nal carotid artery in the mouth region but also that of aberration of the common carotid artery in the neck and may increase the risk of ischemic stroke. Ordinarily, the common carotid artery is covered by the SCM. Advanced age also results in physiologic muscle atrophy. Therefore, careful examination of the neck and mouth, simultaneously with palpation of pulsation (common carotid artery or forarm), is necessary to detect the presence of aberrant carotid arteries.

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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 atropy. 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.

European Journal of Endocrinology **153** 167–176

Introduction

Wolfram syndrome is a rare recessively inherited genetic disorder, which is characteristically associated with juvenile onset diabetes mellitus and progressive optic atropy (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_{cyt}/C_{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.

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ER is a specialized organella 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; Biomeda 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% CO_2 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 prehybridized and hybridized in a buffer containing 50% deionized formamide, 5 × sodium chloride-sodium phosphate-EDTA buffer (750 mmol/l NaCl, 43.25 mmol/l NaH_2PO_4, 6.25 mmol/l EDTA), 2 × Denhardt's solution (0.04% bovine serum albumin, 0.04% Ficoll, 0.04% polyvinylpyrrolidone), and 0.1% soduim 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-proved 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-[^{32}P]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 1×10^5/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,

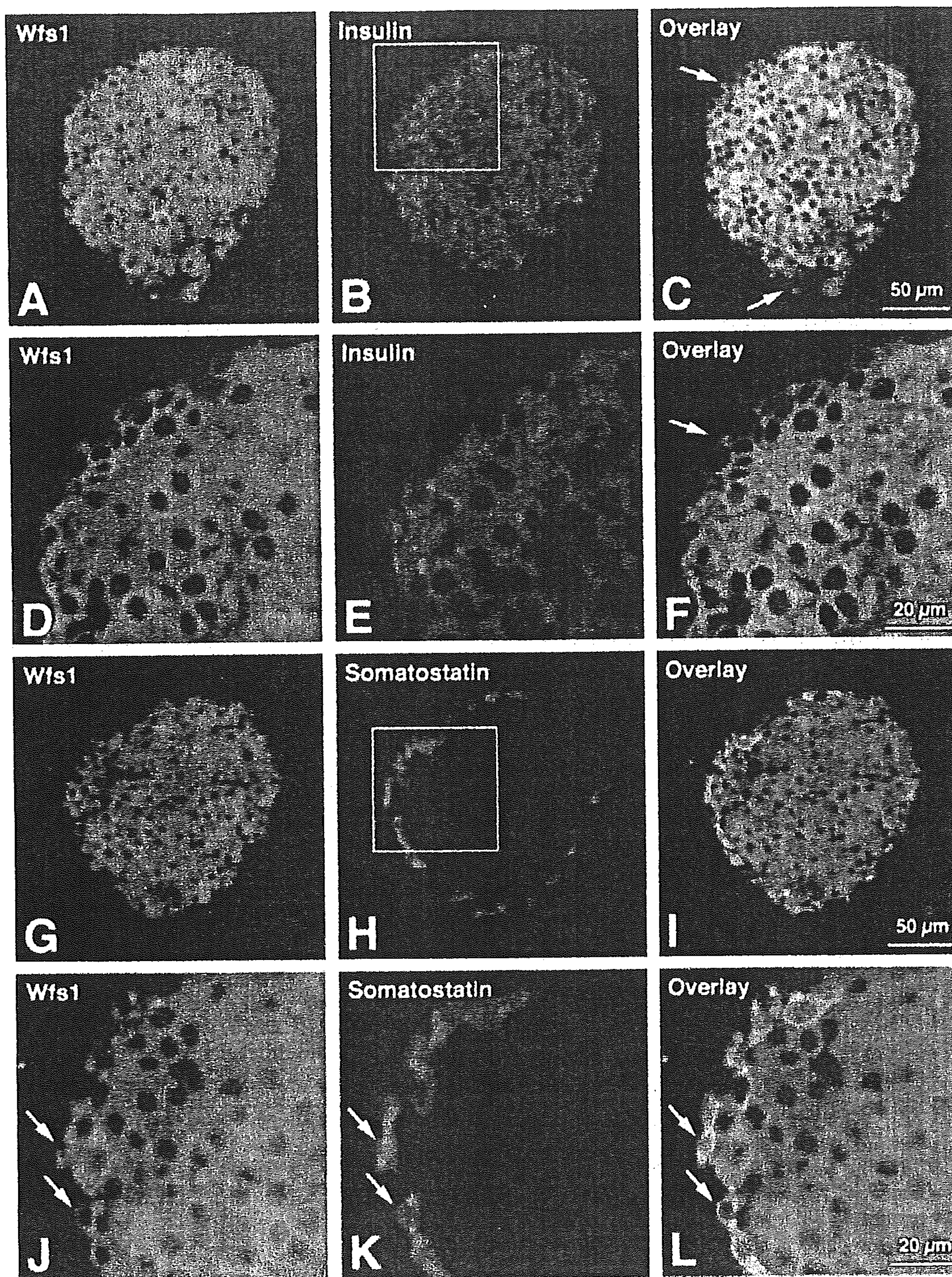

Figure 1 Mouse Wfs1 protein, insulin and somatostatin expression in mouse pancreatic islets. Double immunostaining for mouse Wfs1 (Wfs1: A, D, G, J; Alexa Fluor 488 label; green) and pancreatic hormones (insulin: B, E; somatostatin: H, K; Alexa Fluor 594 label; red) was performed. Panels C, F, I and 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 μm in C and I for A, B, and for G, H; 20 μm in F and L for D, E, and for J, K.

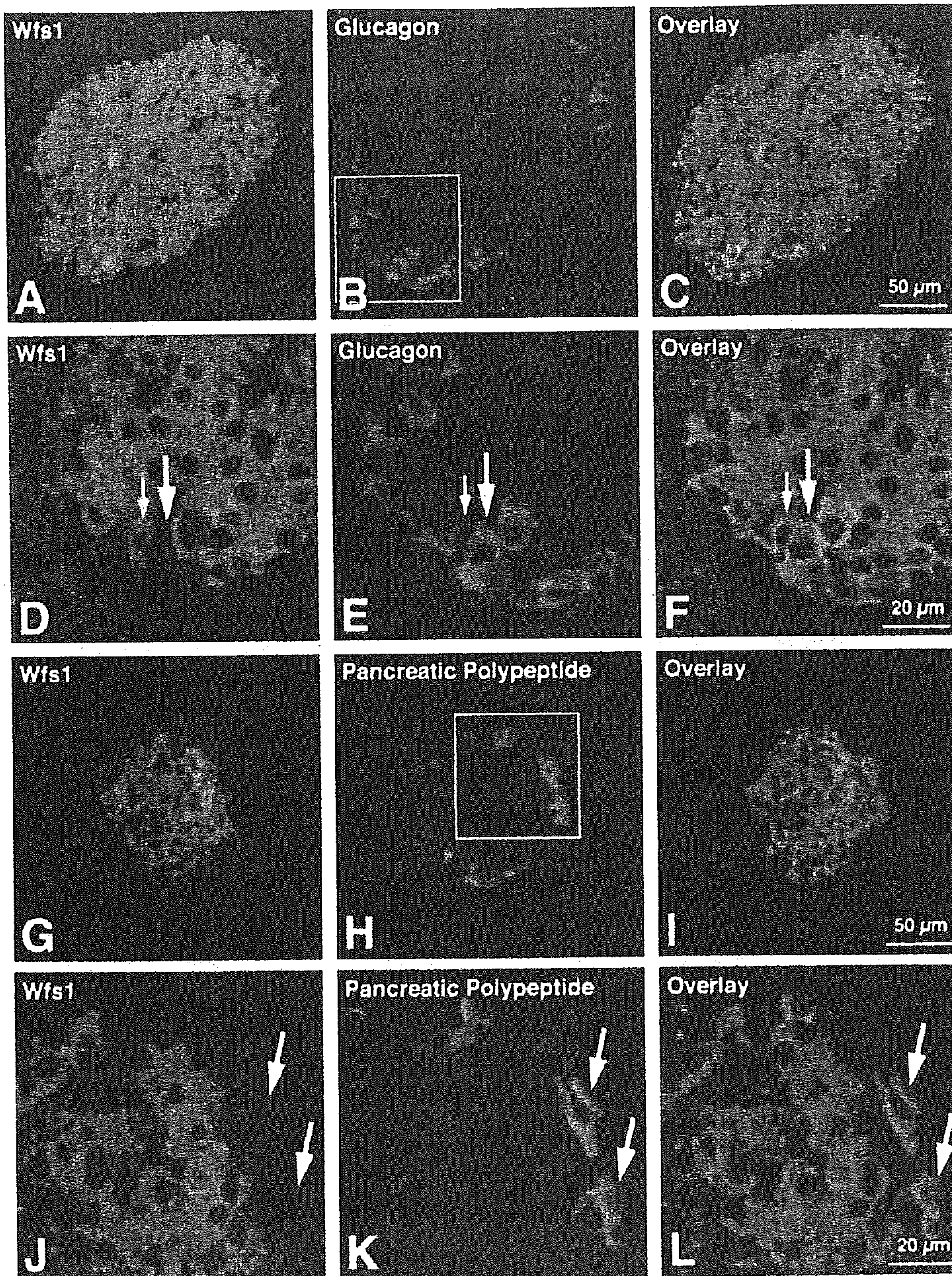

Figure 2 Mouse Wfs1 protein, glucagon and pancreatic polypeptide expression in mouse pancreatic islets. Double immunostaining for mouse Wfs1 (Wfs1: A, D, G, J; Alexa Fluor 488 label; green) and pancreatic hormones (glucagon: B, E; pancreatic polypeptide: H, K; Alexa Fluor 594 label; red) was performed. 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 μm in C and I for A, B, and for G, H; 20 μm 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).

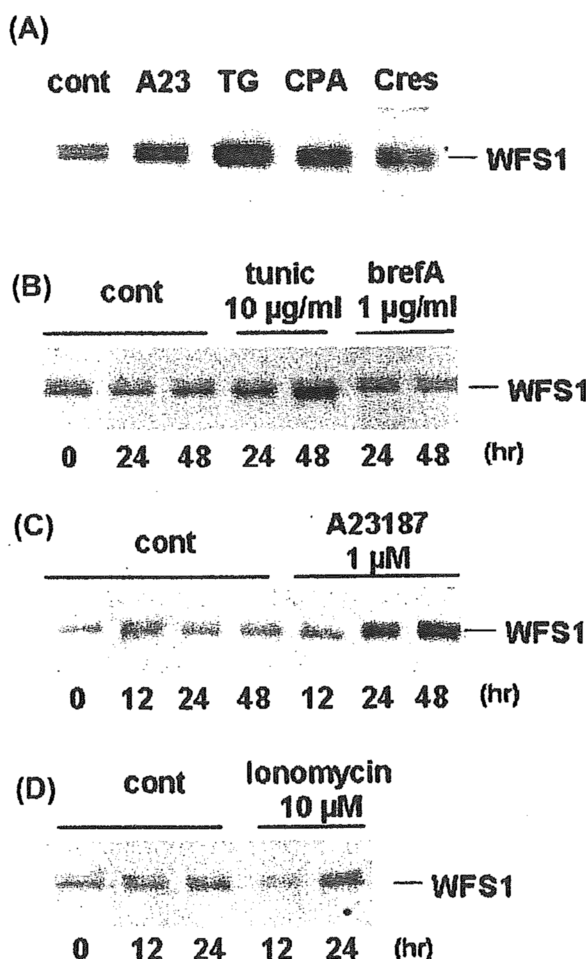

Figure 3 ER stress induces WFS1 protein in fibroblasts. Primary skin fibroblasts were cultured in the presence of (A) A23187 (A23, 1 µmol/l), thapsigargin (TG, 1 µmol/l), cyclopiazonic acid (CPA, 10 µmol/l) and 4-chloro-*m*-cresol (cres, 50 µmol/l) for 48 h. (B–D) Cells were treated for the indicated 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.

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).

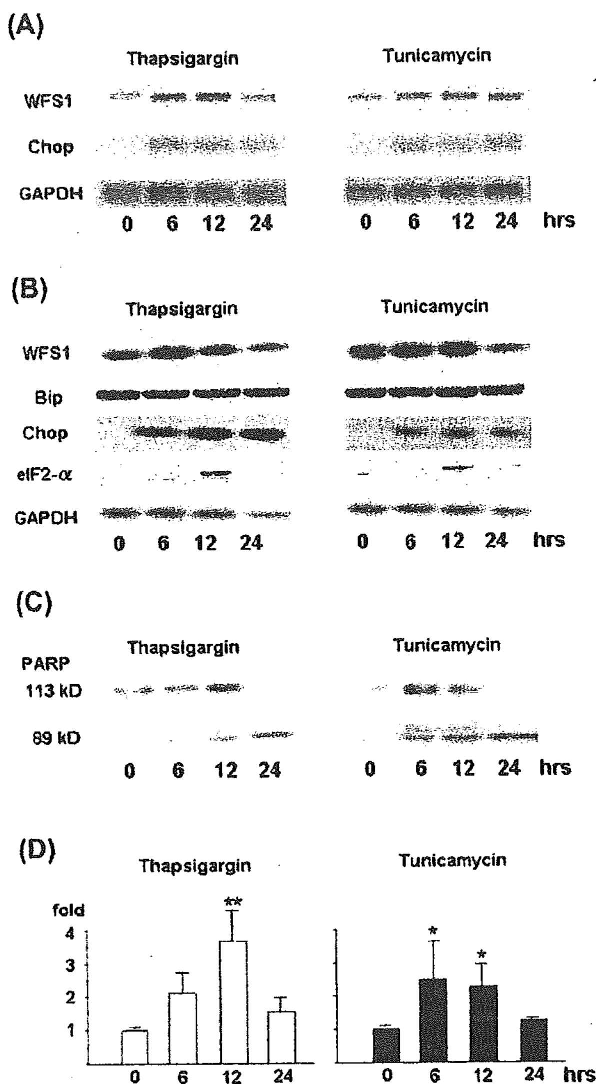

Figure 4 Thapsigargin and tunicamycin increase *Wfs1* mRNA expression in MIN6 cells in association with ER stress and apoptosis induction. MIN6 cells were placed in culture dishes and serum-starved in serum-free DMEM for 12 h, and then treated with thapsigargin (1 μmol/l) or tunicamycin (10 μg/ml) for 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 μg) were separated on 10% SDS-PAGE and analyzed by immunoblotting using anti-Wfs1n, anti-Bip (GRP74), anti-Chop or anti-phosphorylated eIF2-α, (C) Total cell lysates containing equal amounts of protein (50 μg) 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±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 (*$P < 0.05$, **$P < 0.001$).

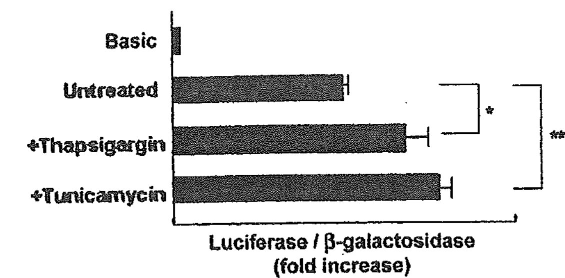

Figure 5 ER stress enhances *WFS1* promoter activity in MIN6 cells. MIN6 cells were transfected with a luciferase reporter plasmid containing a 3.0 kb human *WFS1* gene 5' flanking promoter region (from −3000 to +20) and were exposed to thapsigargin (1 μmol/l) or tunicamycin (10 μg/ml) for 6 h. Beta-galactosidase activity from the co-transfected expression vector pCMVβ 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±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

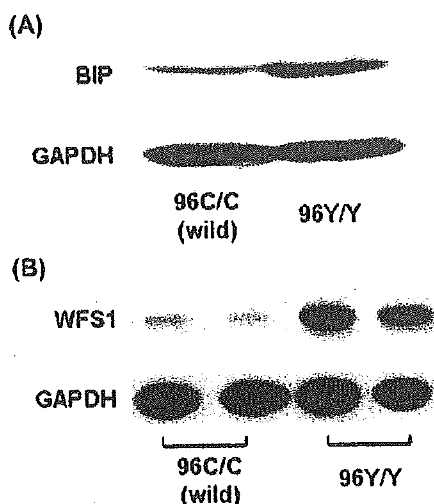

Figure 6 *WFS1* expression is increased in Akita mouse-derived *Ins2*$^{96Y/Y}$ cells. Cell extracts of *Ins2*$^{96Y/Y}$ cells containing equal amounts of protein (50 μg) were separated on 10% SDS-PAGE and analyzed by immunoblotting using (A) anti-Bip, and (B) anti-WFS1n and anti-GAPDH antibodies. Insulinoma 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 (twofold, 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

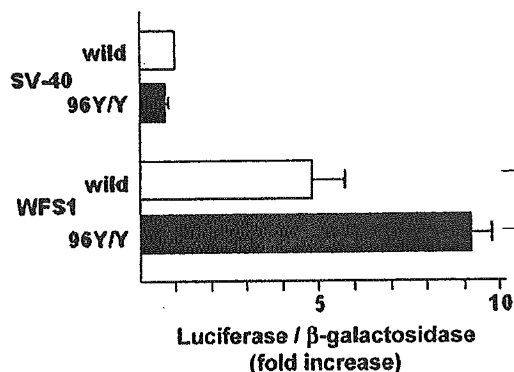

Figure 7 *WFS1* promoter activity is enhanced in *Ins2*$^{96Y/Y}$ cells. *Ins2*$^{96Y/Y}$ cells or wild type (wild) control cells were transfected with a luciferase reporter plasmid containing the 3.0 kb human *WFS1* gene 5′ flanking promoter region (from −3000 to +20), or a control plasmid containing the SV40 promoter-luciferase reporter. The expression vector pCMVβ 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±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 Ca^{2+} levels (7), and islets from *Wfs1*$^{-/-}$ mice exhibited attenuated glucose-stimulated intracellular Ca^{2+} responses (15). Wfs1 protein may be involved in the maintenance of ER and intracellular Ca^{2+} 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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Cell type-specific activation of metabolism reveals that β-cell secretion suppresses glucagon release from α-cells in rat pancreatic islets

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Divisions of [1]Molecular Metabolism and Diabetes and [2]Advanced Therapeutics for Metabolic Diseases, Tohoku University Graduate School of Medicine, Sendai, Miyagi; and [3]Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Mitaka, Tokyo, Japan

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Takahashi, Rui, Hisamitsu Ishihara, Akira Tamura, Suguru Yamaguchi, Takahiro Yamada, Daisuke Takei, Hideki Katagiri, Hitoshi Endou, and Yoshitomo Oka. Cell type-specific activation of metabolism reveals that β-cell secretion suppresses glucagon release from α-cells in rat pancreatic islets. *Am J Physiol Endocrinol Metab* 290: E308–E316, 2006. First published September 27, 2005; doi:10.1152/ajpendo.00131.2005.—Abnormal glucagon secretion is often associated with diabetes mellitus. However, the mechanisms by which nutrients modulate glucagon secretion remain poorly understood. Paracrine modulation by β- or δ-cells is among the postulated mechanisms. Herein we present further evidence of the paracrine mechanism. First, to activate cellular metabolism and thus hormone secretion in response to specific secretagogues, we engineered insulinoma INS-1E cells using an adenovirus-mediated expression system. Expression of the Na^+-dependent dicarboxylate transporter (NaDC)-1 resulted in 2.5- to 4.6-fold ($P < 0.01$) increases in insulin secretion in response to various tricarboxylic acid cycle intermediates. Similarly, expression of glycerol kinase (GlyK) increased insulin secretion 3.8- or 4.2-fold ($P < 0.01$) in response to glycerol or dihydroxyacetone, respectively. This cell engineering method was then modified, using the Cre-*loxP* switching system, to activate β-cells and non-β-cells separately in rat islets. NaDC-1 expression only in non-β-cells, among which α-cells are predominant, caused an increase (by 1.8-fold, $P < 0.05$) in glucagon secretion in response to malate or succinate. However, the increase in glucagon release was prevented when NaDC-1 was expressed in whole islets, i.e., both β-cells and non-β-cells. Similarly, an increase in glucagon release with glycerol was observed when GlyK was expressed only in non-β-cells but not when it was expressed in whole islets. Furthermore, dicarboxylates suppressed basal glucagon secretion by 30% ($P < 0.05$) when NaDC-1 was expressed only in β-cells. These data demonstrate that glucagon secretion from rat α-cells depends on β-cell activation and provide insights into the coordinated mechanisms underlying hormone secretion from pancreatic islets.

pancreatic islet; paracrine regulation; glucagon secretion; cell activation

PANCREATIC ISLETS OF LANGERHANS play a central role in glucose homeostasis. In diabetic patients, not only insulin but also glucagon secretion is impaired. Basal levels of serum glucagon are elevated, and a rise in blood glucose fails to inhibit, and can paradoxically even stimulate, glucagon release in subjects with diabetes (14). In addition, the glucagon secretory response is impaired when circulating glucose drops (11), which may result in life-threatening hypoglycemia in patients treated with insulin. This unresponsiveness to hypoglycemia makes precise glycemic management difficult (8), although strict control is known to be essential for preventing diabetic complications (38). Therefore, it is important to understand the mechanisms whereby glucagon secretion is regulated by nutrients.

Three types of regulatory mechanisms have been proposed by which nutrients, such as glucose, suppress glucagon secretion. The first is a direct action of glucose on α-cells (16, 23). Glucose metabolism in α-cells is considered to generate signals that inhibit glucagon secretion, whereas glucose metabolism increases insulin secretion in β-cells. Therefore, intracellular signaling arising from glucose metabolism might differ between the two cell types, although α-cells also express molecules essential for stimulus-secretion coupling in β-cells, including ATP-sensitive K^+ (K_{ATP}) channels (3, 5). The second mechanism involves modulation by neighboring endocrine cells, such as β- (2, 10, 13, 17, 33, 39) and δ-cells (7, 34). Several molecules, including insulin (2, 13, 33), Zn^{2+} (10, 17), γ-aminobutyric acid (GABA; see Ref. 39), and somatostatin (7, 34), have been postulated to be mediators of these inhibitory effects. Autonomic regulation is the third mechanism (6, 37) and might be clinically important for responses to hypoglycemia, although in humans the glucagon response to hypoglycemia from a transplanted (denerved) pancreas is intact, arguing against this possibility (9).

Studies of stimulus-secretion coupling in β-cells, the predominant cell type of islets, have made great progress in recent decades (20). In contrast, α-cell research has been hampered because of difficulties in getting sufficient numbers of this cell type. Nonetheless, an earlier study has found important characteristics of α-cells (32), and several recent studies have discovered interesting features of this cell type. Characterization of electrical activity and calcium dynamics revealed a unique ion channel composition in α-cells (12). In addition, pyruvate induces glucagon secretion from α-cells (17) but does not stimulate insulin secretion from β-cells. This is probably because α-cells have a transporting system for pyruvate but β-cells do not. This observation suggests that metabolized nutrients can induce exocytosis in α-cells as is the case in β-cells. However, when the pancreas is perfused or islets are stimulated with metabolized nutrients such as glucose, insulin secretion is stimulated, whereas glucagon secretion is suppressed.

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In this study, to gain insight into the regulatory mechanism governing glucagon secretion in islets, we have established a method to activate cellular metabolism in β-cells and non-β-cells separately. For this purpose, we have expressed Na^+-dependent dicarboxylate transporter (NaDC)-1 or glycerol kinase (GlyK) in β-cells and/or non-β-cells. Using this method, we showed rat α-cells to secret glucagon when metabolically activated in the absence of β-cell activation. In addition, basal glucagon secretion was shown for the first time to be suppressed by β-cell activation. These data contribute to our understanding of the regulation of islet hormone secretion, providing insights that are anticipated to be of value in managing hypoglycemia and hyperglycemia in subjects with diabetes.

MATERIALS AND METHODS

Generation of recombinant adenoviruses bearing rat GlyK cDNA (AdRIPHAGlyK and AdCAGlxHAGlyKlx) and NaDC-1 cDNA (AdRIPNaDC and AdCAGlxNaDClx). Rat GlyK cDNA (31) was amplified using rat liver total RNA. An entire coding region was sequenced and subcloned downstream of the hemagglutinin (HA)-epitope sequence. Rat NaDC-1 cDNA was as described previously (36). A *Sph*I-*Spe*I fragment of HA-tagged GlyK (HAGlyK) cDNA and a *Sal*I-*Sma*I fragment of NaDC-1 cDNA were ligated between the 410-bp fragment of the rat insulin 1 promoter and the rabbit β-globin poly(A) signal region. The resulting expression units were used for generation of AdRIPHAGlyK and AdRIPNaDC by the methods described previously (27). Rat GlyK and NaDC-1 cDNA were also subcloned between two *lox*P sequences and ligated under the CAG (a transcriptional unit composed of the cytomegalovirus enhancer, the actin promoter, and the globin intron) promoter unit (28). Recombinant viruses harboring these expression units were then generated (AdCAGlxHAGlyKlx and AdCAGlxNaDClx). AdCAGlacZ (27) expressing β-galactosidase was used as a control adenovirus. AdRIPNCre was renamed from AdInsPNCre generated as described previously (17). Adenovirus titers were measured by the method described previously (27).

Isolation of rat islets and infection with recombinant adenoviruses. Rat islets were prepared by retrograde collagenase infusion through the common bile duct and hand picked under the microscope. Isolated islets were infected with the recombinant adenoviruses at 1.2×10^6 plaque-forming units (PFU)/islet in 1.0 ml medium for 60 min. In the case of combined infection of AdCAGlxNaDClx plus AdRIPNCre or AdCAGlxHAGlyKlx plus AdRIPNCre, the amount of AdRIPNCre was four times greater than the others, with a total amount of 1.2×10^6 PFU/islet.

Immunoblot analysis. INS-1E cells (25) were infected with either AdRIPHAGlyK or AdRIPNaDC at multiplicity of infection (MOI) of 100, cultured for 2 days, and directly dissolved in the SDS sample buffer. Proteins were subjected to SDS-PAGE and were transferred to nitrocellulose membranes. Membranes were probed with rabbit anti-rat NaDC-1 antibody raised against the carboxy-terminal peptide (1:500; see Ref. 36) or with anti-HA tag antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and then incubated for 1 h with anti-rabbit IgG (1:1,000) conjugated with horseradish peroxidase, respectively. Detection was accomplished with chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ).

Immunocytochemical analyses. INS-1E cells infected with either AdRIPHAGlyK or AdRIPNaDC at an MOI of 100 were incubated with anti-rat NaDC-1 antibody (1:500) or with anti-HA tag antibody (1:200) for 1 h at room temperature and then incubated for 1 h with FITC-conjugated anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA). Islets infected with AdRIPHAGlyK, AdCAGlxHAGlyKlx alone, or AdCAGlxHAGlyKlx plus AdRIPNCre were dispersed on coverslips. Cells were then fixed with 4% paraformaldehyde and incubated with anti-HA tag antibody (1:200) followed by incubation with FITC-conjugated anti-rabbit IgG. Insulin and glucagon were also stained using mouse monoclonal antibodies against these hormones (1:1,000; Sigma-Aldrich, Tokyo, Japan) and Texas red-conjugated anti-mouse IgG (1:500; Jackson ImmunoResearch).

Hormone secretion. INS-1E cells (0.2×10^6 cells/well of 24-well plates) or islets (10 islets/tube) infected with recombinant adenoviruses were incubated over a period of 60 min in 1 ml of Krebs-Ringer-bicarbonate-HEPES buffer [140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES (pH 7.4), and 0.1% BSA] containing 2.5 mM glucose plus indicated stimulators. Insulin and glucagon were detected by RIA kits (Linco, St. Louis, MO).

Statistical analyses. Data are presented as means ± SE. Differences between groups were assessed by Student's *t-test* for unpaired data.

RESULTS

Expression of NaDC-1 resulted in cell activation in response to dicarboxylates. We first sought to establish a means of activating metabolism in specific cell types of pancreatic islets to study *1*) the roles of α-cell nutrient metabolism in glucagon secretion and 2) whether activation of neighboring β-cells in response to nutrient metabolism modulates α-cell secretion. It was previously shown that β-cells expressing monocarboxylate transporter (MCT-1) metabolize pyruvate and secrete insulin in response to the monocarboxylate (18). Similarly, insulin secretion is reportedly stimulated in β-cells expressing GlyK in response to glycerol (1, 29). These data suggested that cells normally unresponsive to some nutrients can be activated by expressing protein(s) needed for their metabolism. We tested whether tricarboxylic acid (TCA) cycle intermediates alter insulin and glucagon secretion in isolated rat islets and found α-ketoglutarate, succinate, fumarate, and malate to have no effects on hormone secretion in wild-type islets (Fig. 1). A membrane-permeable analog of succinate, methylsuccinate, is known to stimulate insulin secretion (24), suggesting that inability of TCA cycle intermediates to activate β-cells is attributable to low or no expression of membrane transporters for these compounds. Therefore, to activate cells, a recombinant adenovirus harboring cDNA encoding rat NaDC-1 under the rat insulin promoter (AdRIPNaDC) was constructed, with the aim of activating the cells with TCA cycle intermediates.

We first employed rat insulinoma INS-1E cells to study whether the recombinant adenovirus induces functional expression of NaDC-1, leading to increased cellular metabolism and thereby promoting insulin secretion. Western blotting using an antibody against NaDC-1 showed strong expression of NaDC-1, with the expected protein size, in insulinoma INS-1E cells infected with AdRIPNaDC (Fig. 2*A*). Immunocytochemical analysis revealed strong staining at the cell surface, although weak staining was observed inside the cell, suggesting improper targeting of some expressed membrane proteins because of forced expression (Fig. 2*B*). As shown in Fig. 2*C*, adenovirus-mediated expression of NaDC-1 made INS-1E cells responsive to various TCA cycle intermediates. Citrate, one of the tricarboxylates, existing partly in a divalent form at pH 7.4 (36), could be transported into INS-1E cells expressing NaDC-1, and thereby induced insulin secretion. A nonmetabolizable dicarboxylate, glutarate, failed to induce insulin secretion from INS-1E cells expressing NaDC-1, indi-

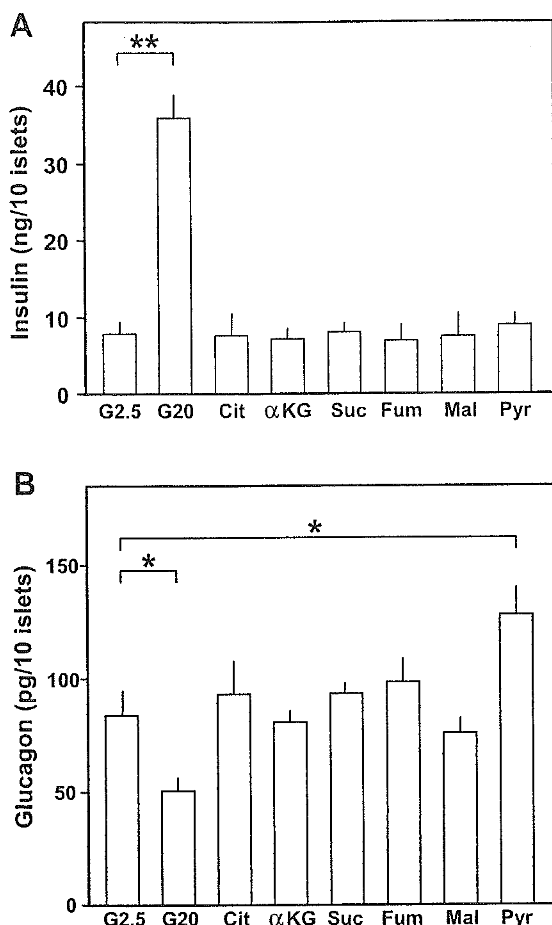

Fig. 1. Tricarboxylic acid (TCA) cycle intermediates had no effects on either insulin or glucagon secretion. Isolated islets were cultured overnight and challenged with glucose (20 mM), pyruvate (10 mM), and various TCA cycle intermediates (10 mM). Insulin (*A*) and glucagon (*B*) secreted during a 60-min incubation were measured. G2.5, 2.5 mM glucose; G20, 20 mM glucose; Cit, citrate; αKG, α-ketoglutarate; Suc, succinate; Fum, fumarate; Mal, malate; Pyr, pyruvate. Data are means ± SE; $n = 3\sim7$. $^*P < 0.05$ and $^{**}P < 0.01$.

cating insulin secretion evoked by dicarboxylates to be the result of activation of metabolism. In addition, insulin secretion evoked by malate was abolished by 2 mM NaN_3, a metabolic inhibitor (data not shown), further supporting this notion.

Expression of GlyK resulted in cell activation in response to glycerol. We also expressed rat GlyK to activate the cellular glycolytic pathway. Glycerol and dihydroxyacetone can enter the glycolytic pathway after GlyK-mediated conversion to glycerol 3-phosphate and dihydroxyacetone phosphate, respectively. Adenovirus-mediated introduction of HAGlyK cDNA resulted in expression of this protein in the INS-1E cell cytosol (Fig. 2, *D* and *E*). Expressed HAGlyK was functional, since INS-1E cells expressing HAGlyK secreted insulin in response to glycerol or dihydroxyacetone (Fig. 2*F*), as was reported in INS-1E cells expressing *Escherichia coli* GlyK (1, 29).

Taken together, these data indicate NaDC-1 and GlyK expressions to be effective in activating cellular metabolism in response to certain nutrients.

Cell type-specific expressions of genes in isolated islets. To study the stimulus-secretion coupling in α-cells and possible cross-talk with other pancreatic endocrine cells, we next sought to express the genes of interest in α- and β-cells separately. As was reported previously (17), the rat insulin 1 promoter has high transcription activity and specificity for β-cell-restricted expression of foreign genes. Therefore, β-cell-specific expression of NaDC-1 or HAGlyK was achieved using recombinant adenovirus vectors with the rat insulin 1 promoter (Fig. 3*A*). When islets were infected with AdRIPHAGlyK, >60% of insulin-positive cells were stained with HA (Fig. 3*D*), but none of the glucagon-positive cells expressed HAGlyK (Fig. 3*E*).

In contrast to the insulin 1 promoter for β-cells, the glucagon promoter (1.6 kbp) did not have high transcriptional activities specific for α-cells when placed in the adenoviral genome (data not shown). To increase expression in α-cells, a dual-adenovirus approach was previously developed (17); one adenovirus produces Cre recombinase under the glucagon promoter, and the other virus expresses the desired genes under the potent CAG promoter unit (28) once the intervening sequence is excised by Cre recombinase (17). Although the strategy increased the expression levels of reporter genes, such as luciferase, in α-cells, it did not significantly increase the activities of cellular enzymes or transporters, such as glucokinase and MCT-1 (data not shown). We then raised the recombinant virus titer. However, a high titer of virus with the glucagon promoter also induced expression in cells other than α-cells (data not shown). We therefore did not employ the glucagon promoter in this study but rather devised a method employing the insulin 1 promoter and the Cre-*loxP* system.

As shown in Fig. 3*C*, a cDNA floxed with *loxP* sequences was placed downstream from the CAG promoter unit (28) that enables transcription in any cell type. This expression unit was then introduced into islet cells, together with the insulin promoter-Cre adenovirus (AdRIPNCre; see Ref. 17). The cDNA was expected to be removed from the unit by the Cre recombinase in the β-cell, allowing expression of the genes of interest in non-β-cells, a cell population where α-cells are predominant. Indeed, when rat islets were infected with AdCAGlxHAGlyKlx and AdRIPNCre, ~70% of glucagon-positive cells was stained with HA (Fig. 3, *H* and *I*). More than 80% of HA-positive cells were observed to be stained with glucagon, and <10% were insulin positive, although HA staining was occasionally observed in somatostatin-positive cells and fibroblast-like cells (data not shown). When islets were infected with AdCAGlxHAGlyKlx alone (Fig. 3*B*), ~60% of β-cells (Fig. 3*F*) and 65% of α-cells (Fig. 3*G*) expressed HAGlyK.

α-Cell activation triggered glucagon secretion when β-cells remained nonactivated. To study the role of nutrient metabolism in glucagon secretion from α-cells, isolated rat islets were infected with AdCAGlxNaDClx plus AdRIPNCre and challenged with succinate or malate. As shown in Fig. 4, *A* and *B*, glucagon secretion was increased by 80%, without changes in insulin secretion. These effects were abolished by 2 mM NaN_3, indicating the observed glucagon secretion to be due to activation of cellular metabolism of the dicarboxylates. When islets were infected with AdCAGlxHAGlyKlx plus AdRIPNCre and then challenged with 10 mM glycerol, insulin secretion did not change (Fig. 4*C*) and glucagon secretion tended to increase, but the differences did not reach statistical significance (Fig. 4*D*).

In contrast, when α-cells were activated together with β-cells by infecting islets with AdCAGlxNaDClx alone, i.e., without AdRIPNCre (Fig. 5, *A* and *B*), insulin secretion was increased by more than threefold in response to 10 mM malate

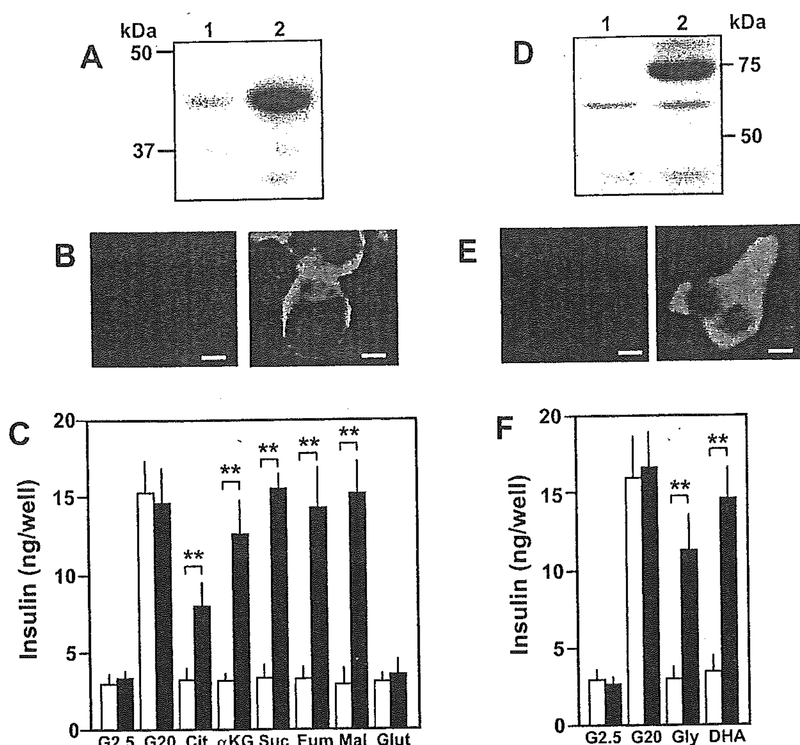

Fig. 2. Adenovirus-mediated Na^+-dependent dicarboxylate transporter (NaDC)-1 or glycerol kinase (GlyK) expression in INS-1E cells. *A*: INS-1E cells infected with either AdCAGlacZ (*lane 1*) or AdRIPNaDC (*lane 2*) were subjected to SDS-PAGE and probed with an anti-NaDC-1 antibody. *B*: INS-1E cells infected with either AdCAGlacZ (*left*) or AdRIPNaDC (*right*) were stained with anti-NaDC-1 antibody. Bars, 4 μm. *C*: INS-1E cells infected with either AdCAGlacZ (open bars) or AdRIPNaDC (filled bars) were challenged with 20 mM glucose or various TCA cycle intermediates (10 mM). Insulin secreted during a 60-min incubation was measured. Data are means ± SE; $n = 5$. $^{**}P < 0.01$. Glut, glutarate. *D*: INS-1E cells infected with either AdCAGlacZ (*lane 1*) or AdRIPHAGlyK (*lane 2*) were subjected to SDS-PAGE and probed with an anti-hemagglutinin (HA) antibody. *E*: INS-1E cells infected with either AdCAGlacZ (*left*) or AdRIPHAGlyK (*right*) were stained with an anti-HA antibody. Bars, 4 μm. *F*: INS-1E cells infected with either AdCAGlacZ (open bars) or AdRIPHAGlyK (filled bars) were challenged with 20 mM glucose, 10 mM glycerol (Gly), or 10 mM dihydroxyacetone (DHA). Insulin secreted during a 60-min incubation was measured. Data are means ± SE; $n = 4$. $^{**}P < 0.01$.

or succinate, whereas glucagon release was unchanged. Similarly, AdCAGlxHAGlyKlx infection increased insulin (by 2.5-fold) but not glucagon secretion with a 10 mM glycerol challenge (Fig. 5, *C* and *D*). These data indicate that α-cell activation dose not lead to glucagon secretion when β-cells are activated simultaneously.

Recent studies have postulated several molecules, including insulin (2, 13, 33), Zn^{2+} (10, 17), and GABA (39), as mediators of β-cell inhibitory effects on glucagon secretion. During succinate stimulation, insulin secreted from β-cells expressing NaDC-1 amounted to ~25 ng/ml (Fig. 5*A*). We therefore examined whether this amount of insulin inhibits glucagon secretion from islets expressing NaDC-1 in α- but not β-cells. As shown in Fig. 6, succinate-stimulated glucagon secretion from islets infected with AdCAGlxNaDClx plus AdRIPNCre was significantly suppressed by 25 ng/ml insulin, indicating that insulin mediates inhibitory effects of β-cells on glucagon secretion. However, the suppression seemed incomplete, sug-

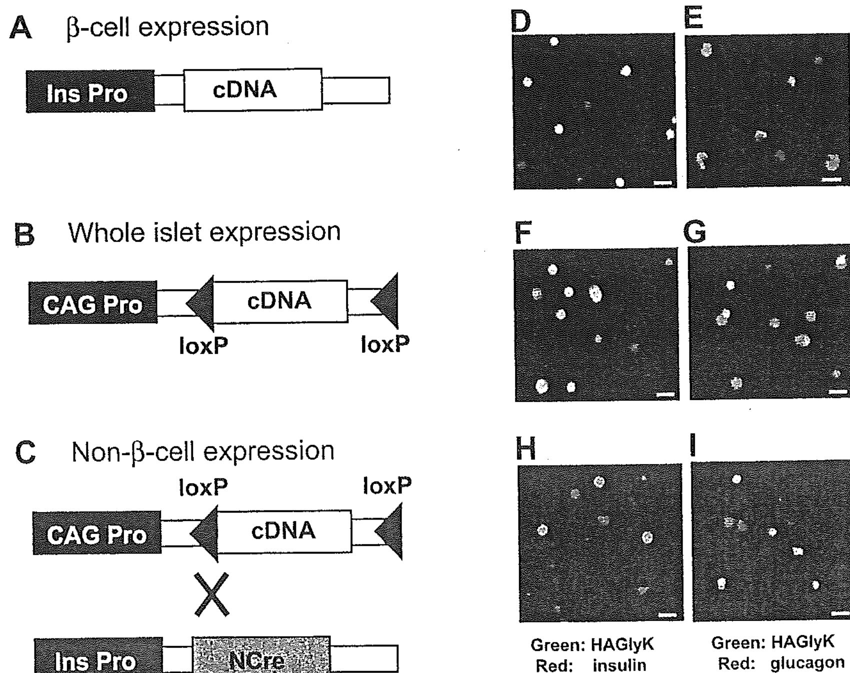

Fig. 3. Selective gene expression method for β- and non-β-cells in islets. *A–C*: schematic representation of adenoviruses for expression in β-cells (*A*), whole islet cells (*B*), and non-β-cells (*C*). NCre, nuclear targeted Cre recombinase; CAG, a transcriptional unit composed of the cytomegalovirus enhancer, the actin promoter, and the globin intron (28). *D* and *E*: islets infected with AdRIPHAGlyK were dispersed and stained with an anti-HA (green) antibody (*D* and *E*) together with anti-insulin (red; *D*) or anti-glucagon (red; *E*) antibody. *F* and *G*: islets infected with AdCAGlxHAGlyKlx alone were dispersed and stained with an anti-HA (green) antibody (*F* and *G*) together with anti-insulin (red; *F*) or anti-glucagon (red; *G*) antibody. *H* and *I*: islets infected with AdCAGlxHAGlyKlx plus AdRIPNCre were dispersed and stained with an anti-HA (green) antibody (*H* and *I*) together with anti-insulin (red; *H*) or anti-glucagon (red; *I*) antibody. Bars, 10 μm. Colocalization resulted in yellow.

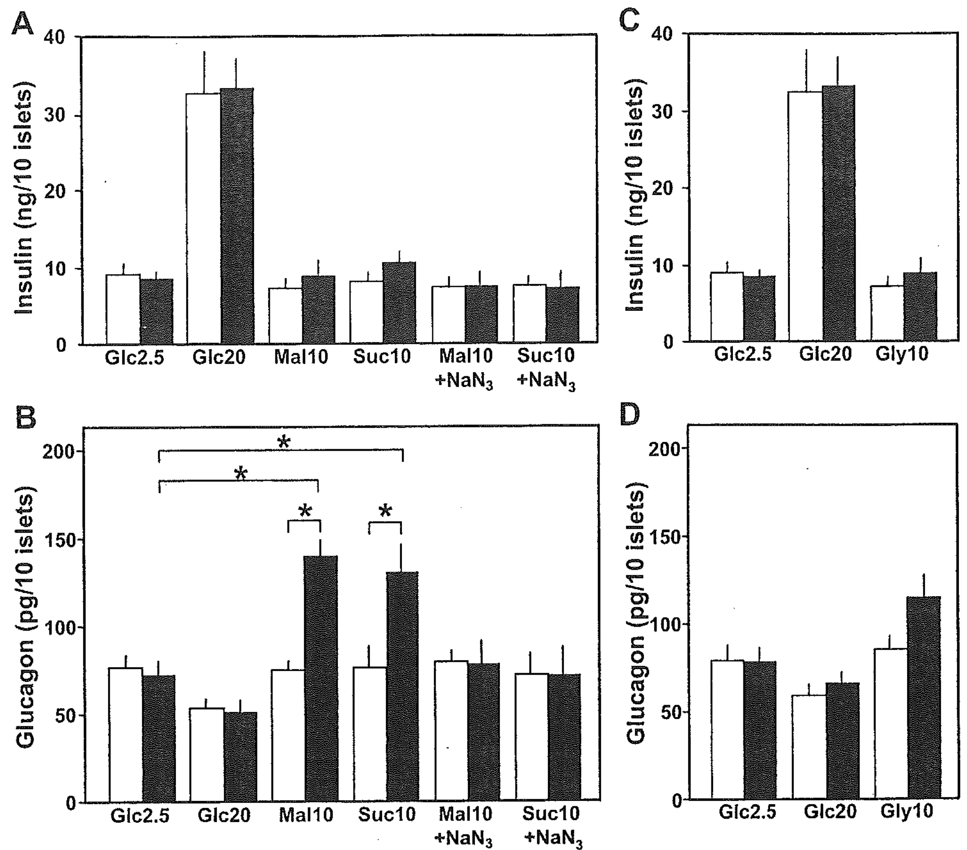

Fig. 4. Selective α-cell activation induced glucagon secretion. *A* and *B*: isolated islets (10 islets/tube) infected with AdCAGlacZ plus AdRIPNCre (open bars) or AdCAGlx-NaDClx plus AdRIPNCre (filled bars) were challenged with 20 mM glucose, 10 mM malate (Mal10), or 10 mM succinate (Suc10) with or without 2 mM NaN_3. Insulin (*A*) and glucagon (*B*) secreted during a 60-min incubation were measured; $n = 3\sim5$. $^*P < 0.05$. *C* and *D*: isolated islets (10 islets/tube) infected with AdCAGlacZ plus AdRIPNCre (open bars) or AdCAGlxHAGlyKlx plus AdRIPNCre (filled bars) were challenged with 20 mM glucose, or 10 mM glycerol (Gly10). Insulin (*C*) and glucagon (*D*) secreted during a 60-min incubation were measured; $n = 4$.

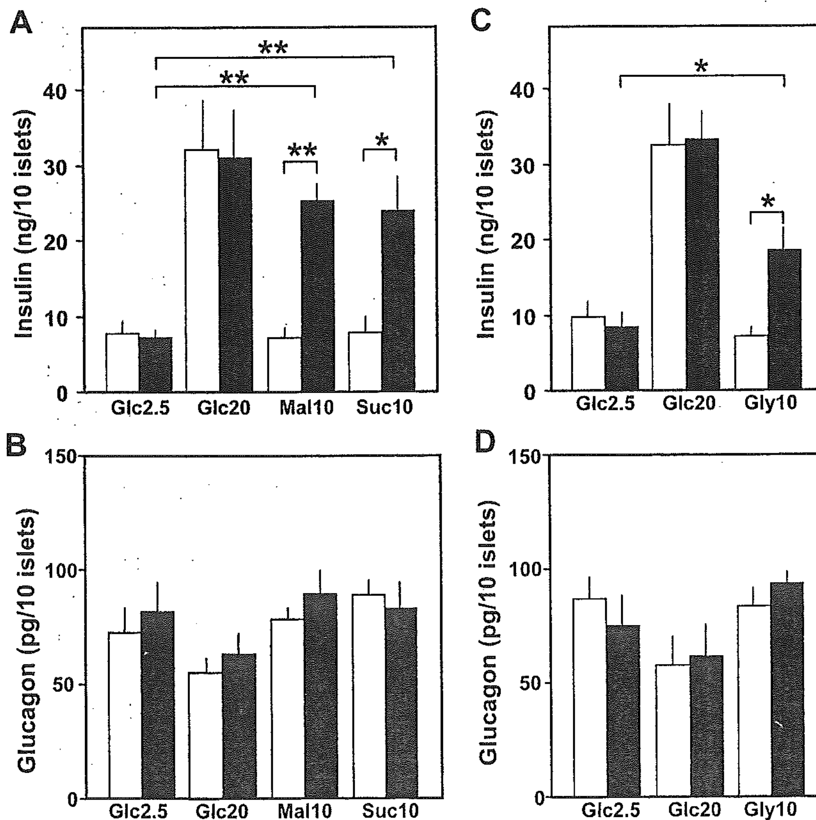

Fig. 5. Simultaneous α- and β-cell activation induced insulin but not glucagon secretion. *A* and *B*: isolated islets (10 islets/tube) infected with AdCAGlacZ (open bars) or AdCA-GlxNaDClx (filled bars) were challenged with 20 mM glucose, 10 mM malate, or 10 mM succinate. Insulin (*A*) and glucagon (*B*) secreted during a 60-min incubation were measured; $n = 4\sim6$. $^*P < 0.05$ and $^{**}P < 0.01$. *C* and *D*: isolated islets (10 islets/tube) infected with AdCAGlacZ (open bars) or AdCAGlxHAGlyKlx (filled bars) were challenged with 20 mM glucose, or 10 mM glycerol. Insulin (*C*) and glucagon (*D*) secreted during a 60-min incubation were measured; $n = 4$. $^*P < 0.05$.

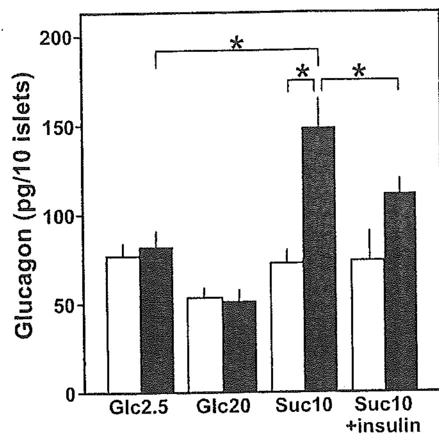

Fig. 6. Insulin suppressed succinate-stimulated glucagon secretion. Isolated islets (10 islets/tube) infected with AdCAGlacZ plus AdRIPNCre (open bars) or AdCAGlxNaDClx plus AdRIPNCre (filled bars) were challenged with 20 mM glucose, 10 mM succinate alone, or 10 mM succinate with 25 ng/ml insulin. Glucagon secreted during a 60-min incubation was measured; $n = 4$. $*P < 0.05$.

gesting another molecule(s) is also important in suppression of glucagon secretion by β-cell activation.

β-Cell activation increased insulin secretion and decreased basal glucagon secretion. We next studied whether basal glucagon secretion was altered by β-cell activation. For this purpose, NaDC-1 was expressed only in β-cells by infecting islets with AdRIPNaDC. When these islets were challenged with 10 mM malate or succinate, insulin secretion more than doubled (Fig. 7*A*). Interestingly, glucagon secretion from the same islets was reduced significantly, by 30% (Fig. 7*B*). Similar modulation of hormone secretion was observed when islets were infected with AdRIPGlyK; insulin secretion was increased significantly, by 1.7-fold, in response to 10 mM glycerol (Fig. 7*C*), whereas glucagon secretion tended to decrease, although not to a statistically significant degree (Fig. 7*D*).

DISCUSSION

Abnormal nutrient-mediated modulation of glucagon secretion is often associated with diabetes mellitus. However, the mechanisms whereby nutrients modulate glucagon secretion remain poorly understood. Paracrine modulation by β- or δ-cells is among the postulated mechanisms. Herein we provide further evidence that glucagon secretion from α-cells is stimulated by nutrient metabolism in the absence of β-cell activation but it is suppressed when β-cells are activated.

We first demonstrated, employing NaDC-1 expression, that TCA cycle intermediates induce insulin secretion from β-cells engineered to transport these substrates. It was previously reported that pyruvate and lactate stimulate insulin secretion from β-cells expressing MCT-1 and lactate dehydrogenase (LDH) but not from normal β-cells (18). This was interpreted as low levels of MCT-1 and LDH expression protecting β-cells

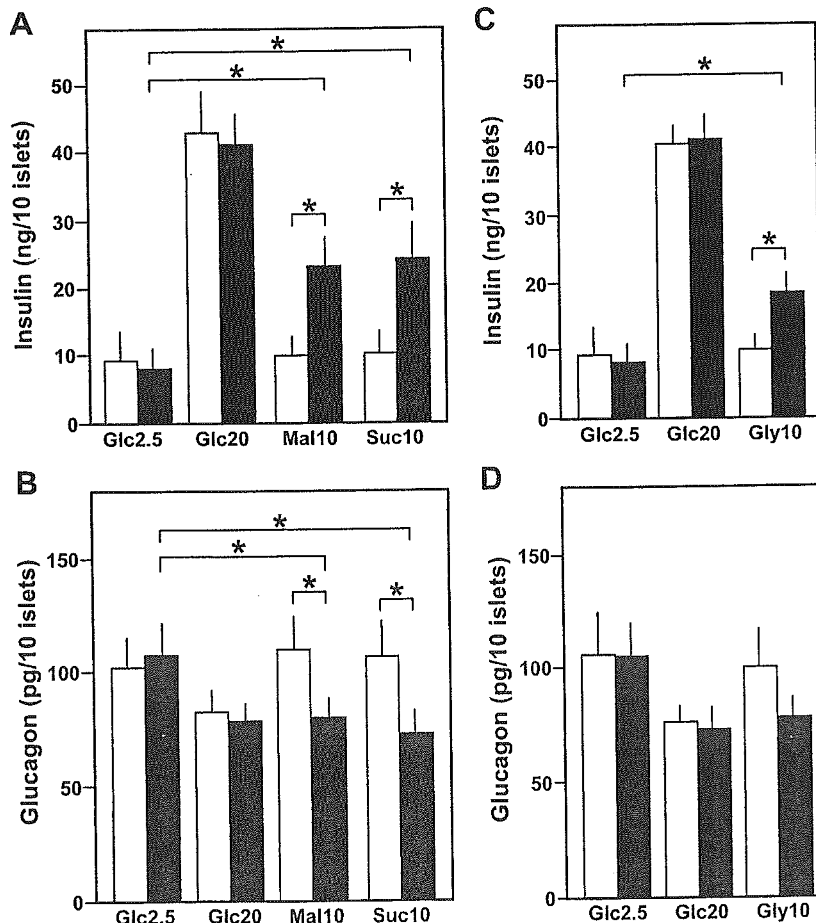

Fig. 7. Selective β-cell activation inhibited glucagon secretion. *A* and *B*: isolated islets (10 islets/tube) infected with AdCAGlacZ (open bars) or AdRIPNaDC (filled bars) were challenged with 20 mM glucose, 10 mM malate, or 10 mM succinate. Insulin (*A*) and glucagon (*B*) secreted during a 60-min incubation were measured. $*P < 0.05$; $n = 5$. *C* and *D*: isolated islets (10 islets/tube) infected with AdCAGlacZ (open bars) or AdRIPHAGlyK (filled bars) were challenged with 20 mM glucose or 10 mM glycerol. Insulin (*C*) and glucagon (*D*) secreted during a 60-min incubation were measured. $*P < 0.05$; $n = 4$.

from the stimulatory effects of pyruvate and lactate, which could otherwise cause undesired insulin secretion in catabolic states, such as during exercise. Similarly, the present data suggest that low levels of NaDC-1 expression protect β-cells from the stimulatory effects of dicarboxylates and confer glucose selectivity on insulin secretion.

A major part of the present study was based on the method we devised to activate cellular metabolism in β-cells and non-β-cells, separately, in primary rat islets by specifically expressing NaDC-1 or GlyK in β-cells and non-β-cells. Employing this innovative approach, we showed TCA cycle intermediates, succinate and malate, to induce glucagon secretion when NaDC-1 is expressed in α-cells. Stimulation of glucagon secretion was previously demonstrated in intact islets challenged with another mitochondrial substrate, pyruvate, which exerts essentially no stimulatory effects on β-cells (17). In subjects with type 1 diabetes, glucose reportedly failed to suppress, or even slightly stimulated, glucagon secretion (14). Abnormal glucagon secretion in response to glucose was also reported in islets from insulin-deficient Chinese hamsters (19). The present data, obtained employing NaDC-1 expression, thus support the concept of α-cells having an inherent capacity to increase glucagon secretion in response to nutrients under certain circumstances, i.e., in the absence of β-cell effects. GlyK expression in α-cells tended to increase glucagon secretion in response to glycerol, but not to a statistically significant degree (Fig. 3*D*). This might be because the coupling of glycolysis and mitochondrial metabolism is less efficient in α-cells than in β-cells, as previously suggested (35). In contrast, dicarboxylates directly stimulate mitochondrial metabolism in cells expressing NaDC-1, thereby possibly producing a significant increase in glucagon secretion.

The concept of α-cells having an inherent capacity to increase glucagon secretion in response to nutrients has been reinforced recently by the demonstration of glucagon secretion stimulated by glucose from purified rat α-cells (10). An earlier study (32), however, reported that glucose inhibited glucagon secretion induced by an amino acid mixture in purified α-cells. Thus direct action on α-cells could be multiple, both inhibitory and stimulatory in nature. Glucose reportedly promotes the filling of the endoplasmic reticulum Ca^{2+} stores in α-cells (23) as in β-cells (20). In the presence of an amino acid mixture, glucose inhibitory effects could attenuate the rise in cytosolic Ca^{2+} induced by amino acids, whereas glucose stimulatory effects could be masked by amino acid-stimulated Ca^{2+} elevation.

Glucagon secretion stimulated by pyruvate was previously shown to be suppressed by activation of β-cells expressing MCT-1 (17). Similar inhibition of activated glucagon secretion by β-cell secretory activities was recently reported in β-cell-specific Foxa2 knockout mice (22). Islets from these mice secreted insulin in response to an amino acid mixture, and, interestingly, the glucagon secretion that is normally seen in the wild-type islets in response to amino acids was abolished in the mutant islets. This result is consistent with the notion that suppression of activated glucagon secretion is attributable to β-cell secretory activities. In the present study, for the first time, we have shown basal glucagon secretion to also be suppressed by β-cell activation. In addition, in NaDC-1-expressing cells, glucose stimulated insulin secretion more potently (an ~4.5-fold increase) than dicarboxylates (an ~2-fold increase; Fig. 7*A*), whereas glucose and dicarboxylates suppressed glucagon secretion to a similar extent (~30%). We speculated that this is because, when islets were challenged with glucose, α-cells were also activated for glucagon secretion, which counteracted the suppressing effect exerted by β-cell secretory activities. Recent studies demonstrated that insulin (2, 13, 33), Zn^{2+} (10, 17), and GABA (39) are candidates for β-cell-derived inhibitory substances of glucagon secretion in rat islets. Our observation of inhibitory effects of insulin on succinate-stimulated glucagon secretion from islets expressing NaDC-1 in α- but not β-cells supports this notion about the role of insulin. To study roles of Zn^{2+} and GABA, it is crucial to determine amounts of these molecules secreted from β-cells during nutrient stimulation. Further studies are needed to elucidate the molecular basis of β-cell inhibitory effects.

Glucagon secretion was reported to depend differentially on Ca^{2+} influx through N- and L-type Ca^{2+} channels (12, 16). N-type Ca^{2+} channels operate predominantly under basal conditions and L-type Ca^{2+} channels in the stimulated state. β-Cell activation suppressed glucagon secretion regardless of whether α-cells were in the basal (Fig. 7*B*) or the stimulated state (Fig. 4*B*; see Refs. 17 and 22), suggesting the suppressed glucagon secretion to possibly be due to direct inhibition of two Ca^{2+} channels or to indirect inhibition of Ca^{2+} channels resulting from prevention of membrane depolarization. The latter could be achieved by opening of $GABA_A$ receptor Cl^- channels in the α-cell (39). In addition, prevention of membrane depolarization is also brought about by activation of K_{ATP} channels, which is reportedly induced by the β-cell secretory products, Zn^{2+} (4, 10) and insulin (10, 21). However, involvement of K_{ATP} channels in regulating glucagon secretion is controversial, since different glucagon responses were demonstrated in the following two mutant islets lacking functional K_{ATP} channels: preserved glucagon responses from islets deficient in one of the K_{ATP} channel subunits, Kir6.2 (26), and no response from islets deficient in another subunit, sulfonylurea receptor 1 (16).

Although inhibition of glucagon secretion by activation of β-cells expressing NaDC-1 supports the paracrine mechanism, it does not exclude a direct inhibitory effect of glucose metabolism on glucagon secretion, especially at relatively low glucose concentrations and in the presence of stimulators of glucagon secretion, such as an amino acid mixture (see above). Two different mechanisms by which glucose directly suppresses glucagon secretion have been proposed. One involves a store-operated current, which controls a depolarizing cascade leading to opening of L-type Ca^{2+} channels in α-cells (23). Thus glucose-induced ATP generation stimulates Ca^{2+} sequestration in endoplasmic reticulum and modulates a store-operated current. Another is based on low K_{ATP} channel activity and the special ion channel composition of the α-cell (5, 15); K_{ATP} channel closure by ATP produced during glucose metabolism causes modest depolarization, which inactivates, instead of activating, voltage-gated Na^+, T- and N-type Ca^{2+}, and A-type K^+ channels participating in action potential generation. Both models are based on data obtained in mouse α-cells, in which the K_{ATP} channel density is much less than

that in rat α-cells. Rat α-cells were calculated to have nearly 100-fold more K_{ATP} channels than mouse α-cells and double the number in rat β-cells (3, 5). K_{ATP} channels couple nutrient metabolism to membrane depolarization. Therefore, in rat α-cells with a greater number of K_{ATP} channels, nutrient metabolism could induce greater changes in membrane potential compared with those in mouse α-cells, thereby allowing glucagon secretion. Thus the importance of paracrine inhibition might be species dependent. It is essential to establish the level of K_{ATP} channel expression in human α-cells and whether this channel contributes to the regulation of glucagon secretion in humans. In this context, it is noteworthy that K_{ATP} channel-blocking agents stimulated glucagon secretion in subjects with insulin-deficient type 1 diabetes (30).

In summary, our findings provide further evidence supporting the concept that α-cell exocytosis can be modulated by β-cells via a paracrine mechanism. Future studies should focus on detailed molecular analyses of stimulus-secretion coupling in α-cells under paracrine regulation. This is a promising approach to identifying new drug targets for treating α-cell abnormalities in diabetic patients.

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GRANTS

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