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.

Acknowledgements

We thank Professor Junichi Miyazaki, Osaka University, Japan, for providing us with MIN6 cells. This study was supported in part by Grants-in-Aid for Scientific Research (14370338 and 16390096 to Y Tanizawa) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, grant no.15591228 (to J Kawano) from the Japan Society for Promotion of Science, and a grant from Takeda Science Foundation.

References

1 Barrett TG & Bundey SE. Wolfram (DIDMOAD) syndrome. *Journal of Medical Genetics* 1997 **34** 838–841.
2 Barrett TG, Bundey SE & Macleod AF. Neurodegeneration and diabetes: UK nationwide study of Wolfram (DIDMOAD) syndrome. *Lancet* 1995 **346** 1458–1463.
3 Inoue H, Tanizawa Y, Wasson J, Behn P, Kalidas K, Bernal-Mizrachi E, Mueckler M, Marshall H, Donis-Keller H, Crock P, Rogers D, Mihuni M, Kumashiro H, Higashi K, Sobue G, Oka Y & Permutt MA. A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome). *Nature Genetics* 1998 **20** 143–148.
4 Strom TM, Hortnagel K, Hofmann S, Gekeler F, Scharfe C, Rabl W, Gerbitz KD & Meitinger T. Diabetes insipidus, diabetes mellitus, optic atrophy and deafness (DIDMOAD) caused by mutations in a novel gene (wolframin) coding for a predicted transmembrane protein. *Human Molecular Genetics* 1998 **7** 2021–2028.
5 Takeda K, Inoue H, Tanizawa Y, Matsuzaki Y, Oba J, Watanabe Y, Shinoda K & Oka Y. WFS1 (Wolfram syndrome 1) gene product: predominant subcellular localization to endoplasmic reticulum in cultured cells and neuronal expression in rat brain. *Human Molecular Genetics* 2001 **10** 477–484.
6 Hofmann S, Philbrook C, Gerbitz K-D & Bauer MF. Wolfram syndrome: structural and functional analyses of mutant and wild-type wolframin, the WFS1 gene product. *Human Molecular Genetics* 2003 **12** 2003–2012.
7 Osman AA, Saito M, Makepeace C, Permutt MA, Schlesinger P & Mueckler M. Wolframin expression induces novel ion channel activity in endoplasmic reticulum membranes and increases intracellular calcium. *Journal of Biological Chemistry* 2003 **26** 52755–52762.
8 Chevet E, Cameron PH, Pelletier MF, Thomas DY & Bergeron JJ. The endoplasmic reticulum: integration of protein folding, quality control, signaling and degradation. *Current Opinion in Structural Biology* 2001 **11** 120–124.
9 Rutkowski DT & Kaufman RJ. A trip to the ER: coping with stress. *Trends in Cell Biology* 2004 **14** 20–28.
10 Oyadomari S, Araki E & Mori M. Endoplasmic reticulum stress-mediated apoptosis in pancreatic β-cells. *Apoptosis* 2002 **7** 335–345.
11 Ron D. Proteotoxicity in the endoplasmic reticulum: lessons from the Akita diabetic mouse. *Journal of Clinical Investigation* 2002 **109** 443–445.
12 Yoshioka M, Kayo T, Ikeda T & Koizumi A. A novel locus, Mody 4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. *Diabetes* 1997 **46** 887–894.
13 Wang J, Takeuchi T, Tanaka S, Kubo SK, Kayo T, Lu D, Takata K, Koizumi A & Izumi T. A mutation in the insulin 2 gene induces diabetes with severe pancreatic β-cell dysfunction in the Mody mouse. *Journal of Clinical Investigation* 1999 **103** 27–37.
14 Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E & Mori M. Targeted disruption of the Chop gene delays endoplasmic reticulum stress–mediated diabetes. *Journal of Clinical Investigation* 2002 **109** 525–532.
15 Ishihara H, Takeda S, Tamura A, Takahashi R, Yamaguchi S, Takei D, Yamada T, Inoue H, Soga H, Katagiri H, Tanizawa Y & Oka Y. Disruption of the WFS1 gene in mice causes progressive β-cell loss and impaired stimulus-secretion coupling in insulin secretion. *Human Molecular Genetics* 2004 **13** 1159–1170.
16 Yamaguchi S, Ishihara H, Tamura A, Yamada T, Takahashi R, Takei D, Katagiri H & Oka Y. Endoplasmic reticulum stress and

N-glycosylation modulate expression of WFS1 protein. *Biochemical and Biophysical Research Communication* 2004 **3** 250–256.
17 Nozaki J, Kubota H, Yoshida H, Naitoh M, Goji J, Yoshinaga T, Mori K, Koizumi A & Nagata K. The endoplasmic reticulum stress response is stimulated through the continuous activation of transcription factors ATF6 and XBP1 in Ins2 + /Akita pancreatic beta cells. *Genes to Cells* 2004 **9** 261–270.
18 Sheng Z, Kawano J, Yanai A, Fujinaga R, Tanaka M, Watanabe Y & Shinoda K. Expression of estrogen receptors (α,β) and androgen receptor in serotonin neurons of the rat and mouse dorsal raphe nuclei; sex and species differences. *Neuroscience Research* 2004 **49** 185–196.
19 Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y & Yamamura K. Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 1990 **127** 126–132.
20 Oka Y, Asano T, Shibasaki Y, Kasuga M, Kanazawa Y & Takaku F. Studies with antipeptide antibody suggest the presence of at least two types of glucose transporter in rat brain and adipocyte. *Journal of Biological Chemistry* 1988 **263** 13432–13439.
21 Li WW, Alexandre S, Cao X & Lee AS. Transactivation of the grp78 promoter by Ca^{2+} depletion. A comparative analysis with A23187 and the endoplasmic reticulum $Ca^{(2+)}$-ATPase inhibitor thapsigargin. *Journal of Biological Chemistry* 1993 **268** 12003–12009.
22 Fujiwara T, Oda K, Yokota S, Takatsuki A & Ikehara Y. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *Journal of Biological Chemistry* 1988 **263** 18545–18552.
23 Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL & Ron D. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes and Development* 1998 **12** 982–995.
24 McCullough KD, Martindale JL, Klotz LO, Aw TY & Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by downregulating Bcl2 and perturbing the cellular redox state. *Molecular and Cellular Biology* 2001 **21** 1249–1259.
25 Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M & Lazebnik YA. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 1995 **376** 37–43.
26 Karasik A, O'Hara C, Srikanta S, Swift M, Soeldner JS, Kahn CR & Herskowitz RD. Genetically programmed selective islet beta-cell loss in diabetic subjects with Wolfram's syndrome. *Diabetes Care* 1989 **12** 135–138.
27 Kinsley BT, Swift M, Dumont RH & Swift RG. Morbidity and mortality in the Wolfram syndrome. *Diabetes Care* 1995 **18** 1566–1570.
28 Gerbitz K-D. Reflexions on a newly discovered diabetogenic gene, wolframin (WFS1). *Diabetologia* 1999 **42** 627–630.
29 Minton JA, Rainbow LA, Ricketts C & Barrett TG. Wolfram syndrome. *Reviews in Endocrine and Metabolic Disorders* 2003 **4** 53–59.
30 Yoshida H, Haze K, Yanagi H, Yura T & Mori K. Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *Journal of Biological Chemistry* 1998 **273** 33741–33749.
31 Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M & Mori K. ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. *Molecular and Cellular Biology* 2000 **20** 6755–6767.
32 Kokame K, Kato H & Miyata T. Identification of ERSE-II, a new cis-acting element responsible for the ATF6-dependent mammalian unfolded protein response. *Journal of Biological Chemistry* 2001 **276** 9199–9205.

Received 24 January 2005
Accepted 7 April 2005

Am J Physiol Endocrinol Metab 290: E308–E316, 2006.
First published September 27, 2005; doi:10.1152/ajpendo.00131.2005.

Cell type-specific activation of metabolism reveals that β-cell secretion suppresses glucagon release from α-cells in rat pancreatic islets

Rui Takahashi,[1] Hisamitsu Ishihara,[1] Akira Tamura,[1] Suguru Yamaguchi,[1] Takahiro Yamada,[1] Daisuke Takei,[1] Hideki Katagiri,[2] Hitoshi Endou,[3] and Yoshitomo Oka[1]

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

Submitted 22 March 2005; accepted in final form 19 September 2005

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.

Address for reprint requests and other correspondence: H. Ishihara, Div. of Molecular Metabolism and Diabetes, Tohoku Univ. Graduate School of Medicine, 2–1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan (e-mail: ishihara-tky@umin.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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-

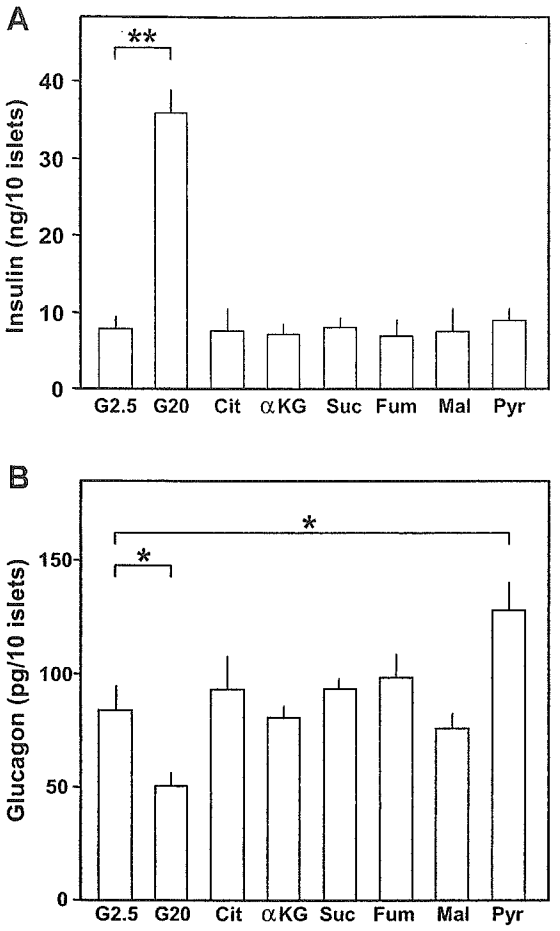


Fig. 1. Tricarboxylic acid (TCA) cycle intermediates had no effects on either insulin or glucagon secretion. Isolated islets were cultured overnight and challenged with glucose (20 mM), pyruvate (10 mM), and various TCA cycle intermediates (10 mM). Insulin (*A*) and glucagon (*B*) secreted during a 60-min 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; $n = 3$~7. *$P < 0.05$ 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

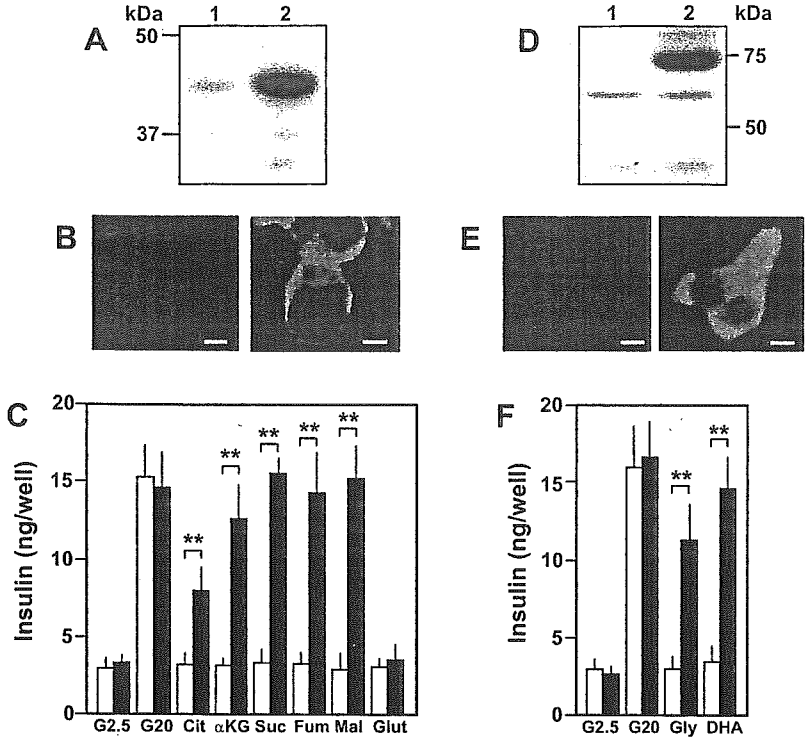


Fig. 2. Adenovirus-mediated Na^+-dependent dicarboxylate transporter (NaDC)-1 or glycerol kinase (GlyK) expression in INS-1E cells. *A*: INS-1E cells infected with either AdCAGlacZ (*lane 1*) or AdRIPNaDC (*lane 2*) were subjected to SDS-PAGE and probed with an anti-NaDC-1 antibody. *B*: INS-1E cells 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-

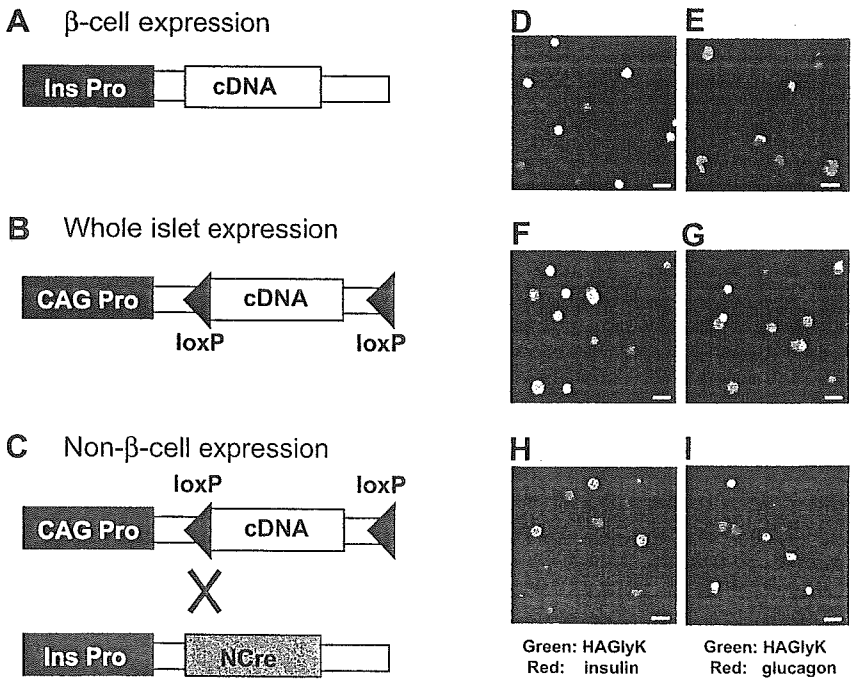


Fig. 3. Selective gene expression method for β- and non-β-cells in islets. *A–C*: schematic representation of adenoviruses for expression in β-cells (*A*), whole islet cells (*B*), and non-β-cells (*C*). NCre, nuclear targeted Cre recombinase; CAG, a transcriptional unit composed of the cytomegalovirus 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 AdCAGlx-HAGlyKlx 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.

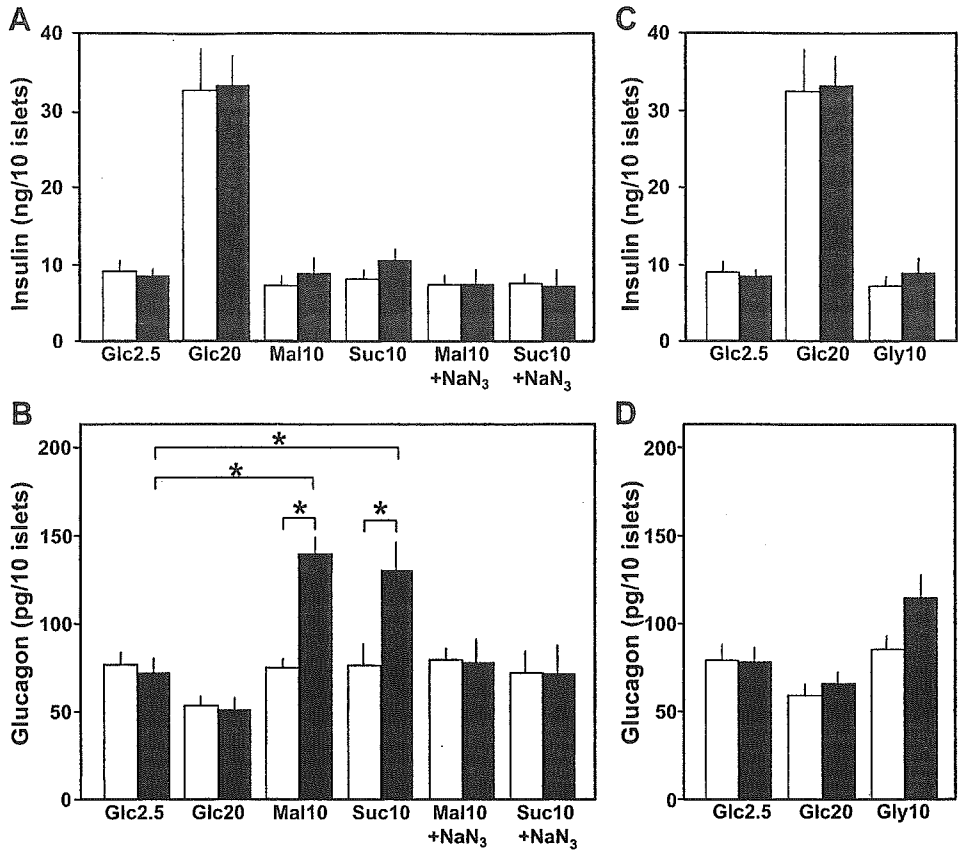


Fig. 4. Selective α-cell activation induced glucagon secretion. *A* and *B*: isolated islets (10 islets/tube) infected with AdCAGlacZ plus AdRIPNCre (open bars) or AdCAGlx-NaDClx plus AdRIPNCre (filled bars) were challenged with 20 mM glucose, 10 mM malate (Mal10), or 10 mM succinate (Suc10) with or 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; $n = 4$.

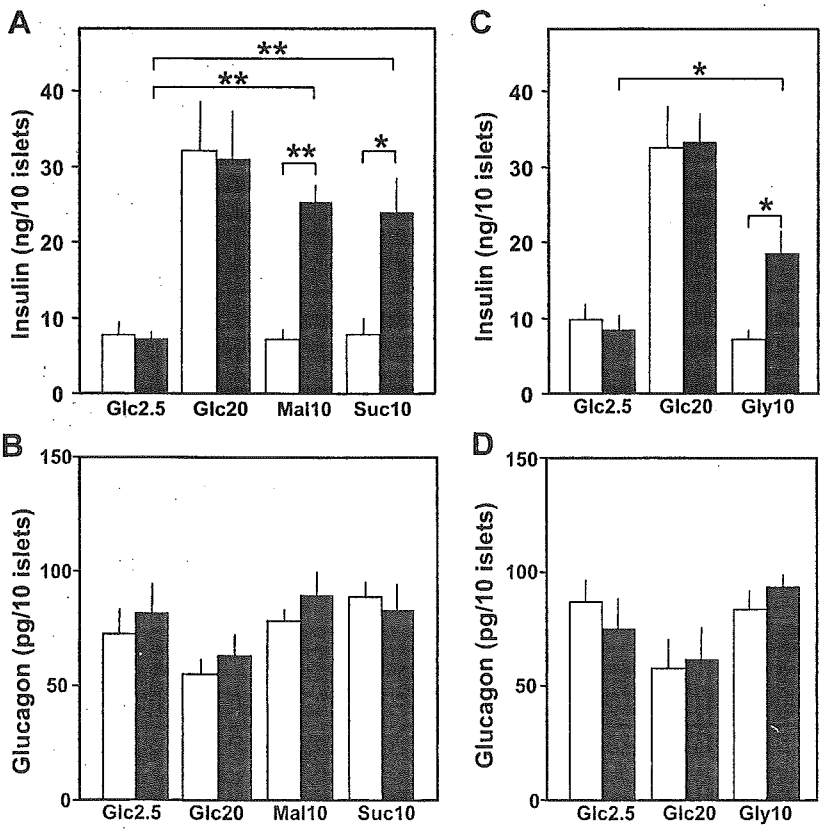


Fig. 5. Simultaneous α- and β-cell activation induced insulin but not glucagon secretion. *A* and *B*: isolated islets (10 islets/tube) infected with AdCAGlacZ (open bars) or AdCA-GlxNaDClx (filled bars) were challenged with 20 mM glucose, 10 mM malate, or 10 mM succinate. Insulin (*A*) and glucagon (*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; $n = 4$. $^*P < 0.05$.

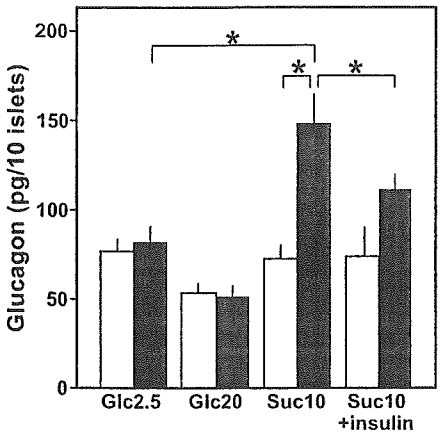


Fig. 6. Insulin suppressed succinate-stimulated glucagon secretion. Isolated islets (10 islets/tube) infected with AdCAGlacZ plus AdRIPNCre (open bars) or AdCAGlxNaDClx plus AdRIPNCre (filled bars) were challenged with 20 mM glucose, 10 mM succinate alone, or 10 mM succinate with 25 ng/ml insulin. Glucagon 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

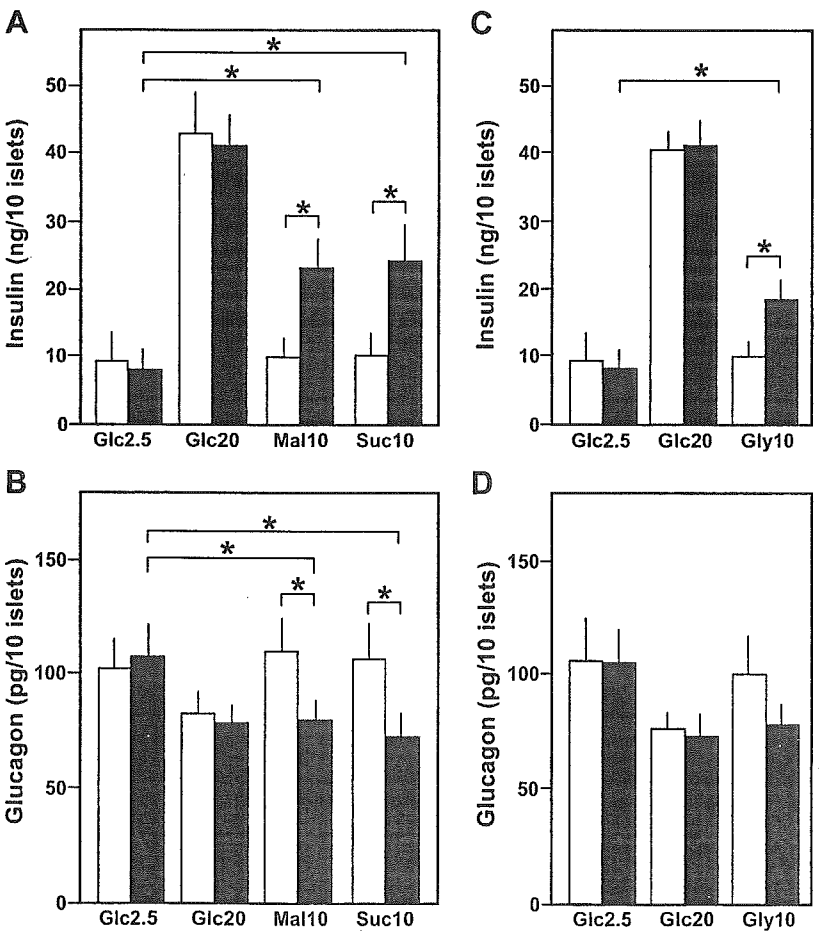


Fig. 7. Selective β-cell activation inhibited glucagon secretion. *A* and *B*: isolated islets (10 islets/tube) infected with AdCAGlacZ (open bars) or AdRIPNaDC (filled bars) were challenged with 20 mM glucose, 10 mM malate, or 10 mM succinate. Insulin (*A*) and glucagon (*B*) secreted during a 60-min 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.

ACKNOWLEDGMENTS

We are grateful to Y. Nagura for expert assistance.

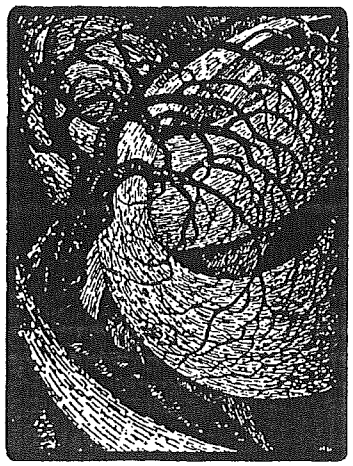
GRANTS

This work was supported by a grant from Suzuken Memorial Foundation to H. Ishihara and a Grant-in-Aid for Scientific Research (15659213) from the Ministry of Education, Science, Sports, and Culture of Japan and a Grant-in-Aid for Research on Human Genome, Tissue Engineering (H17-genome-003) from the Ministry of Health, Labor and Welfare to Y. Oka.

REFERENCES

1. **Antinozzi PA, Ishihara H, Newgard CB, and Wollheim CB.** Mitochondrial metabolism sets the maximal limit of fuel-stimulated insulin secretion in a model pancreatic β-cell: a survey of four fuel secretagogues. *J Biol Chem* 277: 11746–11755, 2002.
2. **Banarer S, McGregor VP, and Cryer PE.** Intraislet hyperinsulinemia prevents the glucagon response to hypoglycemia despite an intact autonomic response. *Diabetes* 51: 958–965, 2002.
3. **Barg S, Galvanovskis J, Gopel SO, Rorsman P, and Eliasson L.** Tight coupling between electrical activity and exocytosis in mouse glucagon-secreting α-cells. *Diabetes* 49: 1500–1510, 2000.
4. **Bloc A, Cens T, Cruz H, and Dunant Y.** Zinc-induced changes in ionic currents of clonal rat pancreatic A-cells: activation of ATP-sensitive K^+ channels. *J Physiol* 529: 723–734, 2000.
5. **Bokvist K, Olsen HL, Hoy M, Gotfredsen CF, Holmes WF, Buschard K, Rorsman P, and Gromada J.** Characterization of sulfonylurea and ATP-regulated K^+ channels in rat pancreatic A-cells. *Pflügers Arch* 438: 428–436, 1999.
6. **Burcelin R and Thorens B.** Evidence that extrapancreatic GLUT2-dependent glucose sensors control glucagon secretion. *Diabetes* 50: 1282–1289, 2001.
7. **Cejvan K, Coy DH, and Efendic S.** Intra-islet somatostatin regulates glucagon release via type 2 somatostatin receptors in rats. *Diabetes* 52: 1176–1181, 2003.
8. **Cryer PE.** Hypoglycaemia: the limiting factor in the glycaemic management of Type I and Type II diabetes. *Diabetologia* 45: 937–948, 2002.
9. **Diem P, Redmon JB, Abid M, Moran A, Sutherland DE, Halter JB, and Robertson RP.** Glucagon, catecholamine and pancreatic polypeptide secretion in type I diabetic recipients of pancreas allografts. *J Clin Invest* 86: 2008–2013, 1990.
10. **Franklin I, Gromada J, Gjinovci A, Theander S, and Wollheim CB.** β-cell secretory products activate α-cell ATP-dependent potassium channels to inhibit glucagon release. *Diabetes* 54: 1808–1815, 2005.
11. **Gerich JE, Langlois M, Noacco C, Karam JH, and Forsham PH.** Lack of glucagon response to hypoglycemia in diabetes: evidence for an intrinsic pancreatic alpha cell defect. *Science* 182: 171–173, 1973.
12. **Gopel SO, Kanno T, Barg S, Weng XG, Gromada J, and Rorsman P.** Regulation of glucagon release in mouse α-cells by K_{ATP} channels and inactivation of TTX-sensitive Na^+ channels. *J Physiol* 528: 509–520, 2000.
13. **Greenbaum CJ, Havel PJ, Taborsky GJ Jr, and Klaff LJ.** Intra-islet insulin permits glucose to directly suppress pancreatic A cell function. *J Clin Invest* 88: 767–773, 1991.
14. **Greenbaum CJ, Progeon RL, and D'Alessio DA.** Impaired β-cell function, incretin effect, and glucagon suppression in patients with type 1 diabetes who have normal fasting glucose. *Diabetes* 51: 951–957, 2002.
15. **Gromada J, Bokvist K, Ding WG, Barg S, Buschard K, Renstrom E, and Rorsman P.** Adrenalin stimulates glucagon secretion in pancreatic A-cells by increasing the Ca2+ current and the number of granules close to the L-type Ca channels. *J Gen Physiol* 100: 217–228, 1997.
16. **Gromada J, Ma X, Hoy M, Bokvist K, Salehi A, Berggren PO, and Rorsman P.** ATP-sensitive K^+ channel-dependent regulation of glucagon release and electrical activity by glucose in wild-type and $SUR1^{-/-}$ mouse α-cells. *Diabetes* 53, *Suppl* 3: S181–S189, 2004.
17. **Ishihara H, Maechler P, Gjinovci A, Herrera PL, and Wollheim CB.** Islet β-cell secretion determines glucagon secretion from the neighboring α-cells. *Nat Cell Biol* 5: 330–335, 2003.
18. **Ishihara H, Wang H, Drewes LR, and Wollheim CB.** Overexpression of monocarboxylate transporter and lactate dehydrogenase alters insulin secretory responses to pyruvate and lactate in β-cells. *J Clin Invest* 104: 1621–1629, 1999.
19. **Iwashima Y, Watanabe K, Eto M, Morikawa A, Takebe T, and Ishii K.** Insulin and glucagon response of the diabetic Chinese hamster in the Asahikawa colony. *Diabetes Res Clin Pract* 1: 87–94, 1985.
20. **Juhl K and Hutton J.** Stimulus-secretion coupling in the pancreatic β-cell. *Adv Exp Med Biol* 552: 66–90, 2004.
21. **Khan FA, Goforth PB, Zhang M, and Satin LS.** Insulin activates ATP-sensitive K(+) channels in pancreatic beta-cells through a phosphatidylinositol 3-kinase-dependent pathway. *Diabetes* 50: 2192–2198, 2001.
22. **Lantz KA, Vatamaniuk MZ, Brestelli JE, Friedman JR, Matschinsky FM, and Kaestner KH.** Foxa2 regulates multiple pathways of insulin secretion. *J Clin Invest* 114: 512–520, 2004.
23. **Liu YJ, Vieira E, and Gylfe E.** A store-operated mechanism determines the activity of the electrically excitable glucagon-secreting α-cell. *Cell Calcium* 35: 357–365, 2004.
24. **MacDonald MJ and Fahien LA.** Glyceraldehyde phosphate and methyl esters of succinic acid. Two "new" potent insulin secretagogues. *Diabetes* 37: 997–999, 1988.
25. **Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, and Maechler P.** Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* 145: 667–678, 2004.
26. **Miki T, Liss B, Minami K, Shiuchi T, Saraya A, Kashima Y, Horiuchi M, Ashcroft F, Minokoshi Y, Roeper J, and Seino S.** ATP-sensitive K+ channels in the hypothalamus are essential for the maintenance of glucose homeostasis. *Nat Neurosci* 4: 507–512, 2001.
27. **Miyake S, Makimura M, Kanegae Y, Harada S, Sato Y, Takamori K, Tokuda C, and Saito I.** Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc Natl Acad Sci USA* 93: 1320–1324, 1996.
28. **Niwa H, Yamamura K, and Miyazaki J.** Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108: 193–199, 1991.
29. **Noel RJ, Antinozzi PA, McGarry D, and Newgard CB.** Engineering of glycerol-stimulated insulin secretion in islet β-cells. *J Biol Chem* 272: 18621–18627, 1997.
30. **Oetergard T, Degn KB, Gall MA, Carr RD, Veldhouis JD, Thomsen MK, Rizza RA, and Schmitz O.** The insulin secretagogues glibenclamide and repaglinide do not influence growth hormone secretion in humans but stimulate glucagon secretion during insulin deficiency. *J Clin Endocrinol Metab* 89: 297–302, 2004.
31. **Okamoto K, Hirano H, and Isohashi F.** Molecular cloning of rat liver glucocorticoid-receptor translocation promoter. *Biochem Biophys Res Commun* 193: 848–854, 1993.
32. **Pipeleers DG, Schuit FC, Van Schravendijk CFH, and Van de Winkel M.** Interplay of nutrients and hormones in the regulation of glucagon release. *Endocrinology* 117: 817–823, 1985.

33. **Ravier MA and Rutter GA.** Glucose or insulin, but not zinc ions, inhibit glucagon secretion from mouse pancreatic α-cells. *Diabetes* 54: 1789–1797, 2005.
34. **Schuit F, Derde MP, and Pipelers D.** Sensitivity of rat pancreatic A and B cell to somatostatin. *Diabetologia* 32: 207–212, 1989.
35. **Schuit F, DeVos A, Farfari S, Moens K, Pipelers D, Brun T, and Prentki M.** Metabolic fate of glucose in purified islet cells. *J Biol Chem* 272: 18572–18579, 1997.
36. **Sekine T, Cha SH, Hosoyamada M, Kanai Y, Watanabe N, Furuta Y, Fukuda K, Igarashi T, and Endou H.** Cloning, functional characterization, and localization of a rat renal Na^+-dicarboxylate transporter. *Am J Physiol Renal Physiol* 275: F298–F305, 1998.
37. **Taborsky GJ Jr, Ahren B, and Havel PJ.** Autonomic mediation of glucagon secretion during hypoglycemia: implications for impaired α-cell responses in type 1 diabetes. *Diabetes* 47: 995–1005, 1998.
38. **The Diabetes Control, and Complications Trial Research Group.** The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329: 977–986, 1993.
39. **Wendt A, Birnir B, Buschard K, Gromada J, Salehi A, Sewing S, Rorsman P, and Braun M.** Glucose inhibition of glucagon secretion from rat α-cells is mediated by GABA released from neighboring β-cells. *Diabetes* 53: 1038–1045, 2004.



WFS1-deficiency increases endoplasmic reticulum stress, impairs cell cycle progression and triggers the apoptotic pathway specifically in pancreatic β-cells

Takahiro Yamada[1], Hisamitsu Ishihara[1*], Akira Tamura[1], Rui Takahashi[1], Suguru Yamaguchi[1], Daisuke Takei[1], Ai Tokita[1], Chihiro Satake[1], Fumi Tashiro[2], Hideki, Katagiri[3], Hiroyuki Aburatani[4], Jun-ichi Miyazaki[2] and Yoshitomo Oka[1]

[1]Division of Molecular Metabolism and Diabetes, Tohoku University Graduate School of Medicine, Sendai, Miyagi 980-8575, Japan. [2]Division of Stem Cell Regulation Research, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan. [3]Division of Advanced Therapeutics for Metabolic Diseases, Tohoku University Graduate School of Medicine, Sendai, Miyagi 980-8575, Japan. [4]Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo 153-8904, Japan.

Address correspondence to: Hisamitsu Ishihara, M.D., Ph.D.

Division of Molecular Metabolism and Diabetes, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan.

Tel. +81 22 717 7611, Fax. +81 22 717 7612.

E-mail, hisamitsu-ishihara@mail.tains.tohoku.ac.jp

SUMMARY

Wolfram syndrome, an autosomal recessive disorder associated with diabetes mellitus and optic atrophy, is caused by mutations in the *WFS1* gene encoding an endoplasmic reticulum (ER) membrane protein. Herein, we report that pancreatic islets of *wfs1*-deficient mice exhibit increases in phosphorylation of RNA-dependent protein kinase-like ER kinase, chaperone gene expressions and active XBP1 protein levels, indicating an enhanced ER stress response. We established *wfs1*-deficient MIN6 clonal β-cells by crossing *wfs1*-deficient mice with mice expressing simian virus 40 large T antigen in β-cells. These cells show essentially the same alterations in ER stress responses as *wfs1*-deficient islets, which were reversed by re-expression of WFS1 protein or overexpression of GRP78, a master regulator of the ER stress response. In contrast, these changes are not observed in heart, skeletal muscle, or brown adipose tissues with WFS1-deficiency. The increased ER stress response was accompanied by reduced BrdU incorporation and increased caspase-3 cleavage, indicating impaired cell cycle progression and accelerated apoptotic processes in the mutant islets. These changes are associated with increased expression of the cell cycle regulator $p21^{CIP1}$ in *wfs1*-deficient islets and clonal β-cells. Treatment of islets with thapsigargin, an ER stress inducer, caused upregulation of $p21^{CIP1}$. In addition, forced expression of $p21^{CIP1}$ resulted in reduced MIN6 β-cell numbers, suggesting the ER stress-induced increase in $p21^{CIP1}$ expression to be involved in β-cell loss in the mutant islets. These data indicate that WFS1-deficiency activates the ER stress response specifically in β-cells, causing β-cell loss through impaired cell cycle progression and increased apoptosis.

INTRODUCTION

Type 2 diabetes is caused by complex interactions between insulin resistance in peripheral tissues and impaired insulin secretion from pancreatic β-cells. There is a general consensus that the latter results from both impaired β-cell function and decreased β-cell mass (1-3). Adult β-cell mass is maintained by a balance between generation and death of β-cells. In patients with type 2 diabetes, new islet formation and β-cell replication are reportedly normal, and an increased rate of apoptosis has been suggested to underlie the loss of β-cell mass (4).

Recent studies using novel mutant mice have led to new insights into endoplasmic reticulum (ER) stress and maintenance of β-cell mass (5,6). The ER stress response, also known as the unfolded protein response (UPR), involves translational attenuation, transcriptional induction of chaperones and folding enzymes, as well as degradation of misfolded proteins, a process called ER-associated degradation (ERAD). When ER stress is strong and cellular survival mechanisms fail to correct the protein-folding defects, an ER stress-mediated apoptotic process is initiated (5-7). Mice with a homozygous null mutation of RNA-dependent protein kinase-like ER kinase (PERK) lose their ability to phosphorylate eukaryotic initiation factor 2α (eIF2α) and fail to attenuate translation in response to ER stress. These mice develop diabetes due to reduced β-cell mass (8). Importantly, mutations of the *EIF2AK3* gene encoding PERK in humans have been recognized as causing Wolcott-Rallison syndrome with diabetes mellitus in early infancy (9). A mouse model in which a Ser51Ala mutation of eIF2α prevents the protein from being phosphorylated by PERK and other eIF2α kinases, also displays a β-cell defect and impaired gluconeogenesis leading to lethal hypoglycemia

(10). Mice with a deletion mutation of $P58^{IPK}$, a cytosolic chaperone, were recently reported to exhibit β-cell failure and diabetes (11). These examples suggest that β-cells, producing large quantities of insulin and thus a greater load on the ER, are especially sensitive to ER stress.

Wolfram syndrome is a rare autosomal recessive disorder characterized by juvenile-onset diabetes mellitus, optic atrophy, diabetes insipidus and sensorineural deafness (12). This syndrome is caused by mutations in the *WFS1* gene (13,14), which encodes an ER resident membrane protein (15). Postmortem studies of the pancreas from subjects with Wolfram syndrome have shown β-cell loss (16). We recently established a line of mutant mice with a disrupted *wfs1* gene and found that these mice also exhibited impaired glucose homeostasis accompanied by a progressive reduction of β-cell mass (17). Thus, the *wfs1*-deficient mouse is a model for studying mechanisms of β-cell loss during the development of diabetes in Wolfram syndrome. We and others have also shown expression of WFS1 protein to be up-regulated by ER stress-inducing agents (18-20). A recent study employing IRE1α knockout and PERK knockout cells suggested that WFS1 is a component of the IRE1 and PERK signaling pathways (20). In addition, *wfs1*-deficient islets have been shown to exhibit increased DNA fragmentation in response to ER stress inducers (17), suggesting β-cell loss in Wolfram syndrome to be attributable to an inability to handle ER stress. A very recent study of islets conditionally lacking the *wfs1* gene in β-cells, demonstrated an increased GRP78 mRNA to GLUT2 mRNA ratio. This observation was interpreted as evidence of an enhanced ER stress response, on the assumption that GLUT2 mRNA levels represented the β-cell number in islets (21).

To further investigate the mechanisms underlying β-cell loss in Wolfram

syndrome, we conducted a systematic study of the UPR in *wfs1*-deficient islets as well as other tissues. We also created β-cell lines with WFS1-deficiency and studied UPR. We found all three UPR subpathways to be activated in *wfs1*-deficient islets and β-cell lines. Furthermore, we demonstrated increased cleavage of caspase-3, a hallmark of apoptosis, and impaired proliferation associated with enhanced expression of the cell cycle regulator $p21^{CIP1}$.

RESULTS

UPR activation in *wfs1*-deficient islets

A systematic study of the UPR was conducted using islets isolated from 6-week-old male *wfs1*-deficient mice with the B6 background. At 6 weeks of age, the β-cell mass of these mice begins to decrease (17). Accumulation of unfolded proteins in the ER is well known to induce dissociation of GRP78 from PERK, resulting in oligomerization and subsequent auto-phosphorylation of PERK. Activated PERK then phosphorylates eIF2α and suppresses general protein translation to reduce the ER load (5-7). In freshly isolated *wfs1*-deficient islets, PERK phosphorylation was increased (Fig. 1A). In addition, eIF2α phosphorylation was slightly but significantly enhanced with no alteration in total eIF2α levels in mutant islets (Fig. 1A). Thus, the ratio of phosphorylated eIF2α over total eIF2α levels analyzed by densitometry was increased by 27 ± 7% ($n = 4$ experiments, $p < 0.05$). These data indicate that one of three subpathways of the UPR arising from PERK phosphorylation is initiated in response to WFS1-deficiency in islets.

ER stress is also sensed by other ER resident proteins, IRE1 and ATF6, in addition to PERK (5-7). Activation of ATF6 via GRP78 dissociation and subsequent cleavage is known to induce the expressions of various chaperone genes, constituting another subpathway of the UPR (5-7). In *wfs1*-deficient islets, GRP94 mRNA levels were increased and those of GRP78 and $P58^{IPK}$ also tended to rise (Fig. 1B). Correspondingly, although the differences failed to reach statistical significance, levels of these chaperone proteins tended to be increased (Fig. 1C), suggesting that the ATF6 subpathway of the UPR is activated in response to WFS1-deficiency.

As shown in Fig. 1D, a shorter form of XBP1 mRNA was increased. This form is produced by 26-nucleotide splicing from primary XBP1 mRNA by the ribonuclease activity of IRE1, increasing active XBP1 protein levels in mutant islets (Fig. 1E). HRD1, a ubiquitin ligase involved in ERAD, is one of the XBP1 target genes (20). In *wfs1*-deficient islets, levels of HRD1 protein were markedly increased (Fig. 1E). In addition, mRNA levels of EDEM (ER-associated degradation-enhancing α-mannosidase-like protein) (23), another target of XBP1, were significantly increased in mutant islets (100 ± 5 arbitrary units (wild type) vs. 136 ± 18 (mutant), $n = 6$, $p < 0.05$). These data indicate that the IRE1-initiated subpathway of the UPR is also activated in *wfs1*-deficient islets.

Establishment of MIN6 β-cell lines deficient in WFS1

To examine the influence of WFS1-deficiency specifically in a homogenous β-cell population, β-cell lines were established by crossing $wfs1^{+/-}$ and $wfs1^{-/-}$ mice (17) with IT6 mice expressing simian virus 40 (SV40) large T antigen under the insulin promoter (24) and were designated MIN6wfs1$^{+/-}$ and MIN6wfs1$^{-/-}$, respectively (see Materials and Methods). IT6 mice were previously reported to develop insulinoma, from which the MIN6 cell line (24), one of the most highly differentiated β-cell lines, was generated. We established two cell lines each for the $wfs1^{+/-}$ and $wfs1^{-/-}$ genotypes. As shown in Fig. 2A, the two cell lines with the $wfs1^{-/-}$ genotype (MIN6wfs1$^{-/-}$-1 and 2) show similar UPR characteristics. Similarly, characteristics of two cell lines with the $wfs1^{+/-}$ genotype (MIN6wfs1$^{+/-}$) were indistinguishable (data not shown). Therefore, only one line of each genotype was used for subsequent analyses. We compared MIN6wfs1$^{-/-}$ with MIN6wfs1$^{+/-}$ at the same passage numbers (passages 5 to 8), but not with the original

MIN6 cells. This is because we were concerned that a difference in passage number between the original MIN6 and MIN6wfs1$^{-/-}$ cells, irrespective of WFS1-deficiency, might affect the protein expression profile, rendering the former an inappropriate control for the latter. After completion of a series of experiments, MIN6wfs1$^{+/-}$ cells reached passages 15 - 20, the same passage of original MIN6 cells we have. The function and survival of MIN6wfs1$^{+/-}$ cells are similar to those of wild-type MIN6 cells at similar passage numbers (data not shown).

Effects of WFS1-deficiency on UPR in β-cell lines

As shown in Fig. 2A, altered expressions of UPR-related proteins observed in *wfs1*-deficient islets were reproduced in MIN6wfs1$^{-/-}$ cells; PERK phosphorylation, as well as expressions of active XBP1 and HRD1, were increased in *wfs1*-deficient MIN6 cells. ATF4 levels were also shown to be increased in these cells. Furthermore, although GRP78 and GRP94 protein levels were similar (Fig. 2B, upper panel), the activity of the GRP78 promoter containing three ER stress response elements was greater in MIN6wfs1$^{-/-}$ cells than in MIN6wfs1$^{+/-}$ cells (Fig. 2B), strongly suggesting activation of the ATF6 subpathway of the UPR in MIN6wfs1$^{-/-}$ cells. To confirm that alterations in UPR-related proteins are due to WFS1-deficiency, wild-type human WFS1 protein was expressed in MIN6wfs1$^{-/-}$ cells. We took advantage of the tetracycline-inducible expression system. MIN6wfs1$^{-/-}$ cells were infected with the Tet-repressor expressing virus (AdCAG-TR) together with a recombinant adenovirus bearing wild-type human WFS1 cDNA under the CMV promoter containing the Tet-operator (AdCTO-WFS1). The cells were then treated with doxycycline (2 μg/ml). As shown in Fig. 2C, when WFS1 expression was restored to levels comparable to those of the original MIN6 cells,

the increase in PERK phosphorylation was prevented. In addition, overexpression of GRP78, a master regulator of the ER stress response, also resulted in normalization of PERK phosphorylation levels (Fig. 2D), clearly indicating the observed alteration in UPR-related proteins to be due to exacerbation of ER stress caused by WFS1-deficiency.

No UPR induction in heart, skeletal muscle or brown adipose tissues from *wfs1*-deficient mice

WFS1 protein is expressed in a variety of non-pancreatic tissues, though less abundantly than in islets (Fig. 3A). Therefore, we also examined expressions of UPR genes in tissues other than pancreatic islets. Cardiac function is reportedly not impaired in subjects with Wolfram syndrome (12) or in *wfs1*-deficient mice (17). Skeletal muscle and brown adipose tissue also appear essentially normal in mutant mice (data not shown). In contrast to islets, no UPR alterations were observed in these tissues from *wfs1*-deficient mice (Fig. 3B-D). Thus, UPR activation is tissue-specific in WFS1-deficiency.

Increased β-cell apoptotic response in *wfs1*-deficient islets

ER stress induces apoptosis through activation of various signaling molecules including JNK and pro-apoptotic proteins, such as CHOP (5-7). CHOP expression was increased at both the mRNA (Fig. 4A) and the protein level (Fig. 4B), in mutant as compared to wild-type islets. In contrast, JNK expression levels and phosphorylation states were not altered in *wfs1*-deficient islets (Fig. 4B). We also found increased levels of cleaved caspase-3, a hallmark of apoptosis, in mutant islets (Fig. 4B). CHOP expression and