was assumed in the highly clustered families in linkage analysis. We adopted all databases available in Endeavour, which prioritized glucokinase regulator (*GCKR*) at the first rank.

2.6. Sequencing

We directly sequenced the coding exons of 6 MODY genes (*HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, and *NEUROD1*) in the 10 index cases. We sequenced *GCKR* including all exons found in the National Center for Biotechnology Information (NCBI) Evidence Viewer (http://www.ncbi.nlm.nih.gov) and the 2-kb promoter region in the index cases from families and in control subjects. We also selected other 7 genes that are highly prioritized within the 11th rank (10.3%) in the linkage region using Endeavour excluding 3 genes with known metabolic functions unrelated to glucose metabolism (Supplementary Table 1). We sequenced the entire coding exons of the 7 genes in the index cases from families included in the linkage analysis. Forward and reverse PCR primers for each exon were selected in an intronic sequence 50 bp away from the intron/exon boundaries and primers to amplify the *GCKR* promoter region were also selected. Sequencing primer data for *GCKR* is shown in Supplementary Table 2. PCR products were run on 2% agarose gel, and the appropriate bands were excised and then purified with the use of the QIAquick Gel Extraction Kit (Qiagen). Sequencing results were analyzed on an ABI Prism 3130 Avant DNA sequencer (Applied Biosystems). Any nucleotide changes identified in sequencing were searched for SNPs (single nucleotide polymorphisms) in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/).

2.7. Genotyping SNPs

If minor allele frequencies (MAF) of nucleotide changes identified in sequencing were unregistered in the HapMap JPT database on dbSNP as of April 2010 and the minor allele appeared in <2 of all subjects, MAF was determined in the expanded population. We defined mutation as MAF <1% [15]. To determine whether each nucleotide change was a mutation or not, we genotyped 105 normoglycemic controls randomly selected from the cohort (Supplementary Table 3), because genotyping of 210 normal chromosomes is necessary to achieve 80% power to detect a polymorphism present in 1% of the population [16]. The PCR-RFLP (restriction fragment length polymorphism) method for *HNF1A* R583G, *GCKR* g.-689G>A, *GCKR* g.-299G>A, *GCKR* E252K and *FOSL2* R198H and Taqman method for *GCKR* g.6859C>G were used.

2.8. Statistical analysis

Frequencies of mutations (MAF<1%) and common nucleotide changes (MAF≥1%) identified in *GCKR* sequencing in the index cases and in normoglycemic controls were compared by the Fisher exact test with SAS software (version 8.2).

2.9. Ethics

The methods used in this study were approved by the Ethics Committee of the Kyoto University Institutional Review Board, and approved written informed consent was obtained from each participant.

3. Results

3.1. Characteristics of family members

Four families with a 3-generation history of diabetes were enrolled in this study (Fig. 1, Table 1). Every family included no less than 1 member that had been diagnosed with diabetes before the age of 50. Sixteen members (6 men, 10 women) had previously been diagnosed with diabetes. Thirteen out of the 16 members with diabetes were lean (BMI<25). Six members were treated with insulin and another 10 members were treated with oral hypoglycemic agents. Twelve family members who had not been diagnosed with diabetes underwent HbA1c measurement and 3 of them had HbA1c level ≥6.0%. These 3 members had already been diagnosed with impaired glucose tolerance before this study and were included as affected members in the study.

3.2. Exclusion of MODY gene mutations in the index cases

For the 10 index cases, we performed direct sequencing in entire coding exons of the MODY genes. The detected missense SNPs were *HNF1A* I27L (rs1169288), *HNF1A* S487N (rs2464196), *HNF1A* R583G, and *HNF4A* T117I (rs1800961) (Supplementary Table 4). *HNF1A* R583G is a mutation that is reported to cause MODY [17], thus we excluded the carrier of the mutation (additional index case #6, Table 1) from further investigation. *HNF1A* I27L and *HNF1A* S487N are common in the general population (MAF = 0.386 and 0.341, respectively in HapMap-JPT). *HNF4A* T117I was associated with late-onset type 2 diabetes but it was not the cause of MODY in a previous report [18].

3.3. Linkage analysis

A total of 30 members (19 affected members) from 4 families were included in the linkage analysis, assuming an autosomal dominant model. The genome-wide linkage results in the screening are shown in Fig. 2. Regions of potential interest by multipoint LOD and HLOD scores were observed on chromosomes 2p24 and 7q34. After fine mapping, 2p25-22 was revealed to be a significant linkage region (Fig. 3, LOD and HLOD = 3.47) while the region on 7q34 was discarded. The size of the region with positive HLOD score was 23.6 Mb (D2S2199–D2S2230). In the region, a haplotype segregated in affected and unaffected members in the pedigrees 1, 2, and 3, but not in the pedigree 4.

3.4. Candidate genes

We searched candidate genes in the implicated linkage region by applying a gene prioritization approach implemented in Endeavour software. We selected 6 MODY genes as training genes. The 2 top-ranked genes were glucokinase regulatory protein (*GCKR*) and nuclear receptor coactivator 1 (*NCOA1*). *GCKR* ranked high in prioritization using gene–gene interaction databases (first rank in 5 out of 7 interaction databases), mainly because the interaction of glucokinase and glucokinase regulatory protein has been demonstrated in previous studies [19,20]. *NCOA1* also ranked high in prioritization using gene–gene interaction databases (second rank in 2 out of 7 interaction databases), because nuclear receptor coactivator 1 has been reported to interact with HNF4α (Hepatocyte Nuclear Factor 4α) as a coactivator [21]. Together with *GCKR* and *NCOA1*, genes that are highly prioritized within the 11th rank (10.3% of annotated genes) were considered candidate genes except 3 genes with well-characterized metabolic functions unrelated to glucose metabolism (Supplementary Table 1).

Fig. 2. Multipoint HLOD and LOD scores in genome-wide linkage analysis for 4 pedigrees.

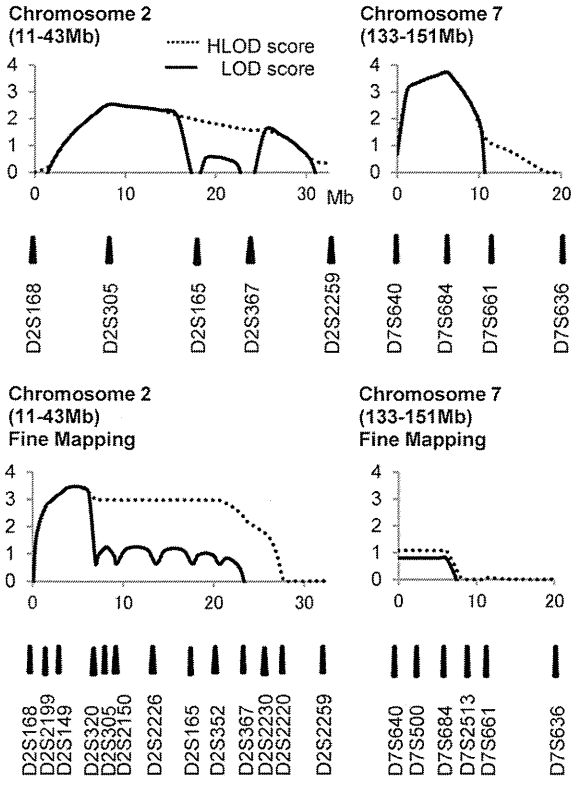

Fig. 3. Multipoint HLOD and LOD scores in fine mapping of D2S168–D2S2259 and D7S640–D7S636.

3.5. Direct sequencing in GCKR and other candidate genes

We performed direct sequencing in exons and the 2-kb promoter region of *GCKR*. Sequencing was performed in 9 index cases from families and in 18 normoglycemic controls in parallel. The 18 control subjects were randomly selected from 206 normoglycemic controls (Supplementary Table 3). Detected sequence changes in the 9 index cases and 18 controls are shown in Table 2. Five nucleotide changes (g.-959 Insertion AATGTTG, E66E, E77G, g.9709G>A, and L446P) were considered to be common variants, because the minor allele was found in not less than 2 subjects out of a total of 27 case and control subjects. To determine whether or not each of the other nucleotide changes (g.-689G>A, g.-299G>A, E252K and g.6859C>G) was a mutation (MAF<1%), genotyping was performed in a total of 105 normoglycemic controls. g.-689G>A, g.-299G>A and g.6859C>G were not detected in the 105 controls, and were regarded as mutations, while E252K was detected in 4 controls out of 105 (MAF = 1.9%) and was regarded as a common change. The number of alleles having mutations was thus significantly larger in the index cases from families than in the controls (3/18 alleles vs. 0/36 alleles, P = 0.033, Fisher exact test).

We performed direct sequencing in the entire coding exons of other 7 candidate genes in index cases from 4 families. One misssense mutation *FOSL2* R198H (MAF = 0.004 in normoglycemic controls) was detected. No other mutations were detected in other 6 genes (Supplementary Table 5).

3.6. Segregation of the mutations with the phenotype in pedigrees

In index cases from the 4 families included in the linkage analysis, 3 sequence changes of *GCKR* were detected (g.-959 Insertion AATGTTG, g.6859C>G and L446P). We tested the segregation of *GCKR* g.6859C>G, a mutation detected in pedigree 3, with the phenotype in the pedigree. Another 2 changes (*GCKR* g.-959 Insertion AATGTG and *GCKR* L446P) were commonly detected in controls (3/36 alleles and 11/36 alleles respectively). In pedigree 3, *GCKR* g.6859C>G was detected in all 9 affected members, but was not detected in the unaffected member (II-7). We performed linkage analysis and haplotype construction in 2p25-22 using the *GCKR* g.6859 genotype together with the microsatellite markers. The parametric multipoint LOD score for pedigree 3 was 2.67 at the *GCKR* g.6859 locus. Haplotype analysis revealed that all affected individuals in pedigree 3 shared a disease haplotype within D2S2199–D2S2230, which includes *GCKR* g.6859G (Fig. 4). In pedigree 3, another sequence change, *GCKR* L446P, was detected, but *GCKR* L446P did not co-segregate with the disease. Haplotype analysis revealed that the minor allele of *GCKR* L446P (g.11169C) resided on a different haplotype than *GCKR* g.6859G in affected subjects III-11, 12, 13, 14 (Fig. 4).

We tested the segregation of *FOSL2* R198H, a mutation detected in pedigree 4, with the phenotype. *FOSL2* R198H was detected in 2 affected subjects (II-2, II-22) but not detected in one subject (II-1).

4. Discussion and conclusions

Recent progress in genome-wide association studies has identified tens of type 2 diabetes susceptibility genes. Even so, only a small

Table 2
Mutations and common nucleotide changes in exons and the promoter of *GCKR* in 9 index cases in families and in 18 controls.

Position	Change	Description	Effect	Detected number of alleles				p^a	Minor allele frequency [MAF]
				Index cases from families (n = 9)		Controls (n = 18)			
				Major	Minor	Major	Minor		
Mutations (MAF<1%)									
Promoter	g.-689G>A			17	1	36	0	0.33	0.000[b]
Promoter	g.-299G>A			17	1	36	0	0.33	0.000[b]
Exon 9	g.6859C>G	Noncoding exon		17	1	36	0	0.33	0.000[b]
Total				15	3	36	0	0.033	
Common changes									
Promoter	g.-959 insAATGTTG			16	2	33	3	1.00	N/D
Exon 2	g.468G>A	Synonymous	E66E	17	1	35	1	1.00	N/D
Exon 3	g.671A>G	Missense	E77G	17	1	33	3	1.00	0.024[c]
Exon 10	g.8817G>A	Missense	E252K	18	0	35	1	1.00	0.019[b]
Exon 11	g.9709G>A	Noncoding exon		17	1	33	3	1.00	0.123[c]
Exon 14	g.11169T>C	Missense	L446P	8	10	25	11	0.087	0.467[c]

GenBank accession no. NT_022184.15.
[a] Fisher exact test.
[b] Frequency in 105 normoglycemic controls.
[c] Frequency in HapMap-JPT.

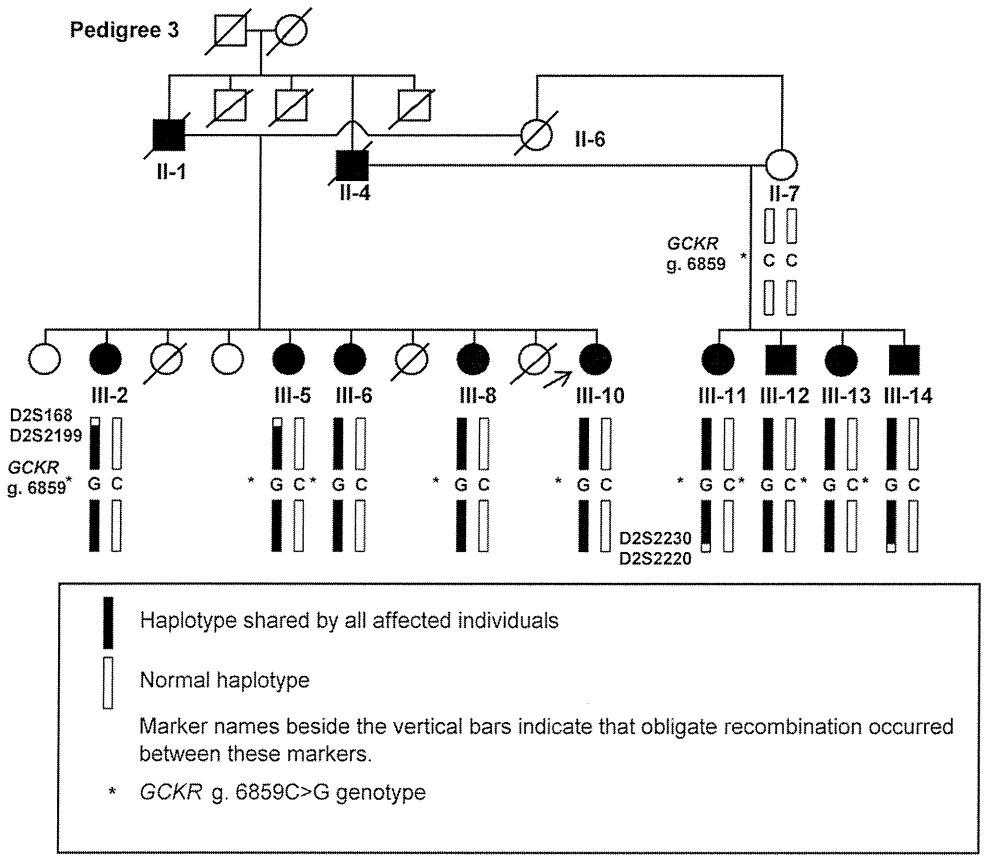

Fig. 4. Haplotype analysis in the D2S168–D2S2259 region and the *GCKR* g.6859C>G genotype for pedigree 3.

portion of the genetic background of diabetes has been explained in the Japanese population. The loci identified in association studies have only very small effect sizes. We hypothesized that rare disease variants with larger effect sizes remain to be discovered that may explain a greater part of the genetic background. Family-based linkage study is an important alternative for the identification of rare disease variants. Indeed, studies with large families with highly clustered diabetes have revealed important mutations involved in MODY and other dominantly inherited diabetes, including a *KCNJ11* mutation [22]. We therefore recruited families with a 3-generation history of diabetes. The validity of our strategy was strengthened by the fact that one case out of the 10 index cases recruited in our study carried a previously reported rare disease variant *HNF1A* R583G.

Our family analysis revealed a significant linkage region on chromosome 2p25-22 that has not been reported in previous Japanese sib-pair analyses [3–5]. Because our approach was based on a higher degree of familial clustering than sib-pair analyses, the linkage region suggested in the present study might well go undetected in sib-pair analyses that include an admixture of sib-pairs with both low and high degrees of familial clustering. In the present study, we conducted a computational approach targeting the linkage region on chromosome 2p25-22. One hundred and six known genes were present in this linkage region. Prioritization of the candidate gene was possible by integrating the information available from multiple publicly available databases [14]. *GCKR* and other 7 gens ranked high in the prioritization, and were selected as candidate genes.

GCKR regulates glucokinase (GCK), the first glycolytic enzyme, in liver. *GCKR*-null mice exhibit elevated postprandial glucose [19]. Adenoviral-mediated overexpression of *GCKR* in mouse liver increases GCK activity and lowers fasting blood glucose. It was suggested that GCKR, a competitive inhibitor of GCK activity, also has a paradoxical role in extending GCK half-life by stabilizing the enzyme [20]. If so, diminished expression of GCKR in human might cause decreased GCK activity in liver and lead to impaired liver glucose uptake, which suggests the *GCKR* mutation as a possible cause of the disease in linked families.

We sequenced entire exons and the 2-kb promoter region of *GCKR* in 9 index 3-generation cases and in 18 control subjects. The rare variants were significantly more frequent in index cases from families than in control subjects. In addition, exonic rare variant g.6859C>G in pedigree 3, which was not detected in 105 control subjects, was clearly segregated in all 9 affected members in pedigree 3. Previous reports have shown the association of common *GCKR* variants with fasting plasma glucose, glucose level after glucose challenge, and diabetes risk in various ethnic groups [23–30]. In Japanese population, a common variant *GCKR* rs780094 is associated with fasting glucose and diabetes risk [27,30]. Our family study suggests the effect of rare *GCKR* variants on diabetes susceptibility that has not been revealed by previous association studies. A recent study has shown the excess of rare *GCKR* variants in individuals with hypertriglyceridemia [31], which supports our idea that rare *GCKR* mutations also affect the diabetes susceptibility.

On the other hand, the only one mutation in other 7 highly prioritized genes was *FOSL2* R198H and it did not co-segregate with the phenotype in the pedigree. Therefore, we tentatively eliminate the possibility that these genes are involved in familial clustering of diabetes patients in the current pedigrees.

Our study has several limitations. First is the large size (23.6 Mb) of the linkage region. Only 4 families could be included in the linkage analysis because we limited the cohort to 3-generation families with ≥2 affected members who donated DNA. Further efforts to recruit large families are needed to narrow down the linkage region. Second, because the *GCKR* g.6859C>G mutation was in a non-coding exon, confirming the relevance of the mutation as the cause of the disease is difficult. Investigation of the effect of the mutation in human liver, where GCKR is predominantly expressed [32], is required, but liver specimens of family

members are currently unavailable. Although we tried to determine the mRNA level in peripheral blood of family members, GCKR mRNA was only barely detectable with the RT-PCR method (data not shown), so comparison of the GCKR mRNA level between affected and unaffected members was not possible. We speculate that the g.6859C>G mutation might affect GCKR function in liver through mRNA transcription or splicing processes [33]. *GCKR* g.-689G>A and g.-299G>A mutations located in the promoter also might affect the expression of GCKR, but TRANSFAC database [34] expected no binding sites of transcription factors at the two promoter mutations.

In conclusion, with systematic investigation we propose that *GCKR* is a susceptibility gene in Japanese families with clustered diabetes. A family-based approach may be a promising strategy to elucidate the complex genetic background of common diseases including type 2 diabetes.

Supplementary materials related to this article can be found online at doi:10.1016/j.ymgme.2010.12.009.

Acknowledgments

This work was supported by a grant for Research on Human Genome Tailor-Made from the Ministry of Health, Labor, and Welfare of Japan, a grant for Intractable Disease Research Program from the Ministry of Health, Labor, and Welfare of Japan, Scientific Research Grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and a grant from Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Cooperation, and by Kyoto University Global COE Program "Center for Frontier Medicine".

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GLP-1 receptor agonist attenuates endoplasmic reticulum stress-mediated β-cell damage in Akita mice

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ABSTRACT

Aims/Introduction: Endoplasmic reticulum (ER) stress is one of the contributing factors in the development of type 2 diabetes. To investigate the cytoprotective effect of glucagon-like peptide 1 receptor (GLP-1R) signaling *in vivo*, we examined the action of exendin-4 (Ex-4), a potent GLP-1R agonist, on β-cell apoptosis in Akita mice, an animal model of ER stress-mediated diabetes.

Materials and Methods: Ex-4, phosphate-buffered saline (PBS) or phlorizin were injected intraperitoneally twice a day from 3 to 5 weeks-of-age. We evaluated the changes in blood glucose levels, bodyweights, and pancreatic insulin-positive area and number of islets. The effect of Ex-4 on the numbers of C/EBP-homologous protein (CHOP)-, TdT-mediated dUTP-biotin nick-end labeling (TUNEL)- or proliferating cell nuclear antigen-positive β-cells were also evaluated.

Results: Ex-4 significantly reduced blood glucose levels and increased both the insulin-positive area and the number of islets compared with PBS-treated mice. In contrast, there was no significant difference in the insulin-positive area between PBS-treated mice and phlorizin-treated mice, in which blood glucose levels were controlled similarly to those in Ex-4-treated mice. Furthermore, treatment of Akita mice with Ex-4 resulted in a significant decrease in the number of CHOP-positive β-cells and TUNEL-positive β-cells, and in CHOP mRNA levels in β-cells, but there was no significant difference between the PBS-treated group and the phlorizin-treated group. Proliferating cell nuclear antigen staining showed no significant difference among the three groups in proliferation of β-cells.

Conclusions: These data suggest that Ex-4 treatment can attenuate ER stress-mediated β-cell damage, mainly through a reduction of apoptotic cell death that is independent of lowered blood glucose levels. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00075.x, 2011)

KEY WORDS: Apoptosis, Endoplasmic reticulum stress, Glucagon-like peptide-1

INTRODUCTION

Type 2 diabetes is a chronic metabolic disorder characterized by the loss of β-cell function and mass. The mechanisms underlying the loss of β-cell function and mass are not fully understood, but recent studies have shown that endoplasmic reticulum (ER) stress is one of the causes of β-cell damage in diabetes[1]. Owing to increased demand for insulin secretion, β-cells show a highly developed ER[1]. The ER has a number of important functions, such as post-translational modification, folding and assembly of newly synthesized secretory proteins[2–4]. Thus, the ER plays an essential role in cell survival. ER function can be impaired by various conditions, including inhibition of protein glycosylation, reduction in formation of disulfide bonds, calcium depletion from the ER lumen, impairment of protein transport from the ER to the Golgi and expression of malfolded proteins[1]. Various physiological or pathological conditions that compromise ER functions are collectively termed ER stress[1–3]. To alleviate ER stress and promote cell survival, an adaptive response, known as unfolded protein response (UPR) is activated. UPR comprises translational attenuation, induction of chaperones and ER stress-associated degradation (ERAD). However, prolonged activation of UPR can ultimately lead to cell death by apoptosis.

Increased demand for insulin secretion under certain conditions, such as chronic hyperglycemia, might result in β-cell overload. Chronic hyperglycemia in diabetes can therefore induce persistent ER stress, cause β-cell dysfunction and finally lead to a reduction in β-cell mass through apoptosis[1].

Glucagon-like peptide 1 (GLP-1) is a physiological incretin, an intestinal hormone released in response to nutrient

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Received 21 July 2010; revised 25 August 2010; accepted 8 September 2010

ingestion that stimulates glucose-dependent insulin secretion. A growing body of evidence suggests that GLP-1 not only increases insulin secretion and upregulates insulin biosynthesis, but also stimulates β-cell proliferation and neogenesis[5–9], and inhibits β-cell apoptosis[9–16], resulting in increased β-cell mass. However, demonstration of an *in vivo* effect in the animal models of type 2 diabetes is problematic, because enhancement of GLP-1R signaling lowers blood glucose levels as result of its insulinotropic action, and it is difficult to evaluate the direct cytoprotective effects of GLP-1 in conditions of similar glucose toxicity.

In the present study, we investigated the cytoprotective effect of GLP-1R signaling *in vivo* on ER stress-mediated apoptotic cell death by using Akita mice, an animal model of ER stress-mediated diabetes mellitus. Akita mice have a point mutation in the insulin 2 gene, resulting in misfolding of insulin that leads to severe ER stress[17,18]. To exclude the possibility that the effect of Ex-4 on β-cells is mediated through improved blood glucose levels, we used three groups of mice: Akita mice treated with phosphate-buffered saline (PBS), Ex-4, or the sodium-coupled glucose transporter inhibitor phlorizin, which decreases blood glucose levels without increasing insulin secretion.

MATERIALS AND METHODS

Experimental Animals

Male C57BL/6 mice and male Akita mice were obtained from Shimizu (Kyoto, Japan). The animals were housed under a light/dark cycle of 12 h with free access to food and water. All experiments were approved by the Kyoto University Animal Care Committee.

In vivo Treatment

The mice were given twice daily intraperitoneal injections of PBS, Ex-4 (24 nmol/kg) or phlorizin (0.3 g/kg) for 2 weeks (from 3 to 5 weeks-of-age). Blood glucose levels were measured every third day by enzyme electrode method using a portable glucose analyzer (Glutest sensor; Sanwakagaku, Nagoya, Japan). Blood samples were collected from tail cuttings from these mice fed *ad libitum*. At the end of the experimental period, blood samples were collected from the inferior vena cava under anesthesia to determine the plasma glycoalbumin levels (Oriental Yeast, Tokyo, Japan). Pancreas samples from each of the animal groups were obtained for histological evaluation, and islets were isolated for measurement of insulin content and RNA extraction.

Evaluation of Pancreatic Insulin-Positive Area and Number of Islets

The pancreas samples were fixed in Bouin's solution. Serial 5-μm paraffin-embedded tissue sections were mounted on slides. After rehydration, sections were incubated with polyclonal rabbit anti-insulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), with a biotinylated goat anti-rabbit antibody (DAKO, Carpinteria, CA, USA), and then with a streptavidin peroxidase conjugate and substrate kit (DAKO) using standard protocols. The total pancreas area and insulin-positive area were quantified on five distal, random, non-overlapping sections from five mice of each group using a BZ-8100 microscope equipped with a BZ-Analyzer (KeyEnce, Osaka, Japan). Insulin-positive areas and the number of islets of each group were adjusted by total pancreas area[15].

Measurement of Insulin Contents of Isolated Islets

Pancreatic islets were isolated by collagenase digestion. To determine insulin contents, islets were homogenized in 400 μL acid ethanol (37% HCl in 75% ethanol, 15:1000 [v/v]) and extracted at 4°C overnight. The acidic extracts were dried by vacuum, reconstituted and subjected to insulin measurement. The amount of immunoreactive insulin was determined by radioimmunoassay (RIA).

Measurement of mRNA Expression of C/EBP-Homologous Protein and BiP in Isolated Islets

Measurement of mRNA expression of C/EBP-homologous protein (CHOP) and BiP was carried out by quantitative reverse transcription polymerase chain reaction (RT–PCR) as described previously[19]. Briefly, total RNA was extracted from isolated islets with an RNeasy mini kit (Qiagen, Valencia, CA, USA) and treated with DNase (Qiagen). cDNA was prepared by SuperScript Reverse Transcriptase system (Invitrogens, Carlsbad, CA, USA) according to the manufacturer's instructions. CHOP mRNA levels and BiP mRNA levels in the islets were measured by quantitative RT–PCR using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The sequences of forward and reverse primers to evaluate

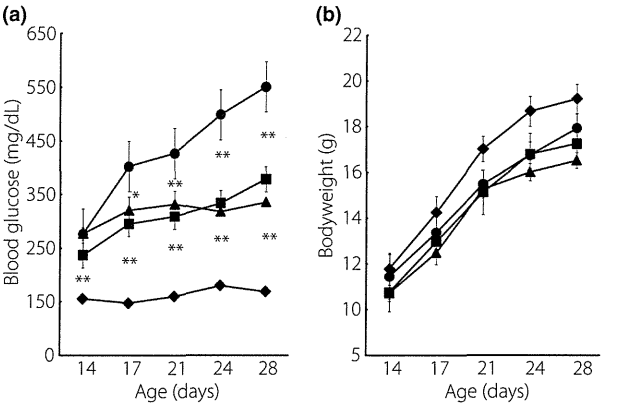

Figure 1 | Ex-4 significantly reduced blood glucose levels in Akita mice. (a) Blood glucose concentration and (b) bodyweight were measured in wild-type C56BL/6 mice (closed diamond, $n = 10$), Akita mice treated with PBS alone (closed circle, $n = 10$), Ex-4 (closed square, $n = 12$) and phlorizin (closed triangle, $n = 10$). Each symbol represents mean ± SE. $^*P < 0.05$, $^{**}P < 0.01$ *vs* PBS-treated Akita mice.

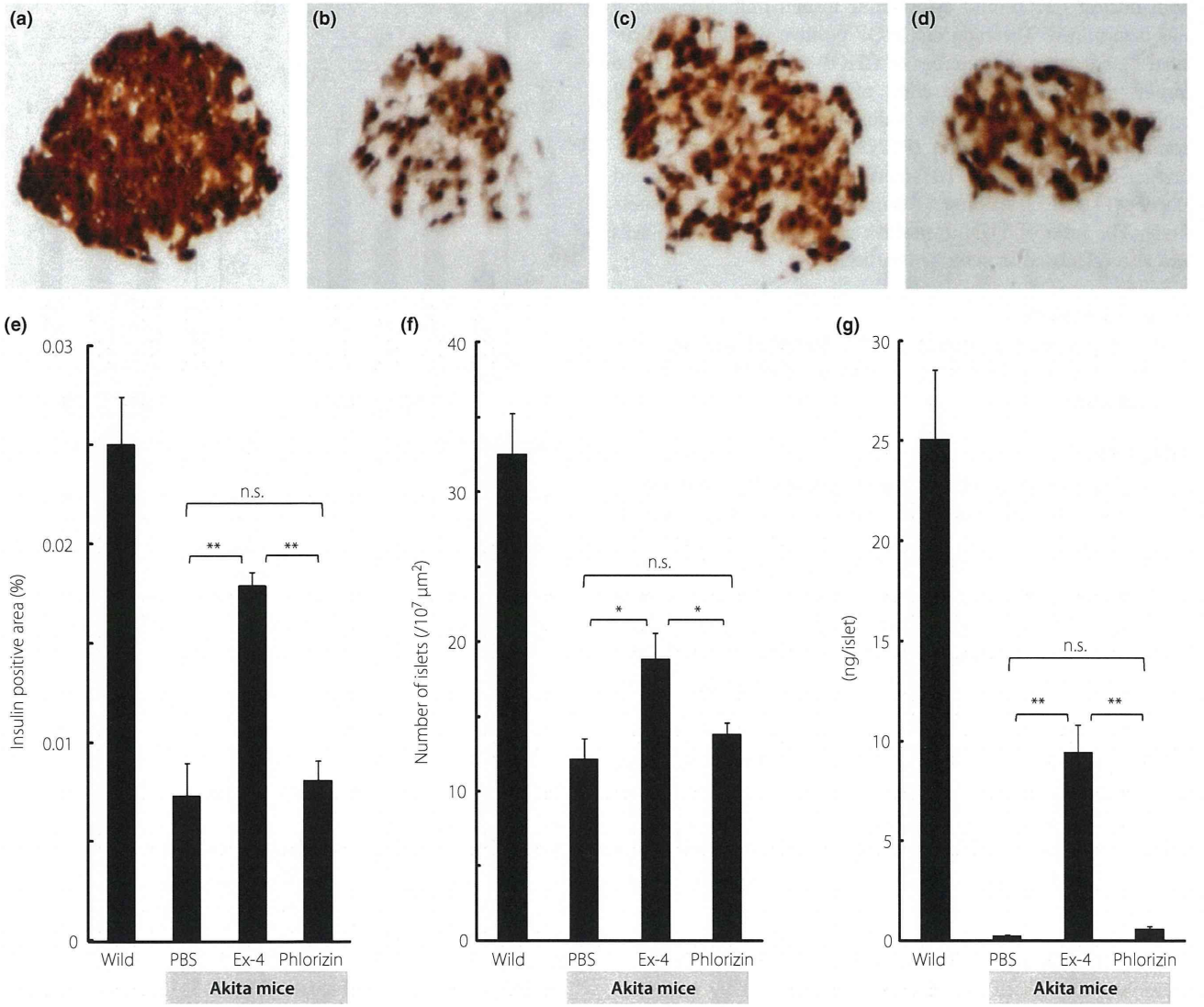

Figure 2 | Ex-4 treatment increased insulin-positive areas, number of islets and insulin content. (a–d) Representative mouse pancreata at 5 weeks-of-age stained with insulin. (a) Wild, (b) Akita mice treated with PBS, (c) Ex-4 or (d) phlorizin. (e) Insulin-positive areas and (f) number of islets were evaluated as described in Materials and Methods (n = 5 for each group). (g) Pancreatic insulin content was measured as described in Materials and Methods, and expressed as ng/islet (n = 5 for each group). Each column represents mean ± SE. *P < 0.05, **P < 0.01.

CHOP expression were 5′-GAGCT- GGAAGCCTGGTATGA-3′ and 5′-GGACGCAGGGTCAAGAGTAG-3′, respectively; the sequences of forward and reverse primers to evaluate BiP expression were 5′-TTTCTGCCATGGTTCTCACTAA-3′ and 5′-GCTGGGCATCATTGAAGTAAG-3′, respectively; and the sequences of forward and reverse primers to evaluate glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression were 5′-AGCTCACTGGCATGGCTTCCG-3′ and 5′-GCCTGCTTC-ACCACCTTCTTGATG-3′, respectively. SYBER Green PCR Master Mix (Applied Biosystems) was prepared for the PCR run. Thermal cycling conditions were denatured at 95°C for 10 min followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Total CHOP and total BiP levels were corrected by GAPDH mRNA levels.

Immunofluorescence Staining

For pancreatic CHOP and insulin immunohistochemistry, the tissues were fixed and embedded in paraffin. Serial 5-μm sections were stained with anti-CHOP/GADD153 (Santa Cruz Biotechnology) and anti-insulin (DAKO) antibodies using standard protocols. Insulin immunopositive areas were measured on five distal, random, non-overlapping sections from five mice of each group using a BZ-8100 fluorescence microscope equipped with a BZ-Analyzer (KeyEnce), and the number of cells showing

both nuclear CHOP and cytoplasmic insulin immunopositivity was determined. The ratio of CHOP-positive β-cells was calculated by adjusting the number of CHOP-positive β-cells by the insulin-positive area[20]. The effect of Ex-4 treatment on β-cell replication and apoptosis was evaluated histologically by proliferating cell nuclear antigen (PCNA) staining (Abcam, Cambridge, MA, USA) and TdT-mediated dUTP-biotin nick-end labeling (TUNEL) staining (Takara Bio, Otsu, Japan), respectively. The ratio of TUNEL-positive and PCNA-positive β-cells was also calculated as described earlier.

Statistical Analysis

Data are presented as means ± SEM. Statistical analyses were carried out by unpaired *t*-test. A *P*-value of <0.05 was considered significant.

RESULTS

Effect of Ex-4 on Hyperglycemia and Bodyweight in Akita Mice

Akita mice showed acute and progressive hyperglycemia at 14 days after birth and thereafter. Twice-daily intraperitoneal injection of Ex-4 from 3 to 5 weeks-of-age significantly reduced blood glucose levels compared with those in PBS-treated mice (Figure 1a). Plasma glucose levels in phlorizin-treated Akita mice were similar to those in Ex-4-treated mice. Plasma glycoalbumin levels were significantly lower in the Ex-4- and phlorizin-treated groups than those in the PBS-treated group, but no significant difference was observed between the Ex-4- and phlorizin-treated groups (12.9 ± 1.5 *vs* 8.7 ± 0.7 *vs* 8.2 ± 0.6, respectively, n = 10–12). Ex-4 treatment or phlorizin treatment did not change bodyweight compared with PBS treatment (Figure 1b). Ex-4 or phlorizin treatment did not change the amount of food intake assessed at 4 weeks-of-age (data not shown).

Effect of Ex-4 on Insulin-Positive Area and Number of Islets

Preservation of β-cell morphology was observed by treatment with Ex-4, as shown in Figure 2a. Quantitative histological analyses showed that Ex-4 treatment significantly increased both the insulin-positive area and the number of islets, whereas there was no significant difference between the PBS-treated group and the phlorizin-treated group (Figure 2b,c).

Effect of Ex-4 on Pancreatic Insulin Content

Figure 2d shows the effect of Ex-4 treatment on insulin content in pancreatic islets. Treatment with Ex-4 significantly increased insulin content in isolated islets, but phlorizin treatment did not.

Quantitative Estimation of CHOP and BiP Expression Levels by Real-Time PCR

The expression levels of CHOP mRNA are shown in Figure 3a, and those of BiP mRNA are shown in Figure 3b. Ex-4 significantly lowered the expression levels of CHOP and BiP mRNA, but there was no significant difference in the expression levels of CHOP or BiP mRNA between the phlorizin- or PBS-treated groups.

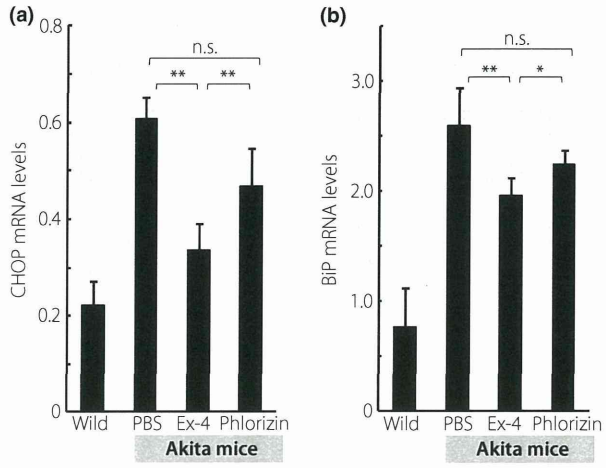

Figure 3 | Ex-4 treatment resulted in a significant decrease in the expression levels of C/EBP-homologous protein (CHOP) mRNA and Bip mRNA in Akita mice. (a) mRNA expression levels of CHOP were evaluated by quantitative real-time polymerase chain reaction (PCR). (b) mRNA expression levels of BiP were evaluated by quantitative real-time PCR. Data are expressed as the ratio to that of glyceraldehyde 3-phosphate dehydrogenase in the same sample (n = 5 for each group). Each column represents mean ± SE. *P < 0.05, **P < 0.01.

Effect of Ex-4 on the Ratio of CHOP-, TUNEL- and PCNA-Positive β-cells

Figure 4a depicts the representative pancreata stained with insulin (red), CHOP (green) and DAPI (blue), respectively. Similarly, Figure 5a shows the representative pancreata stained with insulin (red) and TUNEL (green). Treatment with Ex-4 significantly decreased the ratio of CHOP-positive β-cells and TUNEL-positive β-cells (Figures 4b and 5b), but there was no significant difference in the ratio of CHOP-positive or TUNEL-positive β-cells between the PBS- and phlorizin-treated groups. Figure 6a shows the representative pancreata stained with insulin (red) and PCNA (green). PCNA staining showed no significant difference in proliferation of β-cells among the three groups of Akita mice (Figure 6b). Interestingly, the ratio of PCNA-positive β-cells was increased in all three groups when compared with wild-type C57BL/6 mice.

DISCUSSION

Akita mice are widely used as an animal model of ER stress-mediated diabetes. Akita mice have a point mutation (C96T) in the insulin 2 gene[21] that disrupts the disulfide bond formation between the A and B chains of proinsulin, resulting in a drastic conformational change of the molecule. The unfolded proinsulin accumulates to the ER, causing severe ER stress leading to β-cell apoptosis. In humans, it has recently been shown that a mutation in the insulin gene, which is identical

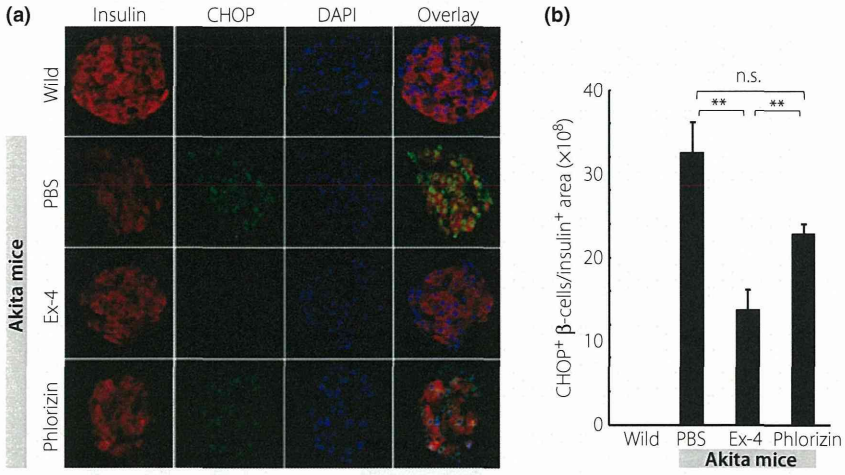

Figure 4 | Ex-4 treatment resulted in a significant decrease in the ratio of C/EBP-homologous protein (CHOP)-positive β-cells in Akita mice. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red), CHOP (green) and DAPI (blue). (b) The number of CHOP-positive β-cells normalized per insulin-positive area was quantified as described in Materials and Methods. Each column represents mean ± SE. $^{**}P < 0.01$.

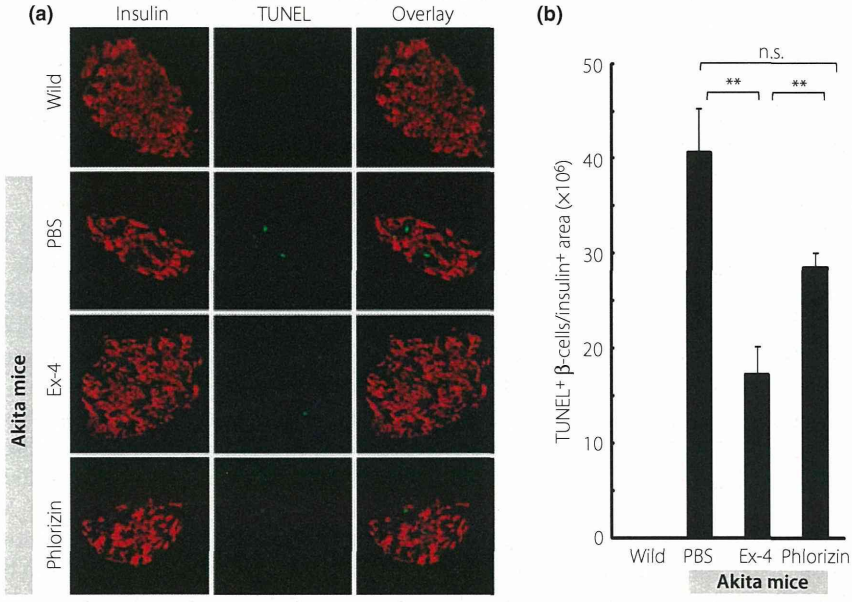

Figure 5 | Ex-4 treatment decreased the ratio of TUNEL-positive β-cells. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red) and TUNEL (green). (b) The number of TUNEL-positive β-cells normalized per insulin-positive area was quantified as described in Materials and Methods. Each column represents mean ± SE. $^{**}P < 0.01$.

to that in the Akita mouse, causes permanent neonatal diabetes within the first month of life that requires lifelong insulin injection[22].

In the present study, we have shown that Ex-4 treatment has a protective effect on β-cells in Akita mice. The insulin-positive area and the number of islets were maintained along with a decreased ratio of CHOP- and TUNEL-positive cells in the islets, showing that the major effect of Ex-4 treatment in the maintenance of β-cell mass is through decreasing β-cell apoptosis in response to ER stress. Because phlorizin decreases blood glucose levels without increasing insulin secretion, it might well reduce ER stress by decreasing the insulin demand. However, in contrast to the Ex-4 treatment, phlorizin treatment failed to show a reduction of ER stress or β-cell protective effects against

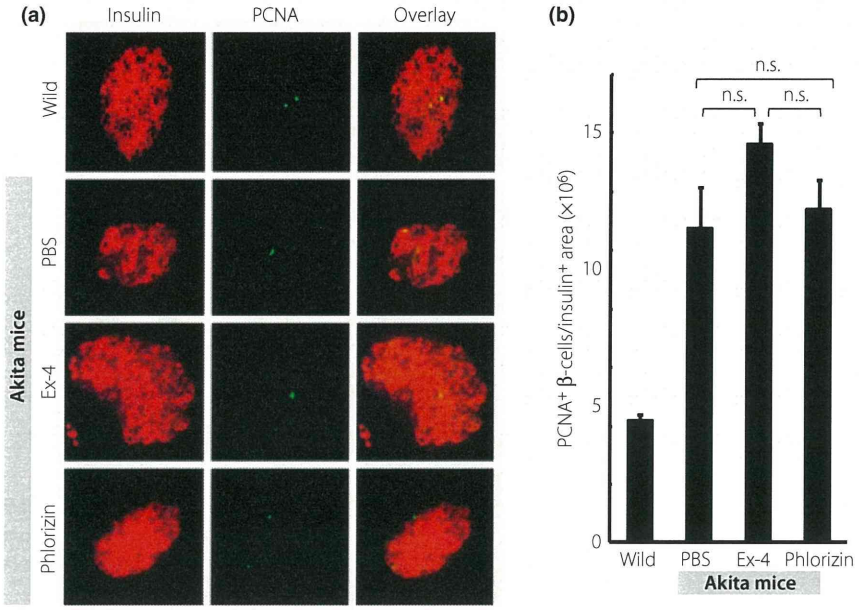

Figure 6 | Ex-4 treatment did not significantly increase the ratio of PCNA-positive β-cells. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red) and PCNA (green). (b) The number of PCNA-positive β-cells normalized per insulin-positive area was quantified as described in Materials and Methods. Each column represents mean ± SE.

apoptosis in our conditions. These findings show that Ex-4 has a direct effect on ER stress-mediated β-cell apoptosis that is independent of decreased insulin demand.

There are several *in vitro* and *in vivo* studies showing that GLP-1R agonists inhibit β-cell apoptosis[9–16], and several molecular mechanisms have been suggested. For example, GLP-1 treatment decreases the expression levels of proapoptotic protein caspase-3 and increases those of anti-apoptotic protein bcl-2 in isolated human islets[10]. It also has been shown that the anti-apoptotic effect of Ex-4 is associated with the activation of protein kinase B/Akt through PKA-dependent phosphorylation of CREB[11]. There are some reports that GLP-1 ameliorates ER stress. Yusta *et al.* found that treatment by Ex-4 reduces blood glucose levels in obese *db/db* mice along with a decrease in the number of CHOP-positive β-cells[20]. Tsunekawa *et al.*[23] reported a beneficial effect of Ex-4 on β-cell damage in calmodulin-over-expressing transgenic (CaMTg) mice that develop diabetes through ER stress-mediated β-cell apoptosis. They found that Ex-4 treatment reduced blood glucose levels while retaining the insulin-positive areas and decreasing the expression levels of CHOP mRNA in CaMTg mice. *In vitro* studies have found that rapid recovery from translational attenuation[19] or upregulation of BiP and JunB[24] accounts for the attenuation of ER stress-mediated β-cell damage by Ex-4 treatment. However, results of chronic Ex-4 treatment in animal models of type 2 diabetes should be carefully interpreted, because enhancement of GLP-1R signaling reduces the blood glucose level by its insulinotropic action. Therefore, the possibility remains that reduced hyperglycemia attenuates persistent ER stress and ameliorates β-cell apoptosis. Our present findings clearly show that Ex-4 treatment attenuates ER stress-mediated β-cell damage in Akita mice through a reduction of apoptotic cell death that is independent of decreased blood glucose levels.

Although several studies have found that the cytoprotective effect of GLP-1R signaling is not only through inhibition of β-cell apoptosis, but also through stimulation of β-cell proliferation[5–9], we did not find any effect of Ex-4 treatment on β-cell proliferation. It is possible that the administration period in the present study was too short to observe β-cell proliferation by Ex-4 or that stimulation of β-cell proliferation does not play a significant role in the cytoprotective effect of GLP-1R signaling in Akita mice. The ratio of PCNA-positive β-cells was increased not only in the Ex-4-treated group of Akita mice, but also in the phlorizin-treated group and the untreated group compared with that in wild-type C57BL/6 mice. Whether or not this result can be attributed to the phenotype of Akita mice requires further study.

Islet mass is reported to be decreased in patients with type 2 diabetes at the time of diagnosis[25]. Although Ex-4 is in clinical use for treatment of type 2 diabetes[26], superiority of Ex-4 over the other antidiabetic drugs has not been shown. Our data confirm the previous findings of a beneficial effect of Ex-4 on glycemic control, but also suggest that Ex-4 has a direct β-cell-protective effect independently of improved glycemic control. Thus, Ex-4 and other GLP-1R agonists might well be more effective than other antidiabetic drugs in clinical use in terms of alleviating β-cell damage and maintaining β-cell mass for diabetic patients.

ACKNOWLEDGEMENTS

This study was supported by Scientific Research Grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and from the Ministry of Health, Labor, Welfare, Japan, and by the Kyoto University Global COE Program 'Center for Frontier Medicine'. There is no conflict of interest for all the authors listed.

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Plasma gastric inhibitory polypeptide and glucagon-like peptide-1 levels after glucose loading are associated with different factors in Japanese subjects

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ABSTRACT

Aims/Introduction: Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are major incretins that potentiate insulin secretion from pancreatic β-cells. The factors responsible for incretin secretion have been reported in Caucasian subjects, but have not been thoroughly evaluated in Japanese subjects. We evaluated the factors associated with incretin secretion during oral glucose tolerance test (OGTT) in Japanese subjects with normal glucose tolerance (NGT).

Materials and Methods: We measured plasma GIP and GLP-1 levels during OGTT in 17 Japanese NGT subjects and evaluated the factors associated with GIP and GLP-1 secretion using simple and multiple regression analyses.

Results: GIP secretion (AUC-GIP) was positively associated with body mass index ($P < 0.05$), and area under the curve (AUC) of C-peptide ($P < 0.05$) and glucagon ($P < 0.01$), whereas GLP-1 secretion (AUC-GLP-1) was negatively associated with AUC of plasma glucose ($P < 0.05$). The insulinogenic index was most strongly associated with GIP secretion ($P < 0.05$); homeostasis model assessment β-cell was the most the strongly associated factor in GLP-1 secretion ($P < 0.05$) among the four indices of insulin secretion and insulin sensitivity.

Conclusions: Several distinct factors might be associated with GIP and GLP-1 secretion during OGTT in Japanese subjects.
(J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00078.x, 2011)

KEY WORDS: Gastric inhibitory polypeptide, Glucagon-like peptide-1, Incretin

INTRODUCTION

Oral glucose administration leads to greater insulin release from pancreatic islets than intravenous glucose loading that yields equivalent glucose levels. Gut hormonal substances released in response to glucose include the incretins, gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which are responsible for 50–60% of postprandial insulin secretion[1]. GIP is secreted on meal ingestion from K-cells in the proximal small intestine, whereas GLP-1 is secreted from L-cells in the distal small intestine and colon, and binds to their respective receptors (GIP receptor [GIPR] and GLP-1 receptor) on the surface of pancreatic β-cells to stimulate insulin secretion by increasing the intracellular adenosine 3′,5′-monophosphate (cAMP) concentration[2–4].

Type 2 diabetes is characterized by both decreased insulin secretion and reduced insulin sensitivity[5–7]. The incretin effect has been shown to be reduced in type 2 diabetic subjects compared with those with normal glucose tolerance (NGT) in previous studies[8,9], suggesting that a reduced incretin effect might be associated with hyperglycemia after food intake and glucose loading in type 2 diabetes. When intravenous infusion of GIP or GLP-1 was carried out in type 2 diabetic subjects, GLP-1 potentiated insulin secretion from pancreatic β-cells, but GIP did not, showing that the GIPR signal is downregulated in β-cells in type 2 diabetes[10]. In studies using rodent models, it was reported that GIPR mRNA and protein expression levels in islets are decreased in the diabetic state[11]. In contrast, in the non-diabetic obese state, GIP plays an important role in maintaining blood glucose levels[12]. The GIP signal might be enhanced as a result of increased GIPR sensitivity of β-cells to GIP or increased GIP secretion from K-cells in the non-diabetic obese state. Indeed, GIP concentrations are reported to be increased in obese rodent models and human subjects compared with those in lean rodents and human subjects, respectively[13–15]. Furthermore, we have previously shown the hypersensitivity of GIPR to GIP in β-cells of high fat-induced obese mice[16]. Plasma GLP-1 concentrations in type 2 diabetic patients are reported to be reduced after meal ingestion and glucose loading[9,17]. However, in other studies it was reported that GLP-1 concentrations did not differ

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Received 12 August 2010; revised 16 September 2010; accepted 16 September 2010

in NGT and type 2 diabetic subjects[18–20]. Thus, the measurement of GIP and GLP-1 concentrations in various metabolic states is important to evaluate the effects of incretin on insulin secretion.

Insulin sensitivity in Asian subjects has been shown to be higher than in Mexican Americans and Caucasians in previous reports[21,22], which is partly as a result of the fact that Asians, including Japanese, are generally less obese. Furthermore, insulin secretion rather than insulin sensitivity is the more important factor in progression from NGT to diabetes in Japanese subjects[23]. We have reported that early-phase insulin secretion is considerably decreased, even in Japanese NGT subjects with 1-h plasma glucose (PG) levels during oral glucose tolerance test (OGTT) of more than 180 mg/dL[24]. Thus, it is especially important to evaluate incretin secretion and determine the factors associated with incretin secretion in Japanese NGT subjects, because GIP and incretin is responsible for more than 50% of postprandial insulin secretion after glucose ingestion. The factors responsible for incretin secretion have been reported in Caucasian subjects, but have not been thoroughly elucidated in Japanese subjects.

In the present study, we evaluated GIP and GLP-1 levels during OGTT and determined the factors involved in GIP and GLP-1 secretion (area under the curve [AUC] of GIP and GLP-1 during OGTT) in Japanese NGT subjects.

MATERIALS AND METHODS

Subjects

We recruited 17 Japanese healthy volunteers. The subjects had no history of hypertension, hyperlipidemia or kidney and liver diseases, and did not take any drugs 2 weeks before the study. The study was designed in compliance with the ethics regulations of the Helsinki Declaration and Kyoto University. Informed consent was obtained from all subjects.

Study Procedure

The subjects' age, height and bodyweight were determined. Blood samples for the measurement of liver and kidney function, HbA_{1c}, serum triglyceride (TG), total cholesterol and high-density lipoprotein (HDL)-cholesterol levels were drawn after an overnight fast. All subjects received OGTT. After the subjects fasted overnight for 10–16 h, standard OGTT with 75 g glucose was given according to the National Diabetes Data Group recommendations[25]. NGT was diagnosed according to World Health Organization (WHO) criteria[26].

Blood samples were collected at −15, 0, 10, 20, 30, 60, 90, 120, 150 and 180 min after glucose loading and were centrifuged at 1800 *g* at 4°C for 10 min. After collecting supernatant of the samples, plasma and serum were stocked at −80°C. Plasma GIP, GLP-1 levels and the various parameters (PG, serum immunoreactive insulin [IRI], serum C-peptide reactivity [CPR], TG, serum free fatty acid [FFA] and plasma glucagon) were measured at the indicated times (plasma GIP and GLP-1 levels were measured at −15, 0, 10, 30, 60, 90, 120 and 180 min after glucose loading, and plasma glucagon levels were measured at −15, 0, 30, 60, 90, 120 and 180 min after glucose loading). The PG levels were measured by glucose oxidase method. Serum IRI levels were measured by two-site radioimmunoassay. Total GIP and total GLP-1 levels were measured using human GIP ELISA kit (Linco Research, St Charles, MO, USA; range of detection from 8.2 pg/mL to 2000 pg/dL) and human GLP-1 ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA; range of detection from 2.4 pg/mL to 1,000,000 pg/dL), respectively, as previously described[27,28]. The AUC of PG, IRI, CPR, TG, FFA, glucagon, total GIP (AUC-GIP) and total GLP-1 (AUC-GLP-1) were calculated. We then analyzed the relationship between the AUC of GIP (GIP secretion) and GLP-1 (GLP-1 secretion) and age, body mass index (BMI) and the parameters during OGTT.

Statistical Analysis

Basal insulin secretion and sensitivity were evaluated by homeostasis model assessment (HOMA) β-cell function and homeostasis model assessment of insulin resistance (HOMA-IR)[29,30], respectively. Early-phase insulin secretion and systemic insulin sensitivity during OGTT were evaluated by insulinogenic index[31] and insulin sensitivity index (ISI) composite[32]. The calculations of the four indices were as follows:

$$\text{HOMO } \beta\text{-cell} = 20 \times \text{fasting IRI level (FIRI) (pmol/L)} / (\text{fasting PG level [FPG] [mmol/L]} - 3.5)$$

$$\text{HOMO-IR} = \text{FIRI (pmol/L)} \times \text{FPG (mmol/L)} / 22.5$$

$$\text{Insulinogenic index} = (30 \text{ min IRI} - \text{FIRI [pmol/L]}) / (30 \text{ min PG} - \text{FPG [mmol/L]})$$

$$\text{IRI composite} = 10{,}000 / (\text{FGP [mg/dL]} \times \text{FIRI [}\mu\text{U/mL]} \times \text{mean OGTT PG [mg/dL]} \times \text{mean OGTT IRI [}\mu\text{U/mL]})^{0.5}$$

All analyses were carried out using statistical analysis software (SPSS version 17.0, IBM, Somers, NY, USA) system. Statistical analysis was carried out by ANOVA with Fisher's PLSD test for changing levels of GIP, GLP-1, and the parameters during OGTT and differences between the two groups were assessed by unpaired *t*-test. We used simple regression analysis to determine the relationship between AUC-GIP or AUC-GLP-1 and the age, BMI and the parameters during OGTT, and we carried out multiple regression analysis to determine the factors most strongly associated with AUC-GIP and AUC-GLP-1, and the indices of insulin secretion and sensitivity. Probability (*P*) values <0.05 were considered statistically significant. Data are presented as mean ± standard error (SE).

RESULTS

Table 1 shows clinical characteristics of the subjects. Mean age was 31.7 ± 1.3 years and mean BMI was 23.1 ± 0.9 kg/m^2. No subjects had liver or kidney dysfunction. HbA_{1c}, FPG, TG, total

Table 1 | Clinical characteristics of the subjects

n (male/female)	17 (14/3)
Age (years)	31.7 ± 1.3
Body mass index (kg/m^2)	23.1 ± 0.9
Fasting plasma glucose (mmol/L)	6.1 ± 0.2
Fasting insulin (pmol/L)	25.2 ± 3.7
HbA_{1c} (%)	4.7 ± 0.0
Triglycerides (mmol/L)	2.00 ± 0.31
Total cholesterol (mmol/L)	4.56 ± 0.16
HDL-cholesterol (mmol/L)	1.51 ± 0.10
Insulinogenic index	66.22 ± 8.54
HOMA β-cell	60.85 ± 8.89
HOMA-IR	0.94 ± 0.15
ISI composite	11.45 ± 1.67

Means ± SE. HDL, high-density lipoprotein; HOMA, homeostasis model assessment; HOMA-IR, homeostasis model assessment of insulin resistance; ISI, insulin sensitivity index.

cholesterol and HDL-cholesterol levels were within normal limits in the fasting state.

The levels of GIP, GLP-1, PG, IRI, CPR, TG, FFA and glucagon after glucose loading were measured (Figure 1). The subjects were diagnosed NGT according to WHO criteria with fasting plasma glucose and 2-h glucose levels below 6.1 and 7.8 mmol/L, respectively. Levels of PG, IRI and CPR were significantly increased from 10 min after glucose loading compared with fasting level (Figure 1a–c). FFA levels were significantly decreased from 10 min after glucose loading (Figure 1d). TG levels were not significantly changed during OGTT (Figure 1e). Glucagon levels were significantly decreased from 30 min after glucose loading (Figure 1f). Total GIP levels were significantly increased from 10 min during OGTT (Figure 1g). Total GLP-1 levels were significantly increased from 10 min during OGTT with peaks at 30 and 120 min (Figure 1h).

We analyzed the relationship between AUC-GIP or AUC-GLP-1 and age, BMI and the several parameters (AUC of PG, IRI, CPR, TG, FFA and glucagon). AUC-GIP were positively related to BMI and AUC of CPR, IRI and glucagon, but AUC-GLP-1 was not related to these factors (Figure 2a–c; AUC data of IRI during OGTT are not shown; $P < 0.05$). In contrast, AUC-GLP-1 was inversely related to AUC of PG (Figure 2d), but AUC-GIP was not.

We then analyzed the relationship between AUC-GIP or AUC-GLP-1 and indices of insulin secretion and insulin sensitivity. AUC-GIP was positively related to insulinogenic index and HOMA-IR, whereas AUC-GLP-1 was positively related to HOMA β-cell function (Figure 3a–c). ISI composite was not related to either AUC-GIP or AUC-GLP-1 (Figure 3d). In addition, multiple regression analysis was carried out to determine the factors strongly associated with AUC-GIP and AUC-GLP. The insulinogenic index was the most strongly associated factor in AUC-GIP (correlation coefficients 0.56, standardized β 0.56, $P < 0.05$) of the four indices; HOMA β-cell function was the strongest factor in AUC-GLP-1 (HOMA β-cell function: correlation coefficients 0.524, standardized β 0.870, $P < 0.01$, ISI composite: correlation coefficients 0.063, standardized β 0.581, $P < 0.05$).

DISCUSSION

In the present study, we estimated the incretin level after glucose loading in Japanese NGT subjects and found that plasma GIP and GLP-1 levels during OGTT are related to different factors.

Incretin action of GIP is reduced in the diabetic state as a result of decreased GIP receptor expression on pancreatic β-cells[11], whereas GIP signaling is enhanced and maintains

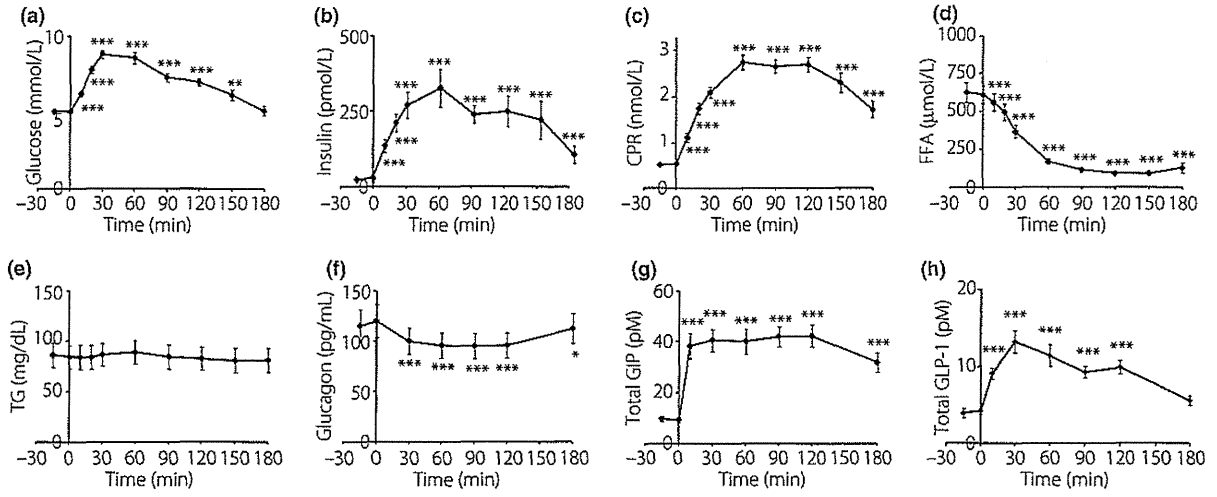

Figure 1 | Concentrations of (a) plasma glucose, (b) serum immunoreactive insulin, (c) serum C-peptide reactivity (CPR), (d) serum free fatty acid (FFA), (e) serum triglyceride (TG), (f) glucagon, (g) total gastric inhibitory polypeptide (GIP) and (h) total glucagon-like peptide-1 (GLP-1) during oral glucose tolerance test in 17 Japanese subjects. Mean ± SE, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the levels at fasting.

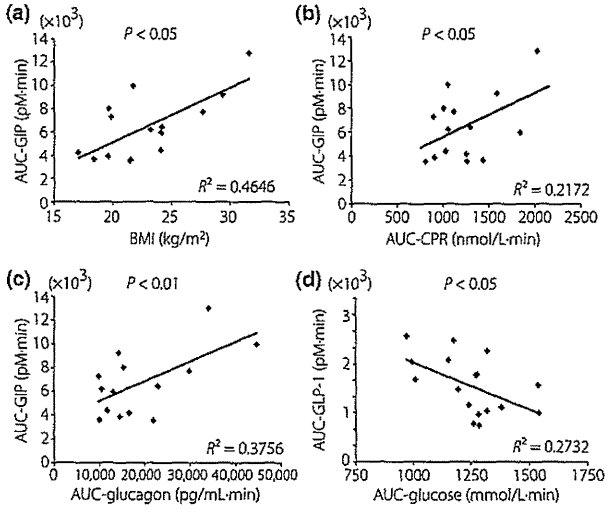

Figure 2 | Simple regression analysis of gastric inhibitory polypeptide secretion (AUC-GIP) and (a) body mass index (BMI), (b) AUC of serum C-peptide reactivity (CPR) and (c) glucagon. (d) Simple regression analysis of glucagon-like peptide-1 secretion (AUC-GLP-1) and AUC of plasma glucose (PG).

glucose homeostasis by compensatory increased insulin secretion in the obese state[15,16]. In some human studies in Caucasians, plasma GIP levels are increased in obese subjects[14,15] and there is a positive relationship between AUC-GIP and AUC of FFA during OGTT[18]. In the present study, AUC-GIP after glucose loading was not associated with AUC of FFA, but was positively associated with BMI, HOMA-IR, and AUC of IRI and CPR after glucose loading. In fact, obese subjects are known to have hyperinsulinemia and insulin resistance[33,34], and BMI was strongly associated with AUC of IRI and CPR. Thus, GIP secretion from K-cells may well be associated with insulin resistance to maintain postprandial hyperinsulinemia in Japanese NGT subjects. It is unknown why there was no correlation between AUC-GIP and AUC-glucose. It might be explained by the fact that GIP secretion is associated with the amount of glucose loading[1], whereas blood glucose levels are maintained within normal levels by GIP-induced compensatory insulin secretion in NGT subjects.

GLP-1 secretions of type 2 diabetes subjects after glucose or meal ingestion are diverse in human studies[9,17–19]. Some studies report that GLP-1 secretion is decreased in Caucasian type 2 diabetes[9,17]. Recently, it is reported that GLP-1 levels after ingestion of glucose and mix meal in Japanese type 2 diabetic subjects were not decreased compared with those in NGT subjects, suggesting that GLP-1 secretion is not decreased in Japanese type 2 diabetes[20,35,36]. Two studies of Caucasian subjects found that AUC-GLP-1 during OGTT is positively associated with age and AUC of glucagon, whereas AUC of GLP-1 is negatively associated with BMI or bodyweight and AUC of FFA[9,18]. In the

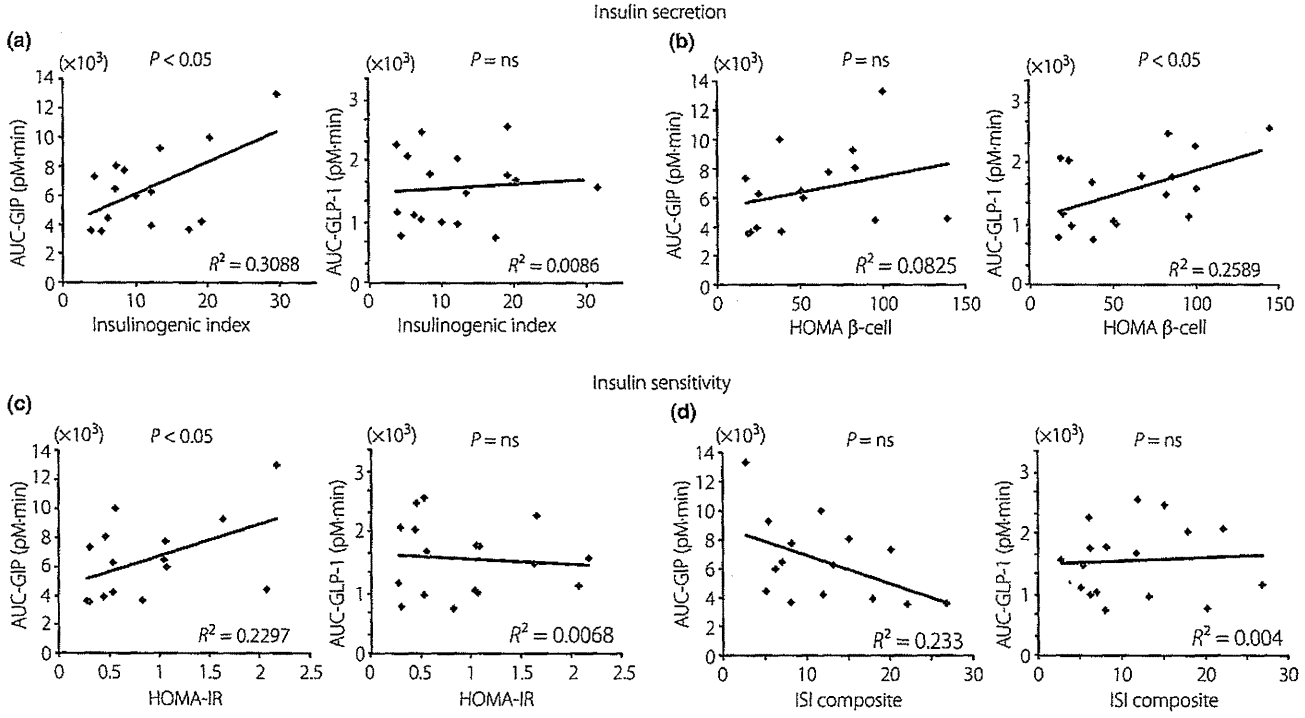

Figure 3 | Relationship between gastric inhibitory polypeptide secretion (AUC-GIP) and glucagon-like peptide-1 secretion (AUC-GLP-1) and the indices of insulin secretion and insulin sensitivity. (a) Insulinogenic index, (b) homeostasis model assessment (HOMA) β-cell function, (c) homeostasis model assessment of insulin resistance (HOMA-IR) and (d) insulin sensitivity index (ISI) composite. Ns, not significant.

present study, AUC-GLP-1 was negatively related to AUC of PG during OGTT, showing that the increase in GLP-1 secretion after glucose loading is associated with a decrease in postprandial glucose levels in Japanese NGT subjects. It has been reported that GLP-1 levels after glucose loading are positively related to gastric empting in Caucasian subjects[37]. Although we did not measure gastric empting of the subjects in the present study, increasing GLP-1 secretion after glucose loading might decrease postprandial glucose levels through gastric emptying. In the present study, BMI and AUC of FFA were not associated with AUC-GLP-1 during OGTT. Obese subjects have higher FFA levels than lean subjects[38]. However, because Japanese subjects are less obese than Caucasian subjects[21], the difference observed in the relationship between AUC-GIP and GLP-1, and AUC of FFA might reflect this ethnic difference in Caucasians and Japanese.

Insulin secretion, rather than insulin sensitivity, is the more important factor in the progression from NGT to type 2 diabetes in Japanese patients[23,39]. Because incretin is an intestinal hormone that induces postprandial insulin secretion[1], we hypothesize that GIP and GLP-1 secretion is more crucial in Japanese subjects than in Caucasian subjects. Indeed, GLP-1 mimetics and DPP-4 inhibitors improve glycemic control better in Japanese type 2 diabetic patients than in Caucasian type 2 diabetic patients in clinical trials[40–43]. We therefore evaluated the correlation between GIP secretion (AUC-GIP) and GLP-1 secretion (AUC-GLP-1), and the indices of insulin secretion and insulin sensitivity in Japanese NGT subjects during OGTT. The values of HOMA β-cell, insulinogenic index, HOMA-IR and ISI composite were similar to those in previous studies of Japanese subjects[24,30,39]. AUC-GIP was positively associated with the insulinogenic index and HOMA-IR, and the insulinogenic index was strongly associated with AUC-GIP, whereas AUC-GLP-1 was associated only with HOMA β-cell among the four indices. It has been reported that early-phase insulin secretion is an important factor in the progression from NGT through impaired glucose tolerance (IGT) to type 2 diabetes[39], and that basal insulin secretion (HOMA β-cell) and insulin resistance are important factors in the progression from NGT through impaired fasting glucose (IFG) to type 2 diabetes in Japanese patients[44]. Thus, enhancing the GIP and GLP-1 signals might be particularly useful in inhibiting the progression of type 2 diabetes in Japanese patients. Recently, variants at the GIP receptor gene locus associated with 2-h glucose levels during OGTT were identified by meta-analysis of genome-wide association studies[45]. In subjects who carry this GIP receptor risk allele, early-phase insulin secretion is decreased. These data seem to support our results that GIP secretion is associated with insulinogenic index in Japanese NGT subjects.

In conclusion, we evaluated plasma GIP and GLP-1 levels during OGTT in Japanese NGT subjects. GLP-1 secretion was associated with PG during OGTT, and basal insulin secretion (HOMA β-cell) and GIP secretion was associated with BMI and early-phase insulin secretion (insulinogenic index). Thus, there might be different factors associated with GIP and GLP-1 secretion during OGTT in Japanese subjects.

ACKNOWLEDGEMENTS

We thank Dr Yutaka Seino (Kansai Electric Power Hospital) for his helpful suggestions. This study was supported by Scientific Research Grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by Kyoto University Global COE Program 'Center for Frontier Medicine', and also by Novo Nordisk Pharma Ltd.

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Relationship of homocysteine and homocysteine-related vitamins to bone mineral density in Japanese patients with type 2 diabetes

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ABSTRACT

Aims/Introduction: To estimate nutritional risk factors for osteoporosis in patients with type 2 diabetes, bone mineral density, homocysteine level, and intakes and levels of Hcy-related vitamins including folate, vitamin B_6 and vitamin B_{12} were analyzed in a cross-sectional study.

Materials and Methods: Lumbar spine and femoral neck bone mineral density, serum concentrations of vitamin B_6, vitamin B_{12}, and folate and plasma homocysteine levels were measured in 125 Japanese patients with type 2 diabetes. Nutrient intake values were evaluated using a food frequency questionnaire.

Results: Homocysteine was inversely correlated with bone mineral density, and with both dietary intake and serum concentration of folate. Intake of green vegetables was correlated with intake and level of folate and homocysteine levels. When the population was analyzed across the quartiles, bone mineral density, serum folate concentration, folate intake and intake of green vegetables were lowest in the highest homocysteine group.

Conclusions: In patients with type 2 diabetes, the nutritional status of folate might affect the homocysteine level, a putative risk factor for osteoporosis. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00088.x, 2011)

KEY WORDS: Osteoporosis, Homocysteine, Folate

INTRODUCTION

Diabetes is becoming increasingly recognized as a risk factor for osteoporotic fracture. Although fracture risk in patients with type 2 diabetes is increased compared with normal subjects, not only in those with low bone mineral density (BMD) but also in those with normal or high BMD[1–3], decreased BMD is a major determinant of fragility fracture.

Patients with type 2 diabetes often follow a calorie-restricted diet, but few studies have investigated the sufficiency of these nutrients for the maintenance of skeletal health. Generally, nutrient intake increases along with energy intake. *Ad libitum* food intake values obtained from a longitudinal study in institutionalized elderly found that intake values of vitamins increased along with increased energy intake[4]. In contrast, implementation of a low-fat, low-energy diet (1000 or 1500 kcal/day) in patients with overweight and hyperlipidemia has been shown to result in a decrease of the intake of certain nutrients, including B-vitamins[5].

Folate, vitamin B_6 and vitamin B_{12} are important enzymatic cofactors in the synthesis of methionine from homocysteine (Hcy), and an elevation of Hcy can be caused by insufficiency of folate, vitamin B_6 or vitamin B_{12}. Numerous studies have linked high circulating Hcy levels and low concentrations of folate or vitamin B_{12} with increased risk of low BMD in non-diabetic subjects[6–14]. The possibility that elevated Hcy is a risk factor for osteoporosis is suggested by studies of patients with homocystinuria, a rare autosomal recessive disease characterized by markedly elevated levels of plasma Hcy, in which early onset of generalized osteoporosis has occurred[15,16]. The underlying pathophysiological mechanism of osteoporosis in patients with elevated Hcy is not completely understood. Hcy has been reported to interfere with cross-links of newly formed collagen[17,18], and consequently with bone mineralization and strength[19], as well as to stimulate osteoclast formation and activity[20,21]. However, there has been no report on the association of Hcy and Hcy-related vitamins with osteoporosis in patients with diabetes. Furthermore, vitamin insufficiency was evaluated only by serum vitamin concentrations in most of these studies, and there has been no comprehensive investigation of the relationship of dietary intake of nutrients and

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Received 28 July 2010; revised 8 November 2010; accepted 9 November 2010