Protection of pancreatic β -cells by exendin-4 may involve the reduction of endoplasmic reticulum stress; *in vivo* and *in vitro* studies

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Abstract

The aim of this study was to investigate the *in vivo* and *in vitro* effects of exendin-4, a potent glucagon-like peptide 1 agonist, on the protection of the pancreatic β -cells against their cell death. In *in vivo* experiments, we used β -cell-specific calmodulin-overexpressing mice where massive apoptosis takes place in their β -cells, and we examined the effects of chronic treatment with exendin-4. Chronic and s.c. administration of exendin-4 reduced hyperglycemia. The treatment caused significant increases of the insulin contents of the pancreas and islets, and retained the insulin-positive area. Dispersed transgenic islet cells lived only shortly, and several endoplasmic reticulum (ER) stress-related molecules such as immunoglobulin-binding protein (Bip), inositol-requiring enzyme-1 α , X-box-binding

protein-1 (XBP-1), RNA-activated protein kinase-like endoplasmic reticulum kinase, activating transcription factor-4, and C/EBP-homologous protein (CHOP) were more expressed in the transgenic islets. We also found that the spliced form of XBP-1, a marker of ER stress, was also increased in β -cell-specific calmodulin-overexpressing transgenic islets. In the quantitative real-time PCR analyses, the expression levels of Bip and CHOP were reduced in the islets from the transgenic mice treated with exendin-4. These findings suggest that excess of ER stress occurs in the transgenic β -cells, and the suppression of ER stress and resultant protection against cell death may be involved in the anti-diabetic effects of exendin-4.

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Introduction

The beneficial effects of glucagon-like peptide 1 (GLP-1) and its related substances such as inhibitors of dipeptidyl peptidase IV, its degrading enzyme, on the pancreatic β -cells have been reported; these agents enhance insulin release, increase (pro)insulin biosynthesis, promote β -cell development/replication, and prevent β -cell exhaustion under the diabetic conditions (Drucker 2003, Leon *et al.* 2005). These effects are considered to result from an increase in the β -cell cAMP content (Thorens 1992), while cAMP-independent mechanisms of GLP-1 have also been suggested in other types of cells (Montrose-Rafizadeh *et al.* 1999).

Multiple pathways which cause β -cell damage have been suggested to participate in the pathogenesis of diabetes mellitus (Donath *et al.* 1999, Mandrup-Poulsen 2003, Scheuner *et al.* 2005). Among them, more attention has recently been directed to endoplasmic reticulum (ER) stress. ER stress is a series of cellular responses induced by several causes such as (over)production of misfolding proteins, disorder in Ca^{2+} dynamics in ER, oxidative stress, and exposure to cytokines. This process includes production of the chaperon proteins, translational attenuation, degradation

of the misfolding proteins, and eventual induction of apoptosis (Oyadomari *et al.* 2002a). A recent pathological study demonstrated that a decrease in the β -cell mass by apoptosis may occur in the human diabetic subjects (Butler *et al.* 2003). β -Cells are considered to be very sensitive to ER stress (Harding & Ron 2002), and resultant apoptotic cell death was reported to occur in diabetic model animals (Oyadomari *et al.* 2002b).

Overexpression of calmodulin in the β -cell *in vivo* causes a sudden onset and persistent hyperglycemia without infiltration of lymphocytes (Epstein *et al.* 1989). We previously demonstrated that Ca^{2+} -dependent apoptosis occurs in the β -cell and suggested that Ca^{2+} /calmodulin-dependent overexpression of nitric oxide may partially participate in the process (Yu *et al.* 2002). Recently, we have reported that the β -cell decay in this model was retarded by chronic administration of pituitary adenylate cyclase-activating polypeptides (PACAP), which increase cAMP production in the β -cell (Tsunekawa *et al.* 2005). We also suggested that the effect of PACAP mainly results from its protecting effect against a decrease in the β -cell mass.

In this paper, we examined the *in vivo* effects of exendin-4, a potent GLP-1 agonist, on the pancreatic β -cell in the

β-cell-specific calmodulin-overexpressing transgenic (CaMTg) mice. We also investigated the underlying mechanism by which exendin-4 protects β-cells from their apoptotic cell death.

Materials and Methods

Production of transgenic animals

CaMTg mice were kindly provided by Prof. A R Means (Duke University, Durham, NC, USA). The methods for production of this model have been described elsewhere (Epstein *et al.* 1989). Briefly, the rat insulin-II promoter/chicken calmodulin cDNA chimeric gene was microinjected into mouse embryos derived from female FVBM mice, followed by the implantation of the embryos into pseudo-pregnant female FVBM mice. The transgenic mice were subsequently bred with the ICR strain (Yu *et al.* 2002). The progeny were housed in a temperature-, humidity-, and light-controlled room (21–23 °C, 12 h light:12 h darkness cycle, and free access to food and water). They were characterized as having blepharostenosis at 2–3 weeks and frank hyperglycemia (>300 mg/dl) with marked hypoinsulinemia at 4 weeks of age. In cases where earlier characterization was necessary, genomic typing was carried out (see below). Male CaMTg mice were bred with normal female ICR mice (Japan SLC, Hamamatsu, Japan) since the female CaMTg mice were almost infertile. The adult transgenic mice exhibited overt hyperphagia, polyposia, and polyuria, while they were only slightly lean. Unless otherwise mentioned, we used non-transgenic (nTg) mice from the same litters as controls. This study has been approved by the Committees on Animal Experiments of Oita University and Nagoya University.

Genomic typing by PCR

Genomic DNA was extracted from the tails of the mice 7 days after birth using the QIAamp DNA Mini Kit (QIAGEN GmbH) and was subjected to PCR amplification of the chicken calmodulin transgene. The specific primer pairs (Table 1) amplified a 200 bp product only in the CaMTg mice. The results were confirmed by a comparison with the later phenotype of these mice such as blepharostenosis and hyperglycemia.

In vivo treatment with exendin-4

Exendin-4 was purchased from Sigma. Osmotic minipumps (model 1002, Alzet, Cupertino, CA, USA) were implanted in the midscapular region at the end of the second week after birth, and exendin-4 (10 µg per pump, equivalent to ≈28 nmol/initial kg BW per day) dissolved in saline was subcutaneously injected during the following 2 weeks. Saline was administered as a control. After the implantation, plasma glucose levels were measured daily using a compact glucose analyzer (MediSafe, Terumo, Tokyo, Japan). Blood samples were collected by tail cutting in the interval from 1000 to 1400 h from these mice fed *ad libitum*. The insulin concentrations in CaMTg serum taken from the canthus were often below the detectable range of the insulin ELISA employed here. Therefore, we took blood samples from the portal vein under anesthesia to determine the serum insulin concentrations. At the end of the experimental period, the pancreata were removed and weighed. For the measurement of the pancreatic insulin content, the pancreata were homogenized in ice-cold acid-ethanol solution (0.18 M HCl in 75% ethanol) and centrifuged. The supernatants

Table 1 Sequences of the PCR primers used in this study, their accession numbers and the sizes of the amplified bands

	Accession number	Forward primer	Reverse primer	Size
Gene				
Primers for genomic typing by PCR				
Calmodulin	DR413558	5'-GAGCCACCATGGCTGATCA-3'	5'-CATGTCCTGTAATTCT GCTTCTGT-3'	164
Primers for RT-PCR				
<i>Bip</i>	AJ002387	5'-GAAAGGATGGTTAATGATGCTGAG-3'	5'-GTCTTCAATGTCCGCATCCTG-3'	232
<i>IRE-1α</i>	AF071777	5'-TGGTCTCCTCTCGGGTTCCTG-3'	5'-AGAAGCAGGCCCATCCACAG-3'	596
<i>XBP-1</i>	BC029197	5'-TTCCTTACCAGCCTCCCTTT-3'	5'-TCCCAGGAGTGGTCTGTACC-3'	396
<i>PERK</i>	NM_010121	5'-CGCTGCTGCTGCTTCTGTTCT-3'	5'-ATCCCAAGGCAGAACAGATATACCTCAG-3'	618
<i>ATF-4</i>	NM_009716	5'-GAAACCTCATGGGTTCTCCA-3'	5'-GCCAATTGGGTTCACTGTCT-3'	292
<i>CHOP</i>	X67083	5'-TATGAGGATCTGCAGGAGGT-3'	5'-TCAGCTAGCTGTGCCACTTT-3'	340
<i>GAPDH</i>	NM_008084	5'-CTTACCACCATGGAGAAGGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'	238
Primers for real-time PCR				
Spliced of XBP-1	AF443192	5'-GTCCGCAGCAGGTGCAG-3'	5'-GTCCGCAGCAGGTGCAG-3'	72
		Double dye probe	5'-AGTTGTACCTCCCCAGAACATCTTCCC-3'	
<i>CHOP</i>	X67083	5'-AGGGCCAACAGAGGTCACAC-3'	5'-GGGCCATAGAACTCTGACTGGA-3'	71
		Double dye probe	5'-CATCCCAAGCCCTCGCTCTCCA-3'	
<i>Bip</i>	AJ002387	5'-AGAACGGCCGCGTGG-3'	5'-CCACATACGACGGCGTGA-3'	63
		Double dye probe	5'-CATAGCCAACGATCAGGGGAACCG-3'	
<i>GAPDH</i>	NM_008084	5'-CTTACCACCATGGAGAAGGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'	238
		Double dye probe	5'-CCTGGCCAAGGTATCCATGACAACCTT-3'	

were diluted and neutralized with Tris-buffered saline (pH 8.0) and subjected to ELISA using a commercialized kit (Shibayagi, Tokyo, Japan). For the histochemical studies, the pancreata were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

Insulin tolerance test

After a 4 h fasting, human insulin (Humulin R, Eli Lilly) at 0.25 U/kg BW was intraperitoneally injected into exendin-4-treated or untreated mice at 4 weeks of age. Plasma glucose levels were then measured at 0, 15, 30, and 45 min after the injection using a compact glucose analyzer.

Measurement of the insulin content of isolated islets and determination of the survival rates of dispersed islet cells

Pancreatic islets were isolated by collagenase digestion. The islet insulin content was measured after acid-ethanol extraction as described above. For the determination of the cell survival rate, isolated islets were dispersed by enzymatic digestion by dispase (Godo Shusei, Tokyo, Japan) and seeded onto a piece of poly-D-lysine-coated cover glass placed on a plastic dish. On the next day of the seeding, the cell number was counted under a phase-contrast microscope (MFA-10100, Nikon, Tokyo, Japan) and was regarded as 100% in each experimental group. The survival rate was expressed as a percentage of the numbers of the surviving cells at 48 and 72 h after seeding to the cell number at 24 h.

Immunofluorescence staining

For the immunohistochemical study, the pancreata fixed in 4% paraformaldehyde were sequentially washed thoroughly in PBS containing 10, 15, and 20% sucrose. They were then embedded in optimal cutting temperature compound (Sakura, Tokyo, Japan) and frozen. The frozen sections were cut into 5 μ m thick slices and incubated for 1 h with anti-insulin guinea pig antibody (Seikagaku Kogyo, Tokyo, Japan) and anti-glucagon rabbit antibody (ZYMED, San Francisco, CA, USA). After washing with PBS, they were further incubated for 1 h in a mixture of rhodamine-conjugated anti-guinea pig IgG antibody (ICN, Costa Mesa, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (Vector, Burlingame, CA, USA). The sections were analyzed with a confocal scanning laser microscope (MR-1024, Japan Bio-Rad). Morphological analysis of the data was carried out by the NIH Image software.

Expression of ER stress-related molecules in the isolated islets

mRNA in the isolated islets was extracted, and the expression levels of several ER stress-related genes (glucose-regulated protein 78/immunoglobulin-binding protein (Bip), inositol-requiring enzyme-1 (IRE-1 α), X-box-binding protein-1

(XBP-1), RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor-4 (ATF-4), C/EBP-homologous protein (CHOP)) were examined by RT-PCR. The sequences of the specific primer pairs are described in Table 1.

For more quantitative analysis, real-time PCR was carried out. Three micrograms of total RNA, extracted from isolated islets using the Trizol reagent (Invitrogen-Life Technologies, Inc.), were reverse transcribed using transcriptor reverse transcriptase (Roche Molecular Biochemicals). After cDNA synthesis, quantitative real-time PCR was performed in 25 μ l reaction mixtures containing qPCR Mastermix (Eurogentec, Seraing, Belgium) using the Mx3000P personal real-time PCR system (Stratagene Japan Co., Tokyo, Japan). Reaction mixtures were incubated for an initial denaturation at 95 °C for 10 min, followed by 40 cycles, each cycle consisting of 95 °C for 15 s and 60 °C for 1 min. The primers and probes of the spliced form of XBP-1, Bip, CHOP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Nippon EGT Co. Ltd (Toyama, Japan). Expression levels of spliced XBP-1, Bip, and CHOP mRNAs were standardized by comparison with that of GAPDH. The sequences of the specific primer pairs are described in Table 1. The specificity of each primer and probe set was confirmed by visualization of a single PCR product by agarose gel electrophoresis.

Statistical analysis

The data were expressed as the mean $\pm$ S.E.M. The statistical significance was estimated by Student's *t*-test or one-way ANOVA, followed by *post hoc* Bonferroni. A *P* value < 0.05 was considered statistically significant.

Results

Effects of chronic administration of exendin-4 on hyperglycemia in the CaMTg mice

CaMTg mice showed an acute and progressive hyperglycemia at 21 days after birth and thereafter. Chronic and s.c. administration of exendin-4 reduced hyperglycemia in CaMTg mice, whereas it did not show any effect on the plasma glucose level in nTg mice (Fig. 1). The exendin-4 treatment did not change BW in both nTg and CaMTg mice (25.8 $\pm$ 0.5 vs 25.3 $\pm$ 0.4 g for nTg mice without and with exendin-4 respectively and 25.0 $\pm$ 0.7 vs 24.4 $\pm$ 0.6 g for CaMTg mice without and with exendin-4 respectively at 4 weeks of age, *n* = 5–12). We also found that the food intake, when assessed at 4 weeks of age, was unchanged by the exendin-4 treatment (data not shown).

Insulin tolerance test

Since some GLP-1-related agents have been reported to improve insulin sensitivity (Gedulin *et al.* 2005), we estimated the effects

Figure 1 Daily profiles of plasma glucose levels in nTg and CaMTg mice treated with exendin-4 or saline. Exendin-4 (28 nmol/initial kg BW per day) was given subcutaneously and continuously over 2 weeks through osmotic minipumps transplanted on day 14 after birth. Blood samples were taken daily by tail cutting, and plasma glucose levels were measured using a compact glucose analyzer. Open circles, CaMTg mice treated with saline ($n=21$). Closed circles, CaMTg mice treated with exendin-4 ($n=13$). Open squares, nTg mice treated with saline ($n=13$). Closed squares, nTg mice treated with exendin-4 ($n=7$). Each symbol represents a mean $\pm$ S.E.M. value. * $P<0.05$, † $P<0.01$, as compared with the corresponding value in the saline-treated CaMTg mice.

of exendin-4 on the insulin sensitivity. As illustrated in Fig. 2, time-dependent decreases in the plasma glucose concentrations after insulin injection (0.25 U/kg BW i.p.) were observed in all groups. Although the glucose levels at time 0 were different, we could not find any difference in the decline between exendin-4-treated and untreated CaMTg or nTg mice.

Figure 2 Lack of effects of the exendin-4 treatment on the insulin tolerance test. After the 2-week *in vivo* treatment with or without exendin-4, human insulin (0.25 U/kg BW) was injected intraperitoneally. The plasma glucose concentrations were measured at 0, 15, 30, and 45 min. Open circles, CaMTg mice treated with saline. Closed circles, CaMTg mice treated with exendin-4. Open squares, nTg mice treated with saline. Closed squares, nTg mice treated with exendin-4. Each symbol represents a mean $\pm$ S.E.M. value; $n=5$.

Figure 3 Effects of exendin-4 treatments on the insulin contents of the pancreata and islets and on insulin release. (A) At the end of the 2-week administration of exendin-4, pancreata from the saline-treated nTg ($n=6$, open column), the exendin-4-treated nTg ($n=3$, shaded column), the saline-treated CaMTg ($n=4$, closed column), and the exendin-4-treated CaMTg mice ($n=8$, hatched column) were homogenized in ice-cold acid-ethanol solution and centrifuged. The supernatants were subjected to ELISA for insulin determination. (B) On day 28 after birth, groups of three pancreatic islets isolated from the saline-treated nTg ($n=24$, open column), the exendin-4-treated nTg ($n=8$, shaded column), the saline-treated CaMTg ($n=25$ closed column), and the exendin-4-treated CaMTg mice ($n=7$, hatched column) were extracted. Their insulin content was measured and expressed as ng/islet. (C) Insulin release from isolated islets was measured by the batch incubation for 30 min with basal (3 mM) or stimulatory (20 mM) glucose. Released insulin was expressed as a rate of release to the islet insulin content. Each column represents a mean $\pm$ S.E.M. value. * $P<0.05$; $n=5$.

Effects of the exendin-4 treatment on the serum insulin concentrations

The serum insulin concentrations were not altered by the exendin-4 treatment both in nTg and CaMTg mice fed *ad libitum* (data not shown). We found that the exendin-4 treatment increased the serum insulin concentrations taken at 15 min after i.p. injection of glucose (2 g/kg BW) in nTg and CaMTg mice to a certain extent, although they did not reach statistical significance (2.88 ± 0.78 and 3.38 ± 0.85 ng/ml for nTg mice without and with the exendin-4 treatment respectively and 0.42 ± 0.15 and 1.54 ± 0.79 ng/ml for CaMTg mice without and with the exendin-4 treatment respectively, $n=3-5$).

Effects of the in vivo treatment with exendin-4 on the pancreatic insulin content

Figure 3 shows the effects of the exendin-4-treatment on the insulin contents in the pancreas and pancreatic islets. The CaMTg pancreatic insulin content was decreased to 11.8% of the nTg control (Fig. 3A) and the CaMTg islet insulin content was also decreased to 6.7% of the control (Fig. 3B) at the end of 4 weeks after birth. Chronic treatment with exendin-4 resulted in significant increases in the pancreatic insulin contents in both nTg and CaMTg mice (Fig. 3A). The treatment also increased the insulin content of isolated islets from nTg and CaMTg mice (Fig. 3B). In insulin secretion experiments using isolated

Figure 4 *In vivo* effects of exendin-4 on the insulin-positive area. (A) Mouse pancreata at 4 weeks of age were immunostained with anti-insulin and anti-glucagon antibodies and visualized by the FITC- and rhodamine-conjugated second antibodies respectively. Bar = 100 μ m. (B) Morphometrical analyses were carried out for 30–80 islets per pancreas from 3 to 5 mice for each group to measure the insulin-positive areas. Each column represents a mean $\pm$ S.E.M. value for the saline-treated nTg (open column), the exendin-4-treated nTg (shaded column), the saline-treated CaMTg (closed column), and the exendin-4-treated CaMTg islets (hatched column). * $P < 0.05$.

islets, glucose stimulation failed to increase insulin secretion from untreated CaMTg islets. The treatment did not ameliorate the unresponsiveness, but it increased the rate of released insulin to the level of the insulin content required for basal glucose (Fig. 3C).

Influence of the exendin-4 treatment in the insulin-positive area

The above effects of the exendin-4 treatment on the insulin contents led us to an idea that the treatment may expand the β -cell insulin-positive area in individual islets. Immunohistochemical approach showed an increase in the insulin-positive areas in the nTg or CaMTg islets treated with exendin-4 (Fig. 4A). Indeed, quantitative morphological analysis suggested that the insulin-positive areas in individual islets were significantly increased by the exendin-4 treatment both in the nTg and CaMTg mice (Fig. 4B). The spatial derangement of the CaMTg islet endocrine cells was, however, unchanged by the treatment with exendin-4 (Fig. 4A). When estimated by 5-bromo-2'-deoxyuridine staining, we could not find any effect of the exendin-4 treatment on β -cell replication in CaMTg mice (data not shown).

Survival rate of the nTg and CaMTg islet cells

In our preliminary experiments, normal islet cells survived well in the presence of 11.1 mM glucose, which agrees with previous report (Efanova *et al.* 1998). With 11.1 mM glucose, the life span for CaMTg islet cells was very short; $\approx 60\%$ of the cells disappeared during the observation from 24 to 72 h after seeding (Fig. 5).

Figure 5 Survival rate of the dispersed islet cells. Dispersed cells obtained from nTg (open columns) and CaMTg islets (closed columns) were seeded onto a plastic dish, and cultured for 3 days. The survival rates were expressed as a percentage of the surviving cells over the cells at 24 h after seeding. Each symbol represents a mean $\pm$ S.E.M. value for 4–5 observations.

Expression of ER stress-related molecules in CaMTg islets

We examined expression levels of the ER stress-related molecules (Bip, IRE-1 α , XBP-1, PERK, ATF-4, and CHOP) in nTg and CaMTg islets at 4 weeks of age by RT-PCR. As illustrated in Fig. 6A, all these ER stress-response genes were more expressed in CaMTg islets. To confirm the induction of ER stress in CaMTg islets, we further carried out quantitative real-time PCR analysis for the spliced form of XBP1 mRNA and found the XBP1 splicing was indeed increased in CaMTg islets ($764 \pm 236\%$ of the expression level in the saline-treated nTg islets; Fig. 6B). These findings suggest that CaMTg islets were exposed to increased ER stress.

Quantitative estimation of Bip and CHOP expression levels by real-time PCR

Among these ER stress-related molecules, we quantified expression levels of Bip and CHOP by quantitative real-time PCR and estimated the effects of the exendin-4 treatment on the expression levels. We estimated these expression levels as a rate to the GAPDH expression, and found Bip and CHOP were more expressed in the CaMTg islets (1682 ± 412 and $9600 \pm 1370\%$ of each expression level in the saline-treated nTg islets for Bip and CHOP respectively, $n=3$). The *in vivo* treatment with exendin-4 significantly lowered the expression levels of both genes in the CaMTg islets (396 ± 201 and $3816 \pm 956\%$ of each expression level in the saline-treated nTg islets for Bip and CHOP respectively, $n=3$; Fig. 7).

Discussion

Exendin-4 is a homolog of GLP-1 originally derived from *Heloderma suspectum* and known to possess a potent anti-diabetic effect through its binding to the GLP-1 receptor (Eng *et al.* 1992). The present study demonstrated that the beneficial effects of exendin-4 were also found in CaMTg diabetic mice, and that its major effect was to maintain the β -cell mass, because 1) the exendin-4 treatment increased the insulin contents of the pancreas and islets and 2) it also expanded the insulin-positive area in both nTg and CaMTg mice. Although the extrapancreatic effects of GLP-1 have been reported to participate in the hypoglycemic effects of exendin-4 (Egan *et al.* 1994, Luque *et al.* 2002, Dardevet *et al.* 2004), we could not find any effect of the treatment on plasma glucose levels in the insulin tolerance test.

Anti-apoptotic effects of the long-acting GLP-1 analog, liraglutide, have also been reported with primary-cultured neonatal rat β -cells, and such effects were reproduced by the adenylate cyclase activator, forskolin (Bregenholt *et al.* 2005). We could not find any effect of the exendin-4 treatment on β -cell replication. Since similar results were obtained by chronic administration of PACAP to CaMTg mice (Tsunekawa *et al.* 2005), we suggest that cAMP production may protect β -cells

Figure 6 Expression levels of ER stress-related molecules by RT-PCR (A) and expression levels of the spliced form of XBP-1 by real-time PCR (B) in CaM transgenic islets. The expression levels of the ER stress-related molecules in mRNA extracted from nTg (open columns) and CaMTg (closed columns) islets were examined by RT-PCR (A) and the expression levels of the spliced form of XBP-1 by real-time PCR (B). Data were expressed as the rates to the expression levels to GAPDH in the same sample. For Fig. 6A, these results are the representative of three experiments with similar results. Statistical significance in Fig. 6B was assessed by Student's *t*-test. **P* < 0.05; *n* = 3. N.D., not determined; A.U., arbitrary units.

against apoptotic cell death in the diabetic mice. One difference between the PACAP and exendin-4 effects on CaMTg mice is the absence of the effects of PACAP on nTg mice. We do not know why the *in vivo* treatment with exendin-4 expanded the insulin-positive area in both nTg and CaMTg mice, whereas that with PACAP only improved the latter (Tsunekawa *et al.* 2005).

A few possible mechanisms of β -cell protection by GLP-1 or exendin-4 have also been reported. Reduction of caspase-3

expression in islets by GLP-1 has been proposed in a diabetic model animal (Wang & Brubaker 2002). Jhala *et al.* (2003) reported that GLP-1 may exhibit its protective effects through the activation of protein kinase B/Akt through cAMP-dependent phosphorylation of cAMP-responsive element-binding protein and resultant activation of the insulin signaling. A recent study using insulin receptor substrate-2 (IRS-2) knockout mice demonstrated that β -cell protection by GLP-1 required the IRS-2 signaling (Park *et al.* 2006).

Figure 7 Effects of the *in vivo* exendin-4 treatment on Bip and CHOP expression levels estimated by quantitative real-time PCR. mRNA was extracted from saline-treated nTg islets (open column), saline-treated CaMTg islets (closed column), and exendin-4-treated CaMTg islets (hatched column) at 4 weeks of age. The expression levels of Bip (A) and CHOP (B) were evaluated by quantitative real-time PCR. Data were expressed as the rates to that of GAPDH in the same sample. Each column represents a mean $\pm$ S.E.M. value for three independent experiments. * $P < 0.05$; † $P < 0.01$; $n = 3$.

We further investigated the underlying mechanism of the β -cell protection by exendin-4 in this study and found that several genes related to ER stress were highly expressed cells as well as the increase of XBP-1 splicing in CaMTg islets. Among them, elevated expression levels of Bip and CHOP in the CaMTg β -cells were reduced by the *in vivo* exendin-4 treatment. It implies that the apoptotic process in the transgenic islet cells may involve excess of ER stress, and exendin-4 may lessen the ER stress and prevent β -cell apoptosis in the transgenic β -cells.

The reason why calmodulin overexpression in the β -cells induces β -cell death remains unclear. It was previously reported that, among the 13 proteins overexpressed in β -cells using the insulin promoter *in vivo*, only calmodulin and major histocompatibility complex molecules produced early onset diabetes (Harrison *et al.* 1989). One possibility is that calmodulin overexpression *per se* may trigger ER stress in CaMTg mice. We previously measured the calmodulin content in the islets in this model (1.3 and 2.1 ng/islet for nTg and CaMTg mice respectively; Yu *et al.* 2002). When we standardized these values by their pancreatic insulin content at this age (Tsunekawa *et al.* 2005), ≈ 4.9 times overexpression of calmodulin was achieved by the gene targeting. The overexpression rate is relatively not very high, but if we consider a fairly high concentration of calmodulin in the intact islets (Sugden *et al.* 1979, Valverde & Malaisse 1984), we speculate that overexpressed calmodulin itself may be a cause of ER stress in the CaMTg β -cells.

Since inhibition of the Ca^{2+} influx prevented high glucose-induced apoptosis of the β -cells (Efanova *et al.* 1998), one possibility is that exendin-4 may reduce Ca^{2+} influx into the β -cells by high glucose. However, the effects of exendin-4 or GLP-1 on the β -cell cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) seem to be complex. GLP-1 has been reported to lower glucose-induced $[\text{Ca}^{2+}]_i$ increase in rat β -cells (Yaekura *et al.* 1998). Protection by exendin-4 of β -cells from apoptotic cell death may, therefore, result from its decreasing effect on the intracellular Ca^{2+} levels. On the other hand, GLP-1 has also been reported to increase $[\text{Ca}^{2+}]_i$ through inhibition of the K_{ATP} channels in the mouse β -cells (Gromada *et al.* 1997) or through activation of Ca^{2+} -induced Ca^{2+} release in INS-1 β cells (Kang & Holz 2003). We have to be careful in the interpretation of these results, because most of these studies are monitoring $[\text{Ca}^{2+}]_i$ during a short duration after application of GLP-1, and the chronic changes in $[\text{Ca}^{2+}]_i$ dynamics after its long-term treatment may be different.

Another possibility is that exendin-4 may act on the intraER Ca^{2+} dynamics. There is a line of evidence suggesting that ER stress is caused by intraER Ca^{2+} depletion. Some isoforms of the sarco(endo)plasmic reticulum Ca^{2+} -ATPases (SERCA) have been expressed in the human and rodent β -cells (Roe *et al.* 1994, Varadi *et al.* 1996). Inhibition of SERCA is known to cause apoptotic cell death in the MIN6 β -cell (Zhou *et al.* 1998). It has recently been reported that cytokines which cause β -cell death down-regulate SERCA2b, deplete intraER Ca^{2+} , and induce ER stress (Cardozo *et al.* 2005). The expression levels of SERCA isoforms are lowered in db/db diabetic mice and IRS-1 null mice (Roe *et al.* 1994, Kulkarni *et al.* 2004). In a genetic approach, the UK prospective diabetes study suggests that sequence variants of SERCA3 are involved in the genetic etiology of Caucasian type 2 diabetes mellitus (Varadi *et al.* 1999). GLP-1, as well as the adenylate cyclase activator forskolin and the membrane-permeable cAMP analog dibutyryl cAMP, is known to increase Ca^{2+} uptake to ER

through activation of ER Ca^{2+} pump in the β -cell (Yaekura & Yada 1998). Therefore, exendin-4 may reduce ER stress through activating the ER Ca^{2+} -pump and increasing Ca^{2+} -uptake into ER. Interestingly, cAMP-dependent phosphorylation of phospholamban has been reported to activate sarco(endo)plasmic reticulum Ca^{2+} pump in endothelial cells (Schildberg *et al.* 2005).

Very recently, it has been demonstrated that *in vivo* treatment with exendin-4 reduced the expression of ER-stress markers in the islets from db/db diabetic mice (Yusta *et al.* 2006). Our present findings also support their finding and further suggests that the GLP-1 agonist may be useful for the maintenance of the β -cells and prevent β -cell exhaustion under diabetic conditions.

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