on two separate occasions from cells derived from the same mutant mouse.

cells. A histopathological study of pancreatic islets from Wolfram syndrome patients showed selective loss of insulin-producing β -cells and an apparent preservation of glucagon-producing α -, somatostatin-producing δ -, and PP-cells (26, 27). The histochemical evidence of Wfs1 protein localization in insulin-producing β -cells might provide a histological background explaining the insulin deficiency caused by *WFS1* mutations in Wolfram syndrome patients and suggests that *WFS1* protein is necessary for β -cell (28, 29), but not δ -cell survival.

We have also presented evidence herein that ER stress induces *Wfs1* gene expression. Treatment of fibroblasts with A23187, ionomycin, thapsigargin, cyclopiazonic acid, 4-chloro-*m*-cresol or tunicamycin increased *Wfs1* protein levels. Chemical insults by these reagents are known to induce ER stress via disruption of Ca^{2+} homeostasis or inhibition of N-linked glycosylation. Thapsigargin and tunicamycin treatments also induced *Wfs1* mRNA expression in a mouse β -cell line, MIN6 cells. In accordance with the mRNA change, thapsigargin increased Wfs1 protein expression. However, the Wfs1 protein level in MIN6 cells did not change with tunicamycin. This is probably due to Wfs1 being an N-glycosylated protein, and inhibition of glycosylation by tunicamycin decreases its stability (6, 16). Increased *Wfs1* expression in association with ER stress was further demonstrated in another β -cell model with ER stress: *Ins2*^{96Y/Y} cells derived from the Akita mouse. The Akita mouse spontaneously develops early-onset non-obese diabetes with a reduced β -cell mass, which is caused by a conformation-altering missense mutation

(Cys96Tyr) in the insulin-2 gene (12, 13). Intramolecular disulfide-bond formation is disrupted in the mutant insulin molecule. It was reported that this misfolded mutant insulin expression constitutively induced ER stress in Akita mouse β -cells (14). We have indeed confirmed increased Bip protein expression in *Ins2*^{96Y/Y} cells as compared with wild type *Ins*^{WT/WT} cells derived from normal littermates. In *Ins2*^{96Y/Y} cells, *Wfs1* mRNA (data not shown) and protein levels (Fig. 6) were both increased. The increased *Wfs1* mRNA (two-fold, data not shown) was consistent with the increased *Wfs1* promoter activity (Fig. 7). Our results provide further evidence, i.e. a detailed analysis, that *Wfs1* expression increases in association with ER stress, especially in the pancreatic β -cells selectively lost in patients with Wolfram syndrome. It is noteworthy that the increase in Wfs1 protein was marked (sixfold) as compared with the modest increase in Bip expression (twofold) in *Ins2*^{96Y/Y} cells. Mechanisms other than ER stress might have further increased Wfs1 protein expression in this cell line.

The increase in *Wfs1* expression is attributable, at least in part, to enhanced *Wfs1* transcription, because both ER stress-inducing chemical insults (MIN6 cells) and intrinsic ER stress (*Ins2*^{96Y/Y} cells) stimulated *WFS1* promoter activity as demonstrated by a transient transfection assay using a human *WFS1* promoter-luciferase reporter construct. A cis-acting ER stress responsive element (ERSE) has been identified in the proximal promoter regions of chaperone-encoding genes. This element consists of a consensus sequence of

Figure 7 *WFS1* promoter activity is enhanced in *Ins2*^{96Y/Y} cells. *Ins2*^{96Y/Y} cells or wild type (wild) control cells were transfected with a luciferase reporter plasmid containing the 3.0 kb human *WFS1* gene 5' flanking promoter region (from -3000 to +20), or a control plasmid containing the SV40 promoter-luciferase reporter. The expression vector pCMV β was co-transfected, and β -galactosidase activity was used to calibrate for transfection efficiency. Results are expressed as fold-increases relative to luciferase/ β -galactosidase activities in *Ins2*^{96Y/Y} cells as compared with control *Ins2*^{WT/WT} cells in four independent experiments (means $\pm$ S.E.), each performed in triplicate. *WFS1* promoter activity was significantly increased in *Ins2*^{96Y/Y} cells as compared with control *Ins2*^{WT/WT} cells (* P = 0.014, Student's *t*-tests).

CCAAT-N9-CCACG (30). The general transcriptional factor, NF-Y/CBF, binds to the CCAAT motif of the ERSE (31). Once ER stress ensues, p50ATF6 (active form of transcriptional factor ATF6) binds to the CCACG motif of the ERSE (31, 32) resulting in transcriptional induction of ER chaperones. Another ERSE (ERSE-II) with a consensus sequence of ATTGG-N-CCACG has also been identified (32). Although there are six CCAAT motifs in the -2800 to -2300 region of the putative human *WFS1* promoter, we found no ERSE consensus sequences within 3 kb upstream from the transcription initiation site. Further studies will be required to elucidate the mechanism of transcriptional regulation of the *Wfs1* gene via ER stress.

The observations made in this study suggest that Wfs1 protein may be involved in the ER stress response pathway, i.e. the unfolded protein response, in which cells respond by inducing chaperones, attenuating protein translation, and inducing apoptosis. Pancreatic β -cells suffer under chronic ER stress, striving to meet the increasing demands of insulin biosynthesis and secretion. In patients with Wolfram syndrome (26) and in *Wfs1* knock-out mice (15), β -cells were selectively lost from pancreatic islets. Moreover, islets from *Wfs*^{-/-} mice were highly susceptible to ER stress (thapsigargin and tunicamycin)-induced apoptosis (15). It is tempting to speculate that Wfs1 protein is upregulated in response to ER stress and that it plays a physiological role in protecting cells from ER stress-induced apoptosis. Loss of function mutations of the *Wfs1* gene may cause β -cell loss due to disruption of this protective function. It was recently reported that Wfs1 protein expressed in oocytes exhibited a cation-selective ion channel activity (7). Expression of Wfs1 protein in oocytes increased cytosolic Ca²⁺ levels (7), and islets from *Wfs1*^{-/-} mice exhibited attenuated glucose-stimulated intracellular Ca²⁺ responses (15). Wfs1 protein may be involved in the maintenance of ER and intracellular Ca²⁺ homeostasis, and its expression is induced under conditions of perturbed homeostasis, including ER stress.

The current findings that Wfs1 protein, which is predominantly expressed in pancreatic islet β -cells, is transcriptionally upregulated by ER stress indicate a link between Wfs1 protein function and ER stress responses. Further investigations utilizing *Wfs1*^{-/-} mice and *Wfs1*^{-/-} β -cells will provide insights into Wfs1 protein function and the pathophysiology of Wolfram syndrome.

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