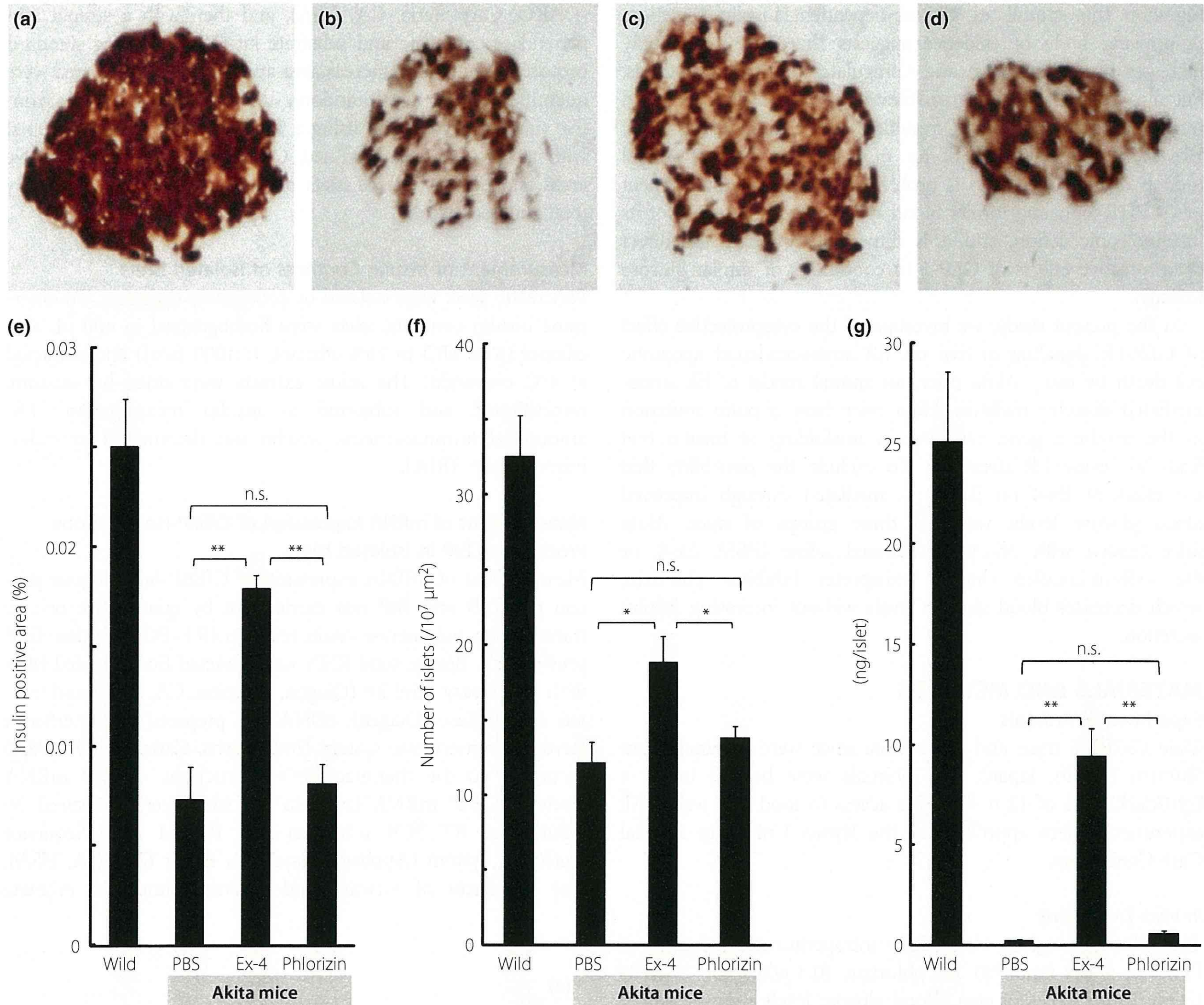

Figure 2 | Ex-4 treatment increased insulin-positive areas, number of islets and insulin content. (a–d) Representative mouse pancreata at 5 weeks-of-age stained with insulin. (a) Wild, (b) Akita mice treated with PBS, (c) Ex-4 or (d) phlorizin. (e) Insulin-positive areas and (f) number of islets were 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′-GCCTGCTTCACCACCTTCTTGATG-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

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

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.

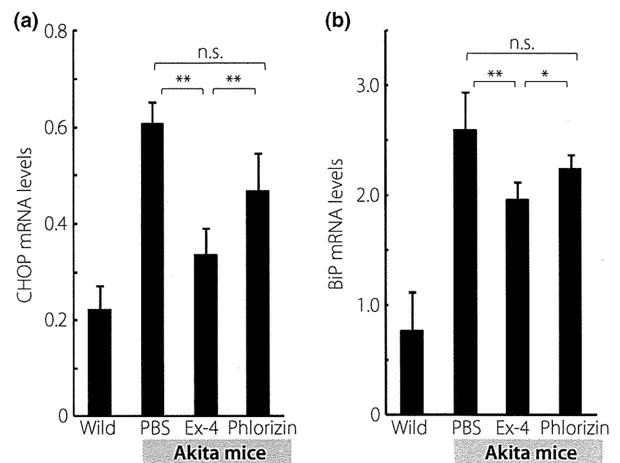

Figure 3 | Ex-4 treatment resulted in a significant decrease in the expression levels of C/EBP-homologous protein (CHOP) mRNA and Bip mRNA in Akita mice. (a) mRNA expression levels of CHOP were evaluated by quantitative real-time polymerase chain reaction (PCR). (b) mRNA expression levels of BiP were 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

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

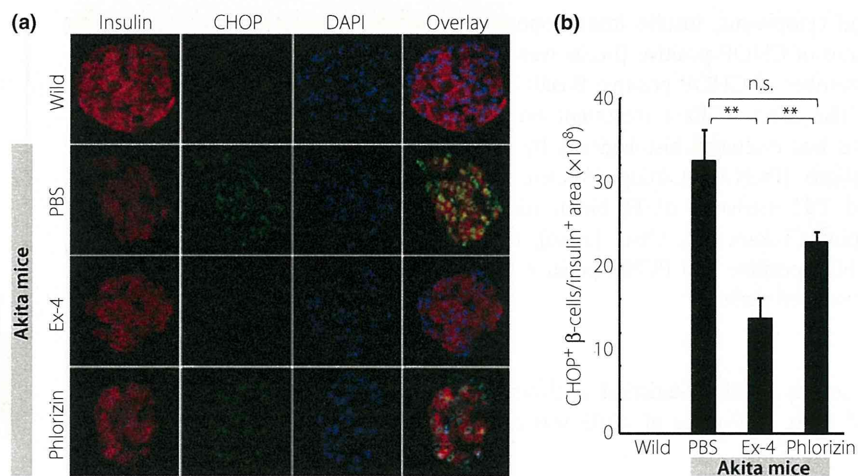

Figure 4 | Ex-4 treatment resulted in a significant decrease in the ratio of C/EBP-homologous protein (CHOP)-positive β-cells in Akita mice. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red), CHOP (green) and DAPI (blue). (b) The number of CHOP-positive β-cells normalized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

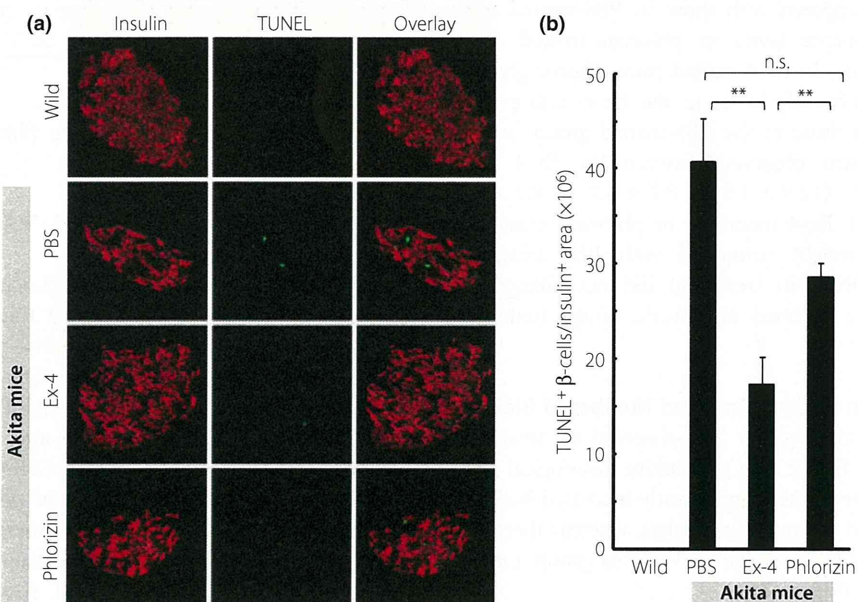

Figure 5 | Ex-4 treatment decreased the ratio of TUNEL-positive β-cells. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red) and TUNEL (green). (b) The number of TUNEL-positive β-cells normalized per insulin-positive area was quantified as described in Materials and Methods. 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

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

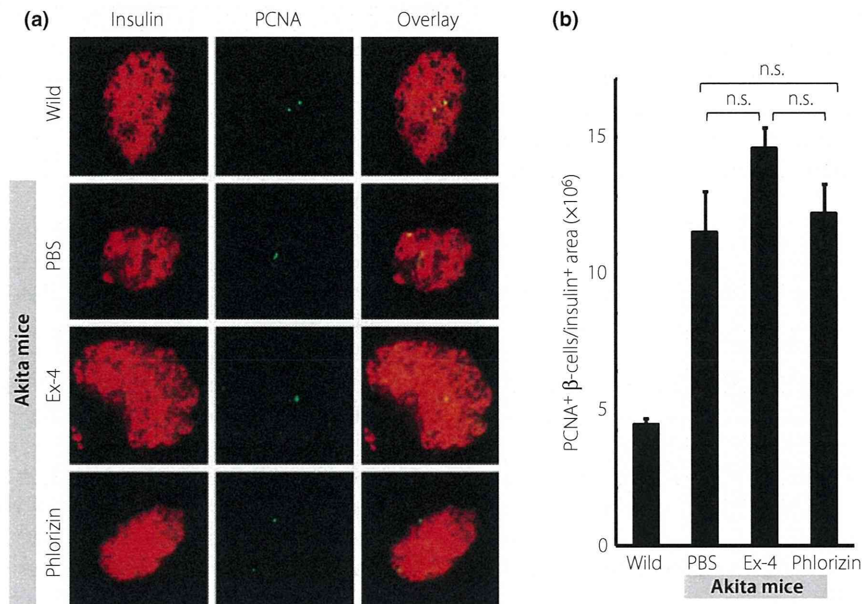

Figure 6 | Ex-4 treatment did not significantly increase the ratio of PCNA-positive β-cells. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red) and PCNA (green). (b) The number of PCNA-positive β-cells normalized per insulin-positive area was quantified as described in Materials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

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

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.

REFERENCES

1. Oyadomari S, Araki E, Mori M. Endoplasmic reticulum stress-mediated apoptosis in pancreatic β-cells. *Apoptosis* 2002; 7: 335–345.
2. Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 1999; 13: 1211–1233.
3. Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* 2000; 101: 451–454.
4. Kaufman RJ, Scheuner D, Schroder M, *et al.* The unfolded protein response in nutrient sensing and differentiation. *Nat Rev Mol Cell Biol* 2002; 3: 411–421.
5. Friedrichsen BN, Neubauer N, Lee YC, *et al.* Stimulation of pancreatic β-cell replication by incretins involves transcriptional induction of cyclin D1 via multiple signalling pathways. *J Endocrinol* 2006; 188: 481–492.
6. Miettinen P, Ormio P, Hakonen E, *et al.* EGF receptor in pancreatic β-cell mass regulation. *Biochem Soc Trans* 2008; 36: 280–285.
7. Jin T, Liu L. The Wnt signaling pathway effector TCF7L2 and type 2 diabetes mellitus. *Mol Endocrinol* 2008; 22: 2383–2392.
8. Zhou J, Pineyro MA, Wang X, *et al.* Exendin-4 differentiation of a human pancreatic duct cell line into endocrine cells: involvement of PDX-1 and HNF3β transcription factors. *J Cell Physiol* 2002; 192: 304–314.
9. Wang Q, Brubaker PL. Glucagon-like peptide-1 treatment delays the onset of diabetes in 8 week-old db/db mice. *Diabetologia* 2002; 45: 1263–1273.
10. Farilla L, Bulotta A, Hirshberg B, *et al.* Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. *Endocrinology* 2003; 144: 5149–5158.
11. Jhala US, Canettieri G, Screaton RA, *et al.* cAMP promotes pancreatic β-cell survival via CREB-mediated induction of IRS2. *Genes Dev* 2003; 17: 1575–1580.
12. Li Y, Hansotia T, Yusta B, *et al.* Glucagon-like peptide-1 receptor signaling modulates β cell apoptosis. *J Biol Chem* 2003; 278: 471–478.
13. Buteau J, El Assaad W, Rhodes CJ, *et al.* Glucagon-like peptide-1 prevents beta cell glucolipotoxicity. *Diabetologia* 2004; 47: 806–815.
14. Bregenholt S, Moldrup A, Blume N, *et al.* The long-acting glucagon-like peptide-1 analogue, liraglutide, inhibits β-cell apoptosis *in vitro*. *Biochem Biophys Res Commun* 2005; 330: 577–584.
15. Park S, Dong X, Fisher TL, *et al.* Exendin-4 uses Irs2 signaling to mediate pancreatic β cell growth and function. *J Biol Chem* 2006; 281: 1159–1168.
16. Toyoda K, Okitsu T, Yamane S, *et al.* GLP-1 receptor signaling protects pancreatic beta cells in intraportal islet transplant by inhibiting apoptosis. *Biochem Biophys Res Commun* 2008; 367: 793–798.
17. Yoshioka M, Kayo T, Ikeda T, *et al.* A novel locus, Mody4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. *Diabetes* 1997; 46: 887–894.
18. Oyadomari S, Koizumi A, Takeda K, *et al.* Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest* 2002; 109: 525–532.
19. Harada N, Yamada Y, Tsukiyama K, *et al.* A novel GIP receptor splice variant influences GIP sensitivity of pancreatic β-cells in obese mice. *Am J Physiol Endocrinol Metab* 2008; 294: E61–E68.
20. Yusta B, Baggio LL, Estall JL, *et al.* GLP-1 receptor activation improves β cell function and survival following induction of endoplasmic reticulum stress. *Cell Metab* 2006; 4: 391–406.
21. Wang J, Takeuchi T, Tanaka S, *et al.* A mutation in the insulin 2 gene induces diabetes with severe pancreatic β-cell dysfunction in the Mody mouse. *J Clin Invest* 1999; 103: 27–37.
22. Stoy J, Edghill EL, Flanagan SE, *et al.* Insulin gene mutations as a cause of permanent neonatal diabetes. *Proc Natl Acad Sci USA* 2007; 104: 15040–15044.
23. Tsunekawa S, Yamamoto N, Tsukamoto K, *et al.* Protection of pancreatic β-cells by exendin-4 may involve the reduction of endoplasmic reticulum stress; *in vivo* and *in vitro* studies. *J Endocrinol* 2007; 193: 65–74.
24. Cunha DA, Ladriere L, Ortis F, *et al.* Glucagon-like peptide-1 agonists protect pancreatic β-cells from lipotoxic endoplasmic reticulum stress through upregulation of BiP and JunB. *Diabetes* 2009; 58: 2851–2862.
25. Butler AE, Janson J, Bonner-Weir S, *et al.* β-cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. *Diabetes* 2003; 52: 102–110.
26. Kendall DM, Riddle MC, Rosenstock J, *et al.* Effects of exenatide (exendin-4) on glycemic control over 30 weeks in patients with type 2 diabetes treated with metformin and a sulfonylurea. *Diabetes Care* 2005; 28: 1083–1091.

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

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

Norio Harada, Akihiro Hamasaki, Shunsuke Yamane, Atsushi Muraoka, Erina Joo, Kazuyo Fujita, Nobuya Inagaki*

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

Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, Japan
*Corresponding author. Nobuya Inagaki Tel.: +81-75-751-3560 Fax: +81-75-751-4244
E-mail address: inagaki@metab.kuhp.kyoto-u.ac.jp
Received 12 August 2010; revised 16 September 2010; accepted 16 September 2010

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

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

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

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

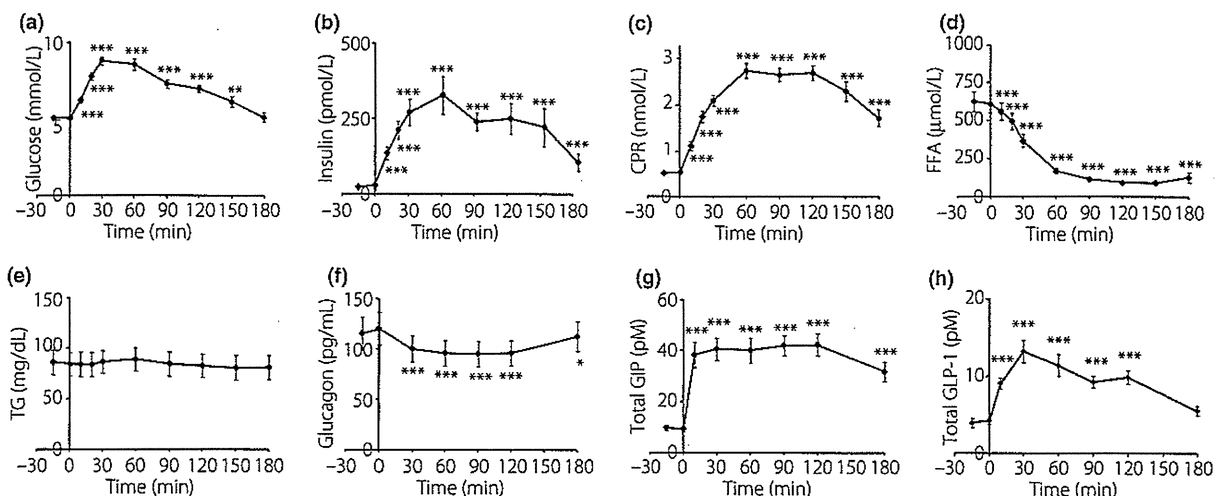

Figure 1 | Concentrations of (a) plasma glucose, (b) serum immunoreactive insulin, (c) serum C-peptide reactivity (CPR), (d) serum free fatty acid (FFA), (e) serum triglyceride (TG), (f) glucagon, (g) total gastric inhibitory polypeptide (GIP) and (h) total glucagon-like peptide-1 (GLP-1) during oral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

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

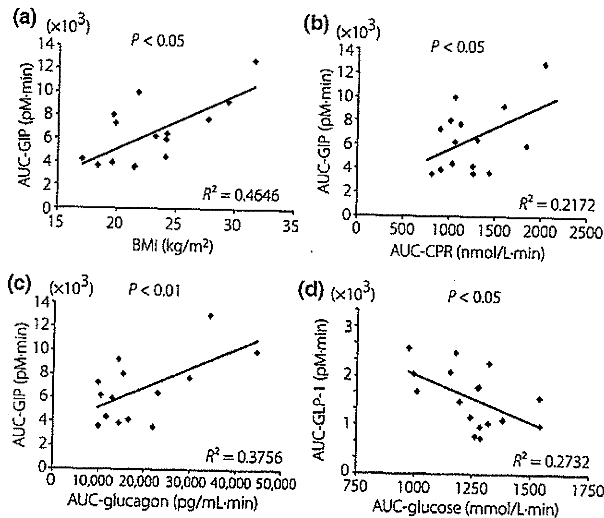

Figure 2 | Simple regression analysis of gastric inhibitory polypeptide secretion (AUC-GIP) and (a) body mass index (BMI), (b) AUC of serum C-peptide reactivity (CPR) and (c) glucagon. (d) Simple regression analysis of glucagon-like peptide-1 secretion (AUC-GLP-1) and AUC of plasma glucose (PG).

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

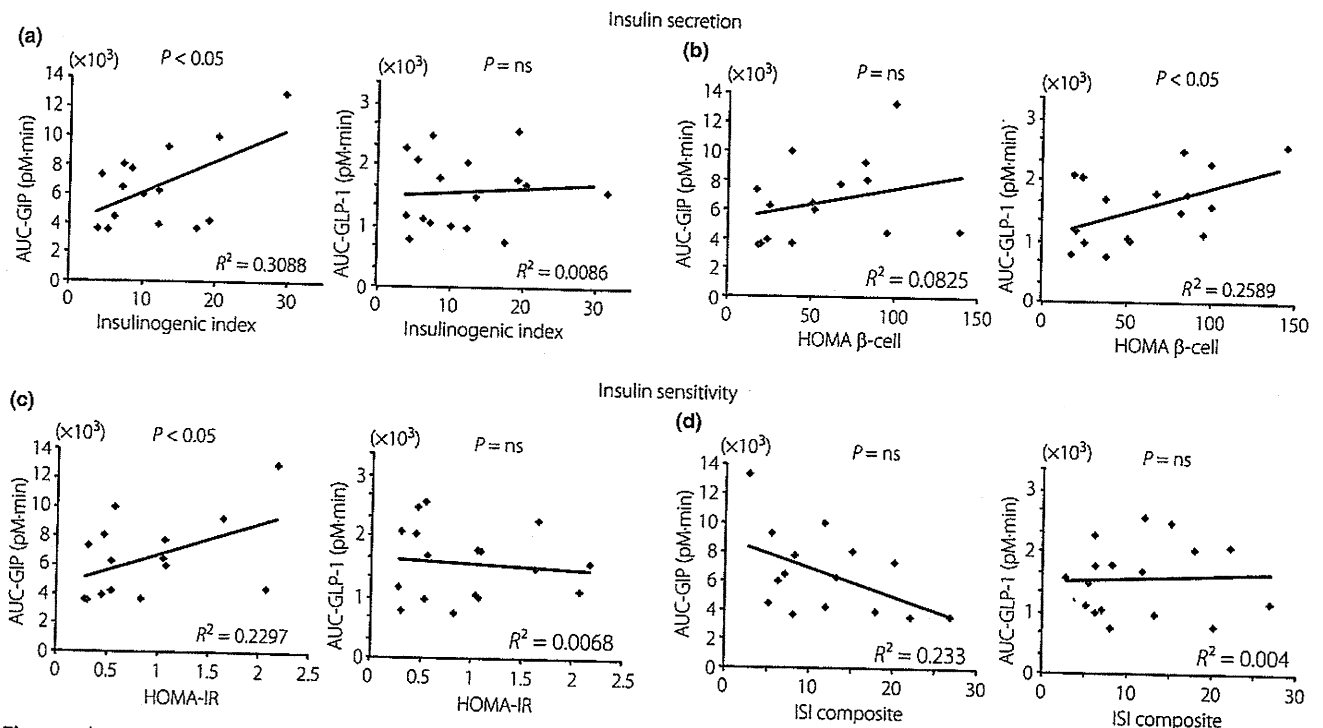

Figure 3 | Relationship between gastric inhibitory polypeptide secretion (AUC-GIP) and glucagon-like peptide-1 secretion (AUC-GLP-1) and the indices of insulin secretion and insulin sensitivity. (a) Insulinogenic index, (b) homeostasis model assessment (HOMA) β-cell function, (c) homeostasis model assessment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

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

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.

REFERENCES

1. Nauck MA, Homberger E, Siegel EG, *et al.* Incretin effects of increasing glucose loads in man calculated from venous insulin and C-peptide responses. *J Clin Endocrinol Metab* 1986; 63: 492–498.
2. Seino Y, Fukushima M, Yabe D. GIP and GLP-1, the two incretin hormone: similalities and differernce. *J Diabetes Invest* 2010; 1: 8–23.
3. Drucker DJ. The biology of incretin hormones. *Cell Metab* 2010; 3: 153–165.
4. Holst JJ. The physiology of glucagon-like peptide 1. *Physiol Rev* 2007; 87: 1409–1439.
5. Mitrakou A, Kelley D, Mokan M, *et al.* Role of reduced suppression of glucose production and diminished early insulin release in impaired glucose tolerance. *N Engl J Med* 1992; 2: 22–29.
6. Haffner SM, Stern MP, Hazuda HP, *et al.* Increased insulin concentrations in nondiabetic offspring of diabetic parents. *N Engl J Med* 1988; 17: 1297–1301.
7. Saad MF, Knowler WC, Pettitt D, *et al.* A two-step model for development of non-insulin-dependent diabetes. *Am J Med* 1991; 90: 229–235.
8. Nauck M, Stöckmann F, Ebert R, *et al.* Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. *Diabetologia* 1986; 29: 46–52.
9. Muscelli E, Mari A, Casolaro A, *et al.* Separate impact of obesity and glucose tolerance on the incretin effect in normal subjects and type 2 diabetic patients. *Diabetes* 2008; 57: 1340–1348.
10. Nauck MA, Heimesaat MM, Orskov C, *et al.* Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. *J Clin Invest* 1993; 91: 301–307.
11. Holst JJ, Gromada J, Nauck MA. The pathogenesis of NIDDM involves a defective expression of the GIP receptor. *Diabetologia* 1997; 40: 984–986.
12. Miyawaki K, Yamada Y, Yano H, *et al.* Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice. *Proc Natl Acad Sci USA* 1999; 96: 14843–14847.
13. Miyawaki K, Yamada Y, Ban N, *et al.* Inhibition of gastric inhibitory polypeptide signaling prevents obesity. *Nat Med* 2002; 8: 738–742.

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

14. Flatt PR, Bailey CJ, Kwasowski P, *et al.* Abnormalities of GIP in spontaneous syndromes of obesity and diabetes in mice. *Diabetes* 1983; 32: 433–435.
15. Creuzfeldt W, Ebert R, Willms B, *et al.* Gastric inhibitory polypeptide (GIP) and insulin in obesity: increased response to stimulation and defective feedback control of serum levels. *Diabetologia* 1978; 14: 15–24.
16. Harada N, Yamada Y, Tsukiyama K, *et al.* A novel GIP receptor splice variant influences GIP sensitivity of pancreatic beta-cells in obese mice. *Am J Physiol Endocrinol Metab* 2008; 294: E61–E68.
17. Vilsbøll T, Krarup T, Deacon CF, *et al.* Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients. *Diabetes* 2001; 50: 609–613.
18. Vollmer K, Holst JJ, Baller B, *et al.* Predictors of incretin concentrations in subjects with normal, impaired, and diabetic glucose tolerance. *Diabetes* 2008; 57: 678–687.
19. Faerch K, Vaag A, Holst JJ, *et al.* Impaired fasting glycaemia vs impaired glucose tolerance: similar impairment of pancreatic alpha and beta cell function but differential roles of incretin hormones and insulin action. *Diabetologia* 2008; 51: 853–861.
20. Yabe D, Kuroe A, Lee S, *et al.* Little enhancement of meal-induced glucagon-like peptide 1 secretion in Japanese: comparison of type 2 diabetes patients and healthy controls. *J Diabetes Invest* 2010; 1: 56–59.
21. Chiu KC, Chuang LM, Yoon C. Comparison of measured and estimated indices of insulin sensitivity and beta cell function: impact of ethnicity on insulin sensitivity and beta cell function in glucose-tolerant and normotensive subjects. *J Clin Endocrinol Metab* 2001; 86: 1620–1625.
22. Mandavilli A, Cyranoski D. Asian's big problem. *Nature Med* 2004; 10: 325–327.
23. Seino Y, Ikeda M, Yawata M, *et al.* The insulinogenic index in secondary diabetes. *Horm Metab Res* 1975; 7: 323–335.
24. Harada N, Fukushima M, Toyoda K, *et al.* Factors responsible for elevation of one hour postchallenge plasma glucose levels in Japanese men. *Diabetes Res Clin Pract* 2008; 81: 284–289.
25. National Diabetes Data Group. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *Diabetes* 1979; 28: 1039–1057.
26. Alberi KG, Zimmeret PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998; 54: 539–553.
27. Narita T, Katsuura Y, Sato T, *et al.* Miglitol induces prolonged and enhanced glucagon-like peptide-1 and reduced gastric inhibitory polypeptide responses after ingestion of a mixed meal in Japanese Type 2 diabetic patients. *Diabet Med* 2009; 26: 187–188.
28. Lim GE, Huang GJ, Flora N, *et al.* Insulin regulates glucagon-like peptide-1 secretion from the enteroendocrine L cell. *Endocrinology* 2009; 150: 580–591.
29. Matthews DR, Hosker JP, Rudenski AS. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; 28: 412–419.
30. Fukushima M, Taniguchi A, Sakai M, *et al.* Homeostasis model assessment as a clinical index of insulin resistance. *Diabetes Care* 1999; 22: 1911–1912.
31. Matsuda M, Defronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euroglycemic insulin clamp. *Diabetes Care* 1999; 22: 1462–1470.
32. Seltzer HS, Allen EW, Herron AL Jr, *et al.* Insulin secretion in response to glycemic stimulus: relation of delayed initial release to carbohydrate intolerance in mild diabetes mellitus. *J Clin Invest* 1967; 46: 323–335.
33. Rabinowitz D, Zierler KL. Forearm metabolism in obesity and its response to intra-arterial insulin. Characterization of insulin resistance and evidence for adaptive hyperinsulinism. *J Clin Invest* 1962; 41: 2173–2181.
34. DeFronzo RA, Ferrannini E. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* 1991; 14: 173–194.
35. Kozawa J, Okita K, Imagawa A, *et al.* Similar incretin secretion in obese and non-obese Japanese subjects with type 2 diabetes. *Biochem Biophys Res Commun* 2010; 393: 410–413.
36. Lee S, Yabe D, Nohtomi K, *et al.* Intact glucagon-like peptide-1 levels are not decreased in Japanese patients with type 2 diabetes. *Endocr J* 2009; 57: 119–126.
37. Wishart JM, Horowitz M, Morris HA, *et al.* Relation between gastric empting of glucose and plasma concentrations of glucagon-like peptide-1. *Peptides* 1998; 19: 1049–1053.
38. Nielsen S, Guo Z, Johnson CM, *et al.* Splanchnic lipolysis in human obesity. *J Clin Invest* 2004; 113: 1582–1588.
39. Suzuki H, Fukushima M, Usami M, *et al.* Factors responsible for development from normal glucose tolerance to isolated postchallenge hyperglycemia. *Diabetes care* 2003; 26: 1211–1215.
40. Madsbad S, Schmitz O, Ranstam J, *et al.* Improved glycemic control with no weight increase in patients with type 2 diabetes after once-daily treatment with the long-acting glucagon-like peptide 1 analog liraglutide (NN2211): a 12-week, double-blind, randomized, controlled trial. *Diabetes Care* 2004; 27: 1335–1342.
41. Aschner P, Kipnes MS, Lunceford JK, *et al.* Effect of the dipeptidyl peptidase-4 inhibitor sitagliptin as monotherapy on glycemic control in patients with Type 2 diabetes. *Diabetes Care* 2006; 29: 2632–2637.
42. Seino Y, Rasmussen MF, Zdravkovic M, *et al.* Dose-dependent improvement in glycemia with once-daily liraglutide

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

without hypoglycemia or weight gain: a double-blind, randomized, controlled trial in Japanese patients with type 2 diabetes. *Diabetes Res Clin Pract* 2008; 81: 161–168.
43. Nonaka K, Kakikawa T, Sato A, *et al.* Efficacy and safety of sitagliptin monotherapy in Japanese patients with type 2 diabetes. *Diabetes Res Clin Pract* 2007; 79: 291–298.
44. Mitsui R, Fukushima M, Nishi Y, *et al.* Factors responsible for deteriorating glucose tolerance in newly diagnosed type 2 diabetes in Japanese men. *Metabolism* 2006; 55: 53–58.
45. Saxena R, Hivert M, Langenberg C, *et al.* Genetic variation in GIPR influences the glucose and insulin responses to an oral glucose challenge. *Nat Genet* 2010; 42: 142–148.

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

ORIGINAL ARTICLE

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

Chizumi Yamada[1], Shimpei Fujimoto[1]*, Kaori Ikeda[1], Yuki Nomura[2], Ami Matsubara[2], Miwako Kanno[2], Kenichiro Shide[2], Kiyoshi Tanaka[3], Eri Imai[4], Tsutomu Fukuwatari[4], Katsumi Shibata[4], Nobuya Inagaki[1]

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

[1]Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, [2]Department of Metabolism and Clinical Nutrition, Kyoto University Hospital, Kyoto, [3]School of Human Culture, The University of Shiga Prefecture, Hikone, and [4]Department of Food and Nutrition, Kyoto Women's University, Kyoto, Japan
*Corresponding author. Shimpei Fujimoto Tel.: +81-75-751-3560
Fax: +81-75-751-4244 E-mail address: fujimoto@metab.kuhp.kyoto-u.ac.jp
Received 28 July 2010; revised 8 November 2010; accepted 9 November 2010

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

serum vitamin concentrations with Hcy and BMD among subjects in the same study.

In the present study, to evaluate nutritional risk factors for osteoporosis in patients with type 2 diabetes, BMD, Hcy level, and intakes and levels of Hcy-related vitamins including folate, vitamin B_6 and vitamin B_{12} were analyzed.

MATERIALS AND METHODS

Study Population

A total of 125 Japanese patients with type 2 diabetes admitted between December 2008 and June 2009 to Kyoto University Hospital were sequentially enrolled in the study. Lateral lumbar X-ray was carried out to exclude those with scoliosis, compression fractures and ectopic calcifications. Subjects with bilateral hip fractures or prosthesis and other diseases that might influence bone metabolism including liver disease, renal dysfunction (serum creatinine above 2 mg/dL), hyperthyroidism, hyperparathyroidism, hypercorticoidism, and hypogonadism were excluded. All subjects were free of drugs that influence bone and calcium metabolism including glucocorticoids, bisphosphonates, calcitonin injection, estrogens, selective estrogen receptor modulators, vitamin D, vitamin K, thiazide diuretics, heparin and anticonvulsants. The number of patients treated with thiazolidinedione and metformin was 7 and 28, respectively. The present study was cross-sectional in design, and was approved by The Ethical Committee of Kyoto University Hospital and complies with the Helsinki Declaration. Written informed consent was obtained from all participants.

Measurement of Bone Mineral Density

BMD was measured by dual-energy X-ray absorptiometry (DXA; Discovery; Hologic, Waltham, MA, USA) at the lumbar spine (L1-L4) and femoral neck. The coefficient of variation of the measurements of BMD was 0.39%. BMD (g/cm^2) was expressed as Z-score calculated on the basis of the normal reference values of the age- and sex-matched Japanese group provided by the DXA system manufacturer. Because male and female patients of different ages were included in the study, comparison of BMD was made based on Z-scores. Fat mass and lean body mass (without bone mineral content) were measured by DXA (Hologic Discovery; Hologic) using whole-body absorptiometry software, and each value was expressed in kilograms.

Biochemical Measurements

Blood samples were obtained after overnight fasting immediately after admission. Glycosylated hemoglobin (HbA_{1c}) was measured by high performance liquid chromatography (HPLC). The value for HbA_{1c} (%) is estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} [Japan Diabetes Society (JDS); %] +0.4%, considering the relational expression of HbA_{1c} (JDS; %) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (NGSP)[22]. Fasting serum C-peptide was measured by ELISA (ST AIA-PACK C-Peptide; Toso Corporation, Tokyo, Japan). Bone-specific alkaline phosphatase (BAP) was measured by enzyme immunoassay (Osteolinks BAP; DS Pharma Biomedical, Suita, Japan), and urine N-terminal cross-linked telopeptide of type-I collagen (uNTx) was measured by ELISA (Osteomark NTx ELISA Urine; Inverness Medical, Waltham, MA, USA). Plasma Hcy levels were determined by HPLC using a thiol-specific fluorogenic reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfate[23], and the upper limit of Hcy was 13.5 nmol/L. As pyridoxal 5′-phosphate (PLP) is the predominant circulating form of vitamin B_6, serum PLP concentrations were measured by HPLC[24,25] for evaluation of vitamin B_6 status. For vitamin B_{12} measurement, 0.2 mmol/L acetate buffer (pH 4.8) was added to the serum samples, and the vitamin B_{12} was converted to cyanocobalamin by boiling with 0.0006% potassium cyanide at acidic pH. Cyanocobalamin was determined by the microbioassay method using *Lactobacillus leichmanii*, ATCC 7830[24,25]. Serum folate was determined by the microbioassay method using *Lactobacillus casei* ATCC 2733[24,25].

Evaluation of Dietary Nutrient Intake

A food frequency questionnaire (FFQ) validated by Takahashi *et al.*[26,27] was used to calculate nutrient intakes. The FFQ used in the present study included questions on the consumption of various food items over the previous 1 or 2 months. Daily nutrient intake was calculated by multiplying the frequency of consumption of each food by the nutrient content of the portion size and summing the products for all food items. The FFQ is validated against 7-day dietary records and the FFQ-estimated nutrient intake values are 72–121% of those of 7-day dietary records[26]. The reproducibility of the FFQ at intervals of 1–2 months is 93–119% for each nutrient[26]. Correlations of dietary folate intake, serum folate concentration, and plasma Hcy level with intakes of various food groups including grain/rice, potato, green vegetables, other vegetables, fruits, seaweeds, beans/soy products, seafood, meats, egg, milk products and oil/fat were evaluated.

Statistical Analysis

Data were expressed as mean ± SD. SPSS statistical software (version 13.0; SPSS, Chicago, IL, USA) was used for all statistical analyses. Pearson's correlation coefficient was calculated as a measure of association by adjusting for age and sex where appropriate. Stepwise multiple linear regression analyses were carried out to determine independent factors for plasma Hcy levels including (i) dietary vitamin B_6, vitamin B_{12} and folate intake values; and (ii) serum PLP, vitamin B_{12} and folate concentrations as independent variables. The relationship between BMD with Hcy and Hcy-related vitamins was further explored using a quartile-based analysis. Statistical differences among the groups were evaluated using analysis of covariance (ANCOVA) adjusted for age and sex, and Dunnett's multiple comparison tests by comparison with the highest Hcy group. $P < 0.05$ was considered significant.

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

RESULTS

Clinical characteristics, laboratory data and nutrient intake of subjects are shown in Table 1. The average serum vitamin B_{12} concentration was 1.45 ± 0.45 pmol/mL (Table 1) and there was no difference between patients taking metformin (1.52 ± 0.49 pmol/mL, $n = 97$) and those without (1.43 ± 0.49 pmol/mL, $n = 28$). Nutrient intake values were significantly positively correlated with total energy intake (Table 2). Dietary vitamin B_6, vitamin B_{12} and folate intake values were positively correlated with serum vitamin B_6, vitamin B_{12} and folate levels, respectively (Table 2). Plasma Hcy levels were negatively correlated with both dietary intake and serum concentration of folate (Table 2). Only vitamin B_6 intake and not vitamin B_6 concentration showed a weak negative correlation with Hcy; the influence of vitamin B_{12} on Hcy elevation was unclear (Table 2). Stepwise multiple linear regression analyses were carried out to determine independent factors for plasma Hcy levels. Dietary folate intake was a significant predictor of Hcy when dietary vitamin B_6, vitamin B_{12} and folate intake values were included as independent variables ($R^2 = 0.088$, β-coefficient = −0.297, $P < 0.001$), and serum folate concentration also was a significant predictor of Hcy when serum PLP, vitamin B_{12} and folate concentrations were included as independent variables ($R^2 = 0.121$, β-coefficient = −0.347, $P < 0.001$). We then evaluated the correlations of folate intake and the concentrations of folate and Hcy with intake of the various food groups determined by FFQ. Dietary folate intake and serum folate concentration were significantly associated with intakes of certain food groups including potato, green vegetables, other vegetables and fruits. Only intake of green vegetables was significantly correlated with the plasma Hcy level (Table 3).

Bone mineral density of lumbar spine (SP-BMD) and femoral neck (FN-BMD) were positively correlated with body mass index (BMI) and fat mass, although no significant correlations were found in diabetes-related parameters including fasting plasma glucose, HbA_{1c} and diabetes duration (Table 4). Both SP-BMD and FN-BMD were positively correlated with fasting serum C-peptide, but these correlations were cancelled when adjusted for BMI. Urinary NTx, a marker of bone resorption, was negatively correlated with FN-BMD. As nutrient intake significantly increases with energy intake, nutrition intakes were also evaluated by adjusting for calories. As a result, calorie-adjusted folate intake was positively correlated with SP-BMD, although the association between calorie-adjusted folate and FN-BMD did not reach statistical significance. There were no significant associations between BMD of both sites and serum concentrations of vitamin B_6, vitamin B_{12} and folate. The plasma Hcy concentration was negatively correlated with both

Table 1 | Background characteristics of the study subjects

Characteristic	
No. subjects	125
Male/female	79 (63.2%)/46 (36.8%)
Age (years)	61.2 ± 12.4
Duration of diabetes (years)	11.2 ± 9.4
Diabetes treatment (diet/OHA/Ins/Ins + OHA)	27 (21.6%)/62 (49.6%)/ 28 (22.4%)/8 (6.4%)
BMI (kg/m^2)	24.9 ± 4.9
Fat mass (kg)	16.5 ± 9.8
Lean body mass (kg)	45.9 ± 9.3
Fasting plasma glucose (mg/dL)	160.2 ± 48.6
HbA_{1c} (%)	9.6 ± 2.2
Fasting serum C-peptide (ng/mL)	1.71 ± 0.89
Serum BAP (U/L)	23.5 ± 8.7
uNTx (nMBCE/mmol Cr)	35.6 ± 19.8
Energy intake (kcal/day)	2073.2 ± 582.5
Protein/fat/carbohydrate intake (g/day)	73.6 ± 19.7/64.4 ± 23.7/ 278.7 ± 80.2
Calcium intake (mg/day)	596.0 ± 213.6
Vitamin D intake (μg/day)	9.21 ± 4.48
Vitamin B_6 intake (mg/day)	1.22 ± 0.34
Vitamin B_{12} intake (μg/day)	8.81 ± 4.65
Folate intake (μg/day)	287.4 ± 100.5
Serum PLP concentration (pmol/mL)	61.3 ± 29.1
Serum vitamin B_{12} concentration (pmol/mL)	1.45 ± 0.45
Serum folate concentration (pmol/mL)	27.5 ± 10.3
Plasma homocysteine concentration (nmol/mL)	11.2 ± 5.1

Data are number of patients (categorized data) or mean ± SD (quantitative data).
BAP, bone-specific alkaline phosphatase; BMI, body mass index; Ins, insulin; OHA, oral hypoglycemic agents; PLP, pyridoxal 5′-phosphate; uNTX, urine N-terminal cross-linked telopeptide of type-I collagen.

Table 2 | Correlations among dietary nutrient intake values, serum concentrations and plasma homocysteine levels adjusted for age and sex

	r	*P*
Correlations of total energy intake with various nutrient intakes		
Vitamin B_6 (mg)	0.521	<0.001
Vitamin B_{12} (μg)	0.253	0.005
Folate (μg)	0.331	<0.001
Correlations of intake values with serum concentrations		
Vitamin B_6	0.192	0.034
Vitamin B_{12}	0.336	<0.001
Folate	0.400	<0.001
Correlations of plasma Hcy levels with B vitamins		
Vitamin B_6 intake (mg)	−0.207	0.022
Vitamin B_{12} intake (μg)	−0.001	0.988
Folate intake (μg)	−0.328	<0.001
Serum PLP concentration (pmol/mL)	0.002	0.982
Serum B_{12} concentration (pmol/mL)	0.001	0.993
Serum folate concentration (pmol/mL)	−0.369	<0.001

Hcy, homocysteine; PLP, pyridoxal 5′-phosphate.

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

Table 3 | Correlations of dietary folate intake, serum folate concentration and plasma homocysteine level with various food groups

	Dietary folate intake		Serum folate concentration		Plasma Hcy level	
	r	*P*	*r*	*P*	*r*	*P*
Grain/rice	−0.076	0.399	−0.086	0.341	−0.056	0.538
Potato	0.470	<0.001	0.220	0.014	0.012	0.895
Green vegetables	0.843	<0.001	0.361	<0.001	−0.207	0.020
Other vegetables	0.620	<0.001	0.197	0.027	0.077	0.390
Fruits	0.338	<0.001	0.206	0.021	0.018	0.839
Seaweeds	0.322	<0.001	0.072	0.426	0.071	0.435
Beans/soy products	0.390	<0.001	0.156	0.083	0.016	0.856
Seafood	0.313	<0.001	0.075	0.407	−0.017	0.848
Meats	0.065	0.474	0.042	0.643	−0.070	0.435
Egg	0.278	0.002	0.068	0.450	−0.056	0.538
Milk products	0.108	0.230	0.113	0.208	−0.035	0.698
Oil/fat	0.145	0.107	0.161	0.073	−0.112	0.214

Hcy, homocysteine.

Table 4 | Correlations of bone mineral density of lumbar spine and femoral neck with diabetes-related parameters, bone turnover markers and B vitamin status

	SP-BMD		FN-BMD	
	r	*P*	*r*	*P*
BMI (kg/m^2)	0.288	0.001	0.463	<0.001
Fasting plasma glucose (mg/dL)	−0.149	0.098	−0.113	0.210
HbA_{1c} (%)	0.098	0.194	0.053	0.556
Diabetes duration (years)	0.082	0.366	0.057	0.528
Fasting serum C-peptide (ng/mL)	0.182	0.045	0.285	0.001
BAP (U/L)	0.112	0.218	−0.061	0.499
uNTx (nMBCE/mmol Cr)	−0.138	0.084	−0.183	0.042
Vitamin B_6 intake (mg)	−0.032	0.727	−0.053	0.559
Vitamin B_6 intake (mg/100 kcal)	0.113	0.211	0.005	0.959
Vitamin B_{12} intake (μg)	0.012	0.899	0.166	0.065
Vitamin B_{12} intake (μg/1000 kcal)	0.054	0.554	0.148	0.102
Folate intake (μg)	0.103	0.256	0.112	0.216
Folate intake (μg/1000 kcal)	0.198	0.027	0.153	0.090
Serum PLP concentration (pmol/mL)	−0.062	0.497	−0.007	0.936
Serum B_{12} concentration (pmol/mL)	0.023	0.799	0.058	0.524
Serum folate concentration (pmol/mL)	0.104	0.248	0.114	0.205
Plasma Hcy concentration (nmol/mL)	−0.278	0.002	−0.201	0.025

BAP, bone-specific alkaline phosphatase; BMI, body mass index; FN-BMD bone mineral density of femoral neck; Hcy, homocysteine; PLP, pyridoxal 5′-phosphate; SP-BMD, bone mineral density of lumbar spine; uNTX, urine N-terminal cross-linked telopeptide of type-I collagen.

SP-BMD and FN-BMD, showing that hyperhomocysteinemia is clearly associated with low BMD in patients with type 2 diabetes (Figure 1).

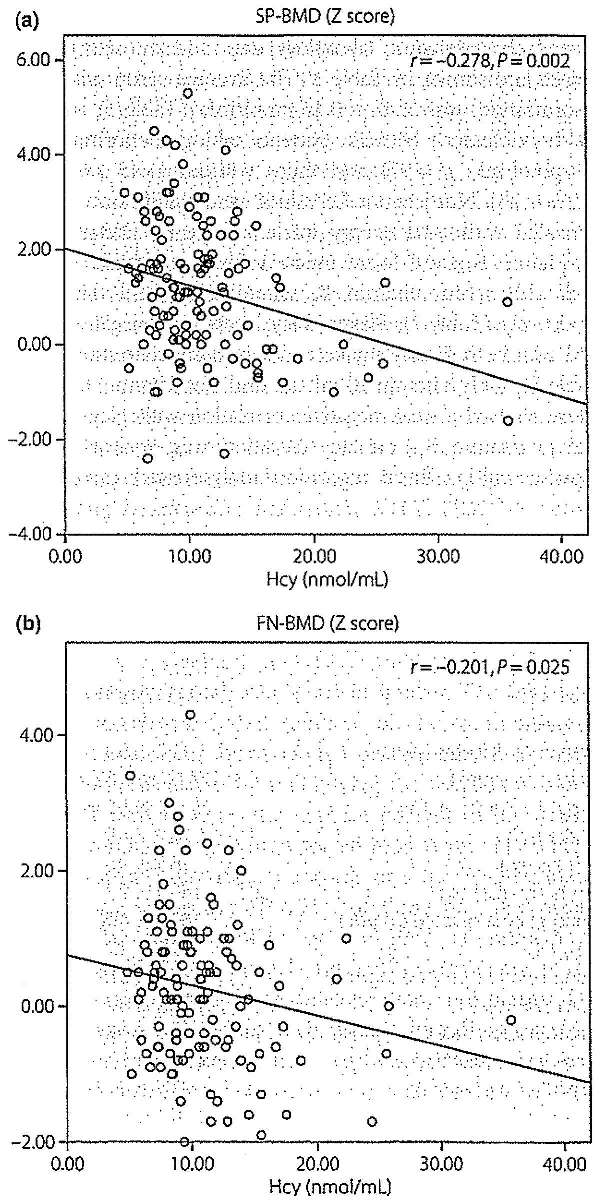

Figure 1 | The relationship between homocysteine (Hcy) and bone mineral density of lumbar spine (SP-BMD) and femoral neck (FN-BMD).

As hyperhomocysteinemia derived from folate insufficiency has been suggested to be involved in low BMD, we compared clinical characteristics of the study population across the quartiles of Hcy (quartile 1, n = 31, Hcy < 8.3 nmol/mL; quartile 2, n = 32, Hcy 8.3 to <9.9 nmol/mL; quartile 3, n = 32, Hcy 9.9 to <12.8 nmol/mL; quartile 4, n = 30, Hcy > 12.8 nmol/mL). There were no significant differences across the quartiles in general clinical characteristics including age, BMI, diabetes-related parameters, energy intake, and vitamin B_6 and vitamin B_{12} status (Table 5). However, SP-BMD and FN-BMD were significantly lower in patients in the highest quartile of Hcy than

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

Table 5 | Comparison of clinical characteristics according to homocysteine quartiles adjusted for age and sex

Hcy concentration (nmol/mL)	Quartile 1 (4.9–8.0)	Quartile 2 (8.1–9.9)	Quartile 3 (10.0–12.8)	Quartile 4 (12.8–35.7)	ANCOVA P
Male/female	17/14	21/11	23/9	18/12	
Age (years)	59.3 ± 13.8	58.1 ± 12.6	63.9 ± 8.7	64.0 ± 13.4	0.212
BMI (kg/m²)	25.0 ± 4.4	25.8 ± 5.0	25.0 ± 5.6	23.8 ± 4.5	0.461
Fasting plasma glucose (mg/dL)	158.8 ± 44.8	162.0 ± 45.8	155.6 ± 50.0	164.6 ± 55.4	0.721
HbA_{1c} (%)	10.1 ± 2.3	9.9 ± 2.5	9.1 ± 1.8	9.4 ± 2.1	0.378
Diabetes duration (years)	9.5 ± 8.4	10.2 ± 9.7	12.6 ± 8.6	12.4 ± 9.0	0.183
SP-BMD (Z score)	1.34 ± 1.43*	1.24 ± 1.38*	1.39 ± 1.24*	0.50 ± 1.18	0.037
FN-BMD (Z score)	0.45 ± 0.99**	0.32 ± 1.23*	0.26 ± 0.96*	−0.27 ± 1.03	<0.001
Energy intake (kcal/day)	2161 ± 543	2145 ± 565	2069 ± 563	1910 ± 650	0.260
Vitamin B_6 intake (mg)	1.31 ± 0.35	1.26 ± 0.36	1.21 ± 0.32	1.09 ± 0.29	0.136
Vitamin B_{12} intake (μg)	8.59 ± 3.44	8.86 ± 4.64	9.49 ± 5.24	8.27 ± 5.21	0.798
Folate intake (μg)	323.5 ± 92.2**	287.2 ± 108.0*	305.0 ± 91.8**	231.7 ± 89.1	0.001
Intake of green vegetables (g/day)	101.9 ± 65.3*	86.1 ± 60.6	89.3 ± 47.5	68.9 ± 49.2	0.043
Serum PLP concentration (pmol/mL)	65.0 ± 33.1	60.0 ± 24.6	58.9 ± 32.9	61.4 ± 26.2	0.943
Serum B_{12} concentration (pmol/mL)	2.39 ± 0.88	2.90 ± 1.61	2.50 ± 0.73	2.53 ± 0.92	0.419
Serum folate concentration (pmol/mL)	33.6 ± 11.5**	26.9 ± 7.6*	26.9 ± 9.0*	21.7 ± 8.7	<0.001
Plasma Hcy concentration (nmol/mL)	6.9 ± 0.9**	9.1 ± 0.5**	11.3 ± 0.8**	17.8 ± 6.1	<0.001

BMI, body mass index; FN-BMD bone mineral density of femoral neck; Hcy, homocysteine; PLP, pyridoxal 5′-phosphate; SP-BMD, bone mineral density of lumbar spine. Mean ± SD, *P < 0.05, **P < 0.01 relative to the highest homocysteine quartile group.

those in patients in the other quartiles. Furthermore, patients in the highest Hcy quartile showed significantly decreased dietary folate intake, serum folate concentration and intake of green vegetables compared with those in the lower Hcy quartiles. Because the caloric intake was similar across the quartiles, the quality of the diet might be poor in the highest Hcy group. Quartile analysis revealed that the highest Hcy group showed the lowest BMD, the lowest serum folate concentration, the lowest folate intake and the lowest intake of green vegetables.

DISCUSSION

In the present study, hyperhomocysteinemia was found to be clearly associated with low BMD in type 2 diabetes patients, as it has been reported to be in non-diabetic subjects[6–14]. Furthermore, folate insufficiency might be one of the important factors in hyperhomocysteinemia, as plasma Hcy levels were negatively correlated with both dietary intake and serum concentration of folate.

Osteoporosis is a multifactorial disease, a major health problem characterized by low BMD, deterioration of bone microarchitecture and increased risk of fracture. Elevation of Hcy is one of the important risk factors for osteoporosis[28,29], and can be caused by insufficiency of Hcy-related vitamins, such as folate, vitamin B_6 and vitamin B_{12}[6–14]. Because dietary risk factors can be improved when recognized, sufficiency of Hcy-related vitamins and its relationship to osteoporosis in patients with type 2 diabetes is of primary concern.

Elevation of Hcy can be caused by insufficiency of folate, vitamin B_6 or vitamin B_{12}, and the plasma Hcy level is considered to be a fairly sensitive index of folate metabolic status compared with that of the other factors in non-diabetic subjects. Previous studies reported hyperhomocysteinemia was observed in 86% of subjects with clinically expressed folate deficiency[30]; folate is a major determinant of Hcy levels in healthy people[31,32] and vitamin B_{12} influences Hcy levels less than folate does[33,34]. Folate, vitamin B_6 and vitamin B_{12} are water-soluble vitamins, which are in general not readily stored and consistent daily intake is important. Usually, folate and vitamin B_6 deficiency develops within a month of insufficient intake. In contrast, it is known that patients with complete loss of intrinsic factor require 3–5 years to become overtly vitamin B_{12} deficient[35]. Vitamin B_{12} is a unique water-soluble vitamin, and because 80% of the 2.5 mg average whole body stock of vitamin B_{12} is reserved in the liver and vitamin B_{12} excreted in the bile and is effectively reabsorbed in the intestine, clinical signs of vitamin B_{12} deficiency take a long time to appear and progress slowly[36]. Some patients in the present study were taking metformin, which is known to inhibit absorption of vitamin B_{12}[37], but there was no difference between the patients taking metformin and those not taking metformin. As to vitamin B_6, only a weak negative correlation between vitamin B_6 intake and Hcy was not enough to conclude that vitamin B_6 is a nutritional risk factor for osteoporosis, and there have been no other studies showing the effect of vitamin B_6 on BMD.

Leafy green vegetables, such as spinach and broccoli, are rich sources of folate. Folate is also contained in a variety of foods including fruits, beans, seaweeds, liver and egg yolk. To investigate the cause of folate insufficiency, we focused particularly on dietary sources of folate. We evaluated the association of dietary folate intake, serum folate concentration, and plasma Hcy level

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

with various food groups, and found that intake of green vegetables correlated well with folate status and Hcy levels. Furthermore, it was revealed by the quartile analysis that the highest Hcy group showed the lowest BMD, the lowest serum folate concentration, the lowest folate intake and the lowest intake of green vegetables. This analysis suggests that insufficient intake of green vegetables, but not insufficient caloric intake, causes folate insufficiency in the group with the highest Hcy.

The strength of the present study is that it is the first study to show that nutritional status of folate might affect the homocysteine level, a putative risk factor for osteoporosis, in Japanese patients with type 2 diabetes. The present study is also meaningful in promoting awareness of the importance of diet quality, because patients with diabetes are at high risk of developing osteoporosis. In contrast, the present study has some limitations. First, the sample size was not large enough for conclusions regarding marginal insignificant *P*-values. We estimated sample size using a correlation coefficient obtained from a previous cross-sectional study assessing the relationship between BMD and plasma Hcy[8]. The correlation coefficient of femoral BMD with Hcy was −0.18 and the sample size was estimated to be $n = 153$ (two-sided $\alpha = 0.1$, $\beta = 0.2$), while we analyzed 125 patients. Second, we only analyzed patients with type 2 diabetes and comparison with non-diabetic subjects is necessary. An unanswered question is whether diabetes modulates the effects of nutritional state of folate on Hcy metabolism, and the effects of Hcy levels on BMD. Finally, a longitudinal study is required to examine the effects of Hcy on rate of BMD loss and risk of fracture for a longer duration in patients with type 2 diabetes. It is also necessary to determine whether encouraging patients with higher Hcy levels to eat more green vegetables is useful as a dietary intervention to improve Hcy levels and BMD.

In conclusion, the present study shows that BMD inversely correlates to plasma Hcy levels in Japanese patients with type 2 diabetes, and that dietary intake and the serum concentration of folate are determinant factors of Hcy levels. When our group was analyzed across quartiles, BMD, serum folate concentration, folate intake and intake of green vegetables were lowest in the highest Hcy group. Taken together, in Japanese patients with type 2 diabetes, a diet low in green vegetables rather than a calorie-restricted diet might be the more important factor in the declining nutritional status of folate that increases the Hcy level, a putative risk factor for osteoporosis.

ACKNOWLEDGEMENTS

This study was supported in part by Diabetes Masters Conference and Japan Diabetes Foundation. The authors declare no conflicts of interest.

REFERENCES

1. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes – a meta-analysis. *Osteoporos Int* 2007; 18: 427–444.
2. Hofbauer LC, Brueck CC, Singh SK, *et al.* Osteoporosis in patients with diabetes mellitus. *J Bone Miner Res* 2007; 22: 1317–1328.
3. Adami S. Bone health in diabetes: considerations for clinical management. *Curr Med Res Opin* 2009; 25: 1057–1072.
4. Lammes E, Törner A, Akner G. Nutrient density and variation in nutrient intake with changing energy intake in multimorbid nursing home residents. *J Hum Nutr Diet* 2009; 22: 210–218.
5. Grzybek A, Klosiewicz-Latoszek L, Targosz U. Changes in the intake of vitamins and minerals by men and women with hyperlipidemia and overweight during dietetic treatment. *Eur J Clin Nutr* 2002; 56: 1162–1168.
6. Gjesdal CG, Vollset SE, Ueland PM, *et al.* Plasma total homocysteine level and bone mineral density: the Hordaland Homocysteine Study. *Arch Intern Med* 2006; 166: 88–94.
7. Baines M, Kredan MB, Usher J, *et al.* The association of homocysteine and its determinants MTHFR genotype, folate, vitamin B_{12} and vitamin B_6 with bone mineral density in postmenopausal British women. *Bone* 2007; 40: 730–736.
8. Golbahar J, Hamidi A, Aminzadeh MA, *et al.* Association of plasma folate, plasma total homocysteine, but not methylenetetrahydrofolate reductase C667T polymorphism, with bone mineral density in postmenopausal Iranian women: a cross-sectional study. *Bone* 2004; 35: 760–765.
9. Golbahar J, Aminzadeh MA, Hamidi SA, *et al.* Association of red blood cell 5-methyltetrahydrofolate folate with bone mineral density in postmenopausal Iranian women. *Osteoporos Int* 2005; 16: 1894–1898.
10. Morris MS, Jacques PF, Selhub J. Relation between homocysteine and B-vitamin status indicators and bone mineral density in older Americans. *Bone* 2005; 37: 234–242.
11. Cagnacci A, Baldassari F, Rivolta G, *et al.* Relation of homocysteine, folate, and vitamin B_{12} to bone mineral density of postmenopausal women. *Bone* 2003; 33: 956–959.
12. Tucker KL, Hannan MT, Qiao N, *et al.* Low plasma vitamin B12 is associated with lower BMD: the Framingham Osteoporosis Study. *J Bone Miner Res* 2005; 20: 152–158.
13. Dhonukshe-Rutten RA, Pluijm SM, de Groot LC, *et al.* Homocysteine and vitamin B_{12} status relate to bone turnover markers, broadband ultrasound attenuation, and fractures in healthy elderly people. *J Bone Miner Res* 2005; 20: 921–929.
14. Dhonukshe-Rutten RA, Lips M, de Jong N, *et al.* Vitamin B-12 status is associated with bone mineral content and bone mineral density in frail elderly women but not in men. *J Nutr* 2003; 133: 801–807.
15. Harpey JP, Rosenblatt DS, Cooper BA, *et al.* Homocystinuria caused by 5,10-methylenetetrahydrofolate reductase deficiency: a case in an infant responding to methionine, folinic acid, pyridoxine, and vitamin B12 therapy. *J Pediatr* 1981; 98: 275–278.
16. Mudd SH, Skovby F, Levy HL, *et al.* The natural history of homocystinuria due to cystathionine beta-synthase deficiency. *Am J Hum Genet* 1985; 37: 1–31.

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

17. Lubec B, Fang-Kircher S, Lubec T, *et al.* Evidence for McKusick's hypothesis of deficient collagen cross-linking in patients with homocystinuria. *Biochim Biophys Acta* 1996; 1315: 159–162.
18. Saito M, Fujii K, Marumo K. Degree of mineralization-related collagen crosslinking in the femoral neck cancellous bone in cases of hip fracture and controls. *Calcif Tissue Int* 2006; 79: 160–168.
19. Krumdieck CL, Prince CW. Mechanisms of homocysteine toxicity on connective tissues: implications for the morbidity of aging. *J Nutr* 2000; 130: 365s–368s.
20. Koh JM, Lee YS, Kim YS, *et al.* Homocysteine enhances bone resorption by stimulation of osteoclast formation and activity through increased intracellular ROS generation. *J Bone Miner Res* 2006; 21: 1003–1011.
21. Herrmann M, Schmidt J, Umanskaya N, *et al.* Stimulation of osteoclast activity by low B-vitamin concentrations. *Bone* 2007; 41: 584–591.
22. The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus. Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus. *J Diabetes Invest* 2010; 1: 212–228.
23. Araki A, Sako Y. Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection. *J Chromatogr* 1987; 422: 43–52.
24. Shibata K, Fukuwatari T, Watanabe T, *et al.* Intra- and inter-individual variations of blood and urinary water-soluble vitamins in Japanese young adults consuming a semi-purified diet for 7 days. *J Nutr Sci Vitaminol (Tokyo)* 2009; 55: 459–470.
25. Fukuwatari T, Yoshida E, Takahashi K, *et al.* Effect of fasting on the urinary excretion of water-soluble vitamins in humans and rats. *J Nutr Sci Vitaminol (Tokyo)* 2010; 56: 19–26.
26. Takahashi K, Yoshimura Y, Kaimoto T, *et al.* Validation of a food frequency questionnaire based on food groups for estimating indivudual nutrient intake. *Jpn J Nutr* 2001; 59: 221–232.
27. Miyaki K, Tohyama S, Murata M, *et al.* Salt intake affects the relation between hypertension and the T-786C polymorphism in the endothelial nitric oxide synthase gene. *Am J Hypertens* 2005; 18: 1556–1562.
28. van Meurs JB, Dhonukshe-Rutten RA, Pluijm SM, *et al.* Homocysteine levels and the risk of osteoporotic fracture. *N Engl J Med* 2004; 350: 2033–2041.
29. Herrmann M, Peter Schmidt J, Umanskaya N, *et al.* The role of hyperhomocysteinemia as well as folate, vitamin B_6 and B_{12} deficiencies in osteoporosis: a systematic review. *Clin Chem Lab Med* 2007; 45: 1621–1632.
30. Savage DG, Lindenbaum J, Stabler SP, *et al.* Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies. *Am J Med* 1994; 96: 239–246.
31. Selhub J, Jacques PF, Wilson PW, *et al.* Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *JAMA* 1993; 270: 2693–2698.
32. Selhub J, Jacques PF, Rosenberg IH, *et al.* Serum total homocysteine concentrations in the third National Health and Nutrition Examination Survey (1991–1994): population reference ranges and contribution of vitamin status to high serum concentrations. *Ann Intern Med* 1999; 131: 331–339.
33. Lindenbaum J, Rosenberg IH, Wilson PW, *et al.* Prevalence of cobalamin deficiency in the Framingham elderly population. *Am J Clin Nutr* 1994; 60: 2–11.
34. Carmel R. *Cobalamin Deficiency, Homocysteine in Health and Disease.* Cambridge University Press, Cambridge, 2001.
35. Chanarin I. *The Megaloblastic Anemias*, 2nd edn. Blackwell Scientific, Oxford, 1979.
36. Food and Nutrition Board, Institute of Medicine. The B vitamins and choline: overview and methods. In: Institute of Medicine. *Dietary Reference Intakes: For Thiamine, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline.* National Academy Press, Washington DC, 1998; 306–356.
37. Adams JF, Clark JS, Ireland JT, *et al.* Malabsorption of vitamin B12 and intrinsic factor secretion during biguanide therapy. *Diabetologia* 1983; 24: 16–18.

© 2010 Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

BJ www.biochemj.org
This is a data-enriched, interactive PDF that provides the gateway to a world of information when opened in Utopia Documents Download FREE software now
BJ Metabolism
Biochem. J. (2011) 435, 421–430 (Printed in Great Britain) doi:10.1042/BJ20101708

Role of mitochondrial phosphate carrier in metabolism–secretion coupling in rat insulinoma cell line INS-1

Yuichi NISHI, Shimpei FUJIMOTO[1], Mayumi SASAKI, Eri MUKAI, Hiroki SATO, Yuichi SATO, Yumiko TAHARA, Yasuhiko NAKAMURA and Nobuya INAGAKI

Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606–8507, Japan

In pancreatic β-cells, glucose-induced mitochondrial ATP production plays an important role in insulin secretion. The mitochondrial phosphate carrier PiC is a member of the SLC25 (solute carrier family 25) family and transports P_i from the cytosol into the mitochondrial matrix. Since intramitochondrial P_i is an essential substrate for mitochondrial ATP production by complex V (ATP synthase) and affects the activity of the respiratory chain, P_i transport via PiC may be a rate-limiting step for ATP production. We evaluated the role of PiC in metabolism–secretion coupling in pancreatic β-cells using INS-1 cells manipulated to reduce PiC expression by siRNA (small interfering RNA). Consequent reduction of the PiC protein level decreased glucose (10 mM)-stimulated insulin secretion, the ATP:ADP ratio in the presence of 10 mM glucose and elevation of intracellular calcium concentration in response to 10 mM glucose without affecting the mitochondrial membrane potential ($\Delta\psi_m$) in INS-1 cells. In experiments using the mitochondrial fraction of INS-1 cells in the presence of 1 mM succinate, PiC down-regulation decreased ATP production at various P_i concentrations ranging from 0.001 to 10 mM, but did not affect $\Delta\psi_m$ at 3 mM P_i. In conclusion, the P_i supply to mitochondria via PiC plays a critical role in ATP production and metabolism–secretion coupling in INS-1 cells.

Key words: inorganic phosphate (P_i), insulin secretion, mitochondria, mitochondrial phosphate carrier (PiC), small interfering RNA (siRNA), solute carrier family 25 (SLC25).

INTRODUCTION

Glucose stimulates insulin secretion by both triggering and amplifying signals in pancreatic β-cells [1]. The triggering pathway includes entry of glucose into β-cells, acceleration of glycolysis in the cytosol and mitochondrial metabolism of products derived from glycolysis, increase in ATP content and ATP/ADP ratio, closure of ATP-sensitive K^+ channels (K_{ATP} channels), membrane depolarization, opening of VDCCs (voltage-dependent Ca^{2+} channels), increase in Ca^{2+} influx through VDCCs, rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and exocytosis of insulin granules. Glucose also exerts its effects by increasing Ca^{2+} efficacy in stimulation–secretion coupling via an amplifying pathway, owing at least in part to the direct effect of increased ATP derived from glucose metabolism on exocytosis. Since depletion of mitochondrial DNA abolishes the glucose-induced ATP elevation, mitochondria are clearly a major source of ATP production in pancreatic β-cells [2,3]. Collectively, in pancreatic β-cells, intracellular glucose metabolism regulates exocytosis of insulin granules according to metabolism–secretion coupling in which glucose-induced mitochondrial ATP production plays an important role.

Almost all of the mitochondrial carrier proteins are embedded in the inner membranes of mitochondria, where they transport solutes across the membrane. They belong to the SLC25 (solute carrier family 25) group of proteins [4]. Several members of the SLC25 group have been reported to play roles in GSIS (glucose-stimulated insulin secretion) in pancreatic β-cells. Overexpression or silencing of AGC1 (aspartate/glutamate carrier 1; SLC25A12 or Aralar1) has been reported to increase or reduce GSIS in INS-1E cells respectively [5,6]. Overexpression of UCP2 (uncoupling protein 2; SLC25A8) by adenovirus vector is known to inhibit GSIS from rat islets [7], whereas GSIS from islets of UCP2-deficient mice is enhanced compared with that from control islets [8]. In addition, down-regulation of OGC (2-oxoglutarate carrier; SLC25A11), CIC (citrate/isocitrate carrier; SLC25A1) and GC1 (glutamate carrier 1; SLC25A22) by siRNA (small interfering RNA) suppress GSIS [9–11].

The mitochondrial phosphate carrier PiC (SLC25A3) is a member of the SLC25 family and transports P_i from the cytosol into the mitochondrial matrix. The *PiC* gene has 9 exons; the 3rd and the 4th exons are called exon 3A and exon 3B respectively. These two exons are alternatively spliced and two isoforms of PiC, PiC-A and PiC-B, are generated [12]. They differ considerably in their kinetic parameters as previously shown in a study using a reconstitution system [13]. The K_m of PiC-A for P_i on the external membrane surface is 3-fold that of PiC-B (PiC-A: ~2.2 mM; PiC-B: ~0.78 mM). The K_m on the internal surface is much higher (PiC-A: ~9.7 mM; PiC-B: ~6.3 mM) than K_m on the external membrane surface. The maximum transport rate of PiC-A is approximately a third that of PiC-B. These isoforms also differ in their tissue distribution. PiC-A is expressed in skeletal muscle and cardiac muscle, whereas PiC-B is expressed ubiquitously [13,14]. A case study of patients with PiC-A deficiency who suffered from lactic acidosis, heart failure and muscle weakness and died within the first year of life, demonstrates the critical significance of this carrier [15].

Since intramitochondrial P_i is an essential substrate for mitochondrial ATP production by complex V (ATP synthase) and affects activity of the respiratory chain [16], the supply of P_i from

Abbreviations used: AAC, ATP/ADP carrier; DAPP, diadenosine pentaphosphate; DIC, dicarboxylate carrier; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GSIS, glucose-stimulated insulin secretion; KRBH, Krebs-Ringer bicarbonate Hepes buffer; RT, reverse transcription; siRNA, small interfering RNA; SLC25, solute carrier family 25; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

[1] To whom correspondence should be addressed (email fujimoto@metab.kuhp.kyoto-u.ac.jp).

© The Authors Journal compilation © 2011 Biochemical Society

Biochemical Journal