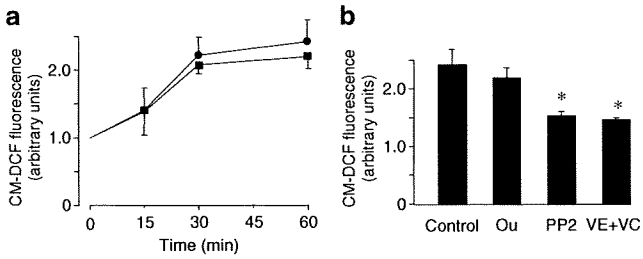

Fig. 8 Effects of ouabain, Src inhibitor and ROS scavenger on ROS production at high glucose in GK islet cells. **a** Effect of 1 mmol/l ouabain on the time-course of high glucose-induced increase of ROS production. After fluorescence measurements at time zero, the dispersed islet cells were incubated for the indicated times with (squares) or without (circles) 1 mmol/l ouabain in the presence of 16.7 mmol/l glucose under Ca^{2+}-depleted conditions. Values are means±SE (n=3) as a ratio of values at time zero. **b** Effects of 1 mmol/l ouabain (Ou), 10 μmol/l PP2 and 100 μmol/l α-tocopherol plus 200 μmol/l ascorbate (VE + VC) on ROS production in the presence of 16.7 mmol/l glucose in GK islet cells. After CM-DCF fluorescence was determined at time zero, islet cells were incubated for 60 min with 16.7 mmol/l glucose in the presence or absence of test materials under Ca^{2+}-depleted conditions, and fluorescence was measured at 60 min. Values are means±SE (n=3) as a ratio of values at time zero. *p<0.05 vs 16.7 mmol/l glucose

glucose may be attributable to $\Delta\Psi_m$-dependence of ROS formation, in which an exponential increase in ROS production is observed above 140 mV in mitochondrial membrane potential [28]. However, in the present study, we show for the first time that there is an increase in mitochondrial ROS production via intracellular signal transduction in pancreatic islets. Thus, ROS production via the signal-transducing function of Na^+/K^+-ATPase does not necessarily require hyperpolarisation of mitochondrial membrane potential, as ouabain increases ROS production while the agent simultaneously inhibits hyperpolarisation of mitochondrial membrane potential.

Src is a 60 kDa membrane-associated non-receptor tyrosine kinase that regulates various signal transduction pathways. Src production is widespread and has been demonstrated in pancreatic islets and in a beta cell line [29–32]. Its catalytic activity is controlled by tyrosine phosphorylation and protein–protein interaction. Phosphorylation of Tyr^{529} on Src holds the kinase in an inactive conformation through an intramolecular interaction with its Src homology 2 domain, whereas phosphorylation of Tyr^{418} activates Src by disrupting the intramolecular interaction and creating the substrate-binding site [33]. The binding of ouabain to the Na^+/K^+-ATPase causes rapid activation of Src in various cells including cardiac myocytes [34], smooth muscle cells [34, 35] and kidney epithelial cells [36] independently of the changes in intracellular ion concentrations. In the present study, ouabain stimulated Tyr^{418} phosphorylation but had no effect on Tyr^{529} phosphorylation, a phenomenon also observed in different types of cells [36]. Since ouabain-induced direct interaction between the Na^+/K^+-ATPase α_1 subunit and Src is observed in kidney epithelial cells [36], ouabain-induced direct interaction between Na^+/K^+-ATPase and Src may well be involved in ouabain-induced Src phosphorylation in pancreatic beta cells.

A signal-transducing function of Na^+/K^+-ATPase via Src activation has been proposed recently in different types of cells including cardiac myocytes, A7r5 cells and HeLa cells [37]. The binding of ouabain to Na^+/K^+-ATPase activates Src, resulting in transactivation of the EGF receptor and increased mitochondrial production of ROS independently of changes in intracellular ion concentrations. In the present study, PP2, a specific Src inhibitor that reduces Src kinase activity and Tyr^{418} phosphorylation in rat islets [32], was found to decrease ouabain-induced ROS production, indicating that this signal-transducing function of Na^+/K^+-ATPase plays a role in regulating mitochondrial ROS production in islets. However, the involvement of the transactivation of the EGF receptor in this pathway in islets remains unknown.

In a previous study, we found that ouabain reduces not only the increment in ATP content and the hyperpolarisation of mitochondrial membrane potential by glucose, but also the increment in O_2 consumption by glucose [17]. Since increased O_2 consumption occurs in uncoupling [38], ouabain-induced suppression of mitochondrial ATP production clearly is not mediated by uncoupling, and the suppression may derive from direct or indirect effects on the respiratory chain. Ouabain (1 mmol/l) was found to reduce glucose oxidation in the presence of 16.7 mmol/l glucose in islets in medium containing a physiological level of Ca^{2+} [39]. In the present study, 1 mmol/l ouabain also suppressed glucose oxidation in the presence of 16.7 mmol/l glucose in Ca^{2+}-depleted conditions. Since ouabain-induced suppression of glucose oxidation was restored by ROS scavenger and by Src inhibitor, increased ROS production derived from Src activation may well suppress mitochondrial metabolism in the Krebs cycle, in which CO_2 is released in the reaction mediated by dehydrogenases. This is supported by the fact that administration of 50 μmol/l H_2O_2, a concentration nearly equivalent to the 1 mmol/l ouabain-induced increase in ROS production [17], to mitochondria reduced activity of Krebs cycle enzymes including aconitase, α-ketoglutarate dehydrogenase and succinate dehydrogenase, whose activities declined 96%, 39% and 37%, respectively [40]. Considered together, these findings suggest that ouabain-induced mitochondrial ROS suppresses mitochondrial metabolism in the Krebs cycle, subsequently reducing NADH supply to the respiratory chain, hyperpolarisation of mitochondrial membrane potential, O_2 consumption and ATP production.

We then investigated the role of ROS generated by Src activation in impaired glucose-induced insulin secretion in

diabetes. One of the characteristics of type 2 diabetes is that the insulin secretory response of beta cells to glucose is selectively impaired [41]. In the GK rat, a genetic model of type 2 diabetes mellitus [42], glucose-induced insulin secretion is selectively impaired [43]. On single-channel recording, the glucose sensitivity of the beta cell K_{ATP} channel is remarkably reduced in GK rats, while the inhibitory effect of ATP on channel activity is not significantly different in control and GK rats [5]. The intracellular ATP elevation induced by high glucose is impaired in GK rats [44] as well as in patients with type 2 diabetes [45]. Thus, the impaired insulinotrophic action of glucose in beta cells of GK rats may be attributable to insufficient closure of the K_{ATP} channel because of deficient ATP production derived from impaired glucose metabolism. While there is evidence that islets in GK rats (a diabetes model) and human type 2 diabetes are oxidatively stressed [46, 47], the association between oxidative stress and impaired intracellular ATP elevation in islets is unclear. In the present study, both Src inhibitor and ROS scavenger restored the impairment in high glucose-induced insulin release and ATP elevation in GK islets but had no such effects in control islets. Moreover, Src inhibitor reduced the high glucose-induced increase in ROS generation in GK islet cells but had no effect on that in control islet cells. Ouabain had no effect on ATP content and ROS production in the presence of high glucose despite the prominent recovery effect of Src inhibitor in GK islets, suggesting that Src is endogenously activated independently of ouabain. Taken together, these results indicate that ROS generated by Src activation plays an important role in impaired glucose-induced insulin secretion derived from impaired glucose metabolism in GK islets.

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References

1. Maechler P, Wollheim CB (2001) Mitochondrial function in normal and diabetic β-cells. Nature 414:807–812
2. Kennedy ED, Maechler P, Wollheim CB (1998) Effects of depletion of mitochondrial DNA in metabolism secretion coupling in INS-1 cells. Diabetes 47:374–380
3. Tsuruzoe K, Araki E, Furukawa N et al (1998) Creation and characterization of a mitochondrial DNA-depleted pancreatic β-cell line: impaired insulin secretion induced by glucose, leucine, and sulfonylureas. Diabetes 47:621–631
4. Takehiro M, Fujimoto S, Shimodahira M et al (2005) Chronic exposure to β-hydroxybutyrate inhibits glucose-induced insulin release from pancreatic islets by decreasing NADH contents. Am J Physiol 288:E372–E380
5. Tsuura Y, Ishida H, Okamoto Y et al (1993) Glucose sensitivity of ATP-sensitive K^+ channels is impaired in β-cells of the GK rat. A new genetic model of NIDDM. Diabetes 42:1446–1453
6. Hughes SJ, Faehling M, Thorneley CW, Proks P, Ashcroft FM, Smith PA (1998) Electrophysiological and metabolic characterization of single β-cells and islets from diabetic GK rats. Diabetes 47:73–81
7. Anello M, Lupi R, Spampinato D et al (2005) Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients. Diabetologia 48:282–289
8. Nabe K, Fujimoto S, Shimodahira M et al (2006) Diphenylhydantoin suppresses glucose-induced insulin release by decreasing cytoplasmic H^+ concentration in pancreatic islets. Endocrinology 147:2717–2727
9. Radu RG, Fujimoto S, Mukai E et al (2005) Tacrolimus suppresses glucose-induced insulin release from pancreatic islets by reducing glucokinase activity. Am J Physiol 288: E365–E371
10. Patane G, Anello M, Piro S, Vigneri R, Purrello F, Rabuazzo AM (2002) Role of ATP production and uncoupling protein-2 in the insulin secretory defect induced by chronic exposure to high glucose or free fatty acids and effects of peroxisome proliferator-activated receptor-γ inhibition. Diabetes 51:2749–2756
11. Joseph JW, Koshkin V, Saleh MC et al (2004) Free fatty acid-induced β-cell defects are dependent on uncoupling protein 2 expression. J Biol Chem 279:51049–51056
12. Maechler P, Jornot L, Wollheim CB (1999) Hydrogen peroxide alters mitochondrial activation and insulin secretion in pancreatic beta cells. J Biol Chem 274:27905–27913
13. Krippeit-Drews P, Kramer C, Welker S, Lang F, Ammon HP, Drews G (1999) Interference of H_2O_2 with stimulus-secretion coupling in mouse pancreatic β-cells. J Physiol 514:471–481
14. Bindokas VP, Kuznetsov A, Sreenan S, Polonsky KS, Roe MW, Philipson LH (2003) Visualizing superoxide production in normal and diabetic rat islets of Langerhans. J Biol Chem 278:9796–9801
15. Sakai K, Matsumoto K, Nishikawa T et al (2003) Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic β-cells. Biochem Biophys Res Commun 300:216–222
16. Lambert AE, Henquin JC, Malvaux P (1974) Cationic environment and dynamics of insulin secretion. IV. Effect of ouabain. Horm Metab Res 6:470–475
17. Kajikawa M, Fujimoto S, Tsuura Y et al (2002) Ouabain suppresses glucose-induced mitochondrial ATP production and insulin release by generating reactive oxygen species in pancreatic islets. Diabetes 51:2522–2529
18. Kometiani P, Li J, Gnudi L, Kahn BB, Askari A, Xie Z (1998) Multiple signal transduction pathways link Na^+/K^+-ATPase to growth-related genes in cardiac myocytes. J Biol Chem 273:15249–15256
19. Xie Z, Kometiani P, Liu J, Li J, Shapiro JI, Askari A (1999) Intracellular reactive oxygen species mediate the linkage of Na^+/K^+-ATPase to hypertrophy and its marker genes in cardiac myocytes. J Biol Chem 274:19323–19328
20. Liu J, Tian J, Haas M, Shapiro JI, Askari A, Xie Z (2000) Ouabain interaction with cardiac Na^+/K^+-ATPase initiates signal cascades independent of changes in intracellular Na^+ and Ca^{2+} concentrations. J Biol Chem 275:27838–27844
21. Fujimoto S, Ishida H, Kato S et al (1998) The novel insulinotropic mechanism of pimobendan: direct enhancement of the exocytotic process of insulin secretory granules by increased Ca^{2+} sensitivity in β-cells. Endocrinology 139:1133–1140

22. Fujimoto S, Tsuura Y, Ishida H et al (2000) Augmentation of basal insulin release from rat islets by preexposure to a high concentration of glucose. Am J Physiol 279:E927–E940
23. Kaneto H, Xu G, Song KH et al (2001) Activation of the hexosamine pathway leads to deterioration of pancreatic β-cell function through the induction of oxidative stress. J Biol Chem 276:31099–31104
24. Takahashi H, Tran PO, LeRoy E, Harmon JS, Tanaka Y, Robertson RP (2004) D-Glyceraldehyde causes production of intracellular peroxide in pancreatic islets, oxidative stress, and defective beta cell function via non-mitochondrial pathways. J Biol Chem 279:37316–37323
25. Tsubouchi H, Inoguchi T, Inuo M et al (2005) Sulfonylurea as well as elevated glucose levels stimulate reactive oxygen species production in the pancreatic β-cell line, MIN6—a role of NAD(P)H oxidase in β-cells. Biochem Biophys Res Commun 326:60–65
26. Yu BP (1994) Cellular defenses against damage from reactive oxygen species. Physiol Rev 74:139–162
27. Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552:335–344
28. Kadenbach B (2003) Intrinsic and extrinsic uncoupling of oxidative phosphorylation. Biochim Biophys Acta 1604:77–94
29. Ohnishi M, Tokuda M, Masaki T et al (1994) Changes in annexin I and II levels during the postnatal development of rat pancreatic islets. J Cell Sci 107:2117–2125
30. Tejedo JR, Ramirez R, Cahuana GM, Rincon P, Sobrino F, Bedoya FJ (2001) Evidence for involvement of c-Src in the anti-apoptotic action of nitric oxide in serum-deprived RINm5F cells. Cell Signal 13:809–817
31. Tejedo JR, Cahuana GM, Ramirez R et al (2004) Nitric oxide triggers the phosphatidylinositol 3-kinase/Akt survival pathway in insulin-producing RINm5F cells by arousing Src to activate insulin receptor substrate-1. Endocrinology 145:2319–2327
32. Cheng H, Straub SG, Sharp GW (2007) Inhibitory role of Src family tyrosine kinases on Ca^{2+}-dependent insulin release. Am J Physiol 292:E845–E852
33. Roskoski R Jr (2004) Src protein-tyrosine kinase structure and regulation. Biochem Biophys Res Commun 324:1155–1164
34. Haas M, Askari A, Xie Z (2000) Involvement of Src and epidermal growth factor receptor in the signal-transducing function of Na^+/K^+-ATPase. J Biol Chem 275:27832–27837
35. Aydemir-Koksoy A, Abramowitz J, Allen JC (2001) Ouabain-induced signaling and vascular smooth muscle cell proliferation. J Biol Chem 276:46605–46611
36. Haas M, Wang H, Tian J, Xie Z (2002) Src-mediated inter-receptor cross-talk between the Na^+/K^+-ATPase and the epidermal growth factor receptor relays the signal from ouabain to mitogen-activated protein kinases. J Biol Chem 277:18694–18702
37. Xie Z, Cai T (2003) Na^+-K^+-ATPase-mediated signal transduction: from protein interaction to cellular function. Mol Interv 3:157–168
38. Hutton JC, Malaisse WJ (1980) Dynamics of O_2 consumption in rat pancreatic islets. Diabetologia 18:395–405
39. Sener A, Malaisse WJ (1991) Hexose metabolism in pancreatic islets. Regulation of D-[6–^{14}C]glucose oxidation by non-nutrient secretagogues. Mol Cell Endocrinol 76:1–6
40. Nulton-Persson AC, Szweda LI (2001) Modulation of mitochondrial function by hydrogen peroxide. J Biol Chem 276:23357–23361
41. Leahy JL, Bonner-Weir S, Weir GC (1992) Beta-cell dysfunction induced by chronic hyperglycemia. Current ideas on mechanism of impaired glucose-induced insulin secretion. Diabetes Care 15:442–455
42. Kimura K, Toyota T, Kakizaki M, Kudo M, Takebe K, Goto Y (1982) Impaired insulin secretion in the spontaneous diabetes rats. Tohoku J Exp Med 137:453–459
43. Portha B, Serradas P, Bailbe D, Suzuki K, Goto Y, Giroix MH (1991) β-cell insensitivity to glucose in the GK rat, a spontaneous nonobese model for type II diabetes. Diabetes 40:486–491
44. Hughes SJ, Faehling M, Thorneley CW, Proks P, Ashcroft FM, Smith PA (1998) Electrophysiological and metabolic characterization of single β-cells and islets from diabetic GK rats. Diabetes 47:73–81
45. Anello M, Lupi R, Spampinato D et al (2005) Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients. Diabetologia 48:282–289
46. Ihara Y, Toyokuni S, Uchida K et al (1999) Hyperglycemia causes oxidative stress in pancreatic β-cells of GK rats, a model of type 2 diabetes. Diabetes 48:927–932
47. Sakuraba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S (2002) Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese type II diabetic patients. Diabetologia 45:85–96

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GLP-1 receptor antagonist as a potential probe for pancreatic β-cell imaging

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ABSTRACT

We examined exendin(9-39), an antagonist of glucagon-like peptide-1 (GLP-1) receptor (GLP-1R), as a potential probe for imaging of pancreatic β-cells. To evaluate *in vitro* receptor specificity, binding assay was performed using dispersed mouse islet cells. Binding assay showed competitive inhibition of [^{125}I]BH-exendin(9-39) binding by non-radioactive exendin(9-39). To assess *in vivo* selectivity, the biodistribution was evaluated by intravenous administration of [^{125}I]BH-exendin(9-39) to mice. Radioactivity of harvested pancreas reached highest levels at 60 and 120 min among organs examined except lung. Pre-administration of excess non-radioactive exendin(9-39) remarkably and specifically blocked the radioactivity of pancreas. After [^{125}I]BH-exendin(9-39) injection into transgenic mice with pancreatic β-cells expressing GFP, fluorescent and radioactive signals of sections of pancreas were evaluated with an image analyzer. Imaging analysis showed that the fluorescent GFP signals and the radioactive signals were correspondingly located. Thus, the GLP-1R antagonist exendin(9-39) may serve as a useful probe for pancreatic β-cell imaging.

Introduction

Type 1 diabetes is an autoimmune disease in which the pancreatic β-cells are almost destroyed, which leads to loss of endogenous insulin secretion. Insulin therapy is therefore required for survival in subjects with type 1 diabetes [1]. Type 2 diabetes is characterized by impaired insulin secretion and insulin resistance, and the pathogenesis is well known to be dependent on a reduction in β-cell function [2]. While a decrease in β-cell mass has been reported in American and Asian type 2 diabetic subjects compared with non-diabetic subjects [3–5], the decrease is small at onset in European subjects, suggesting that the decrease might occur only after onset of the disease [6]. It is therefore unknown whether a decrease in β-cell mass contributes to the development of hyperglycemia that leads to type 2 diabetes. Thus, accurately measuring changes in β-cell mass *in vivo* during diabetes progression is important not only for understanding the pathogenesis but also for facilitating early diagnosis and developing improved treatments for both type 1 and type 2 diabetes.

However, it is difficult to identify islets ranging in size from 50–500 μm in diameter and scattered throughout the pancreas, which is surrounded by abdominal organs. To quantify β-cell mass non-invasively, appropriate probes that can specifically bind to pancreatic β-cells are required. There are previous reports using probes targeting the proteins in β-cells, including sulfonylurea receptor 1 (SUR1) and monoamine transporter 2 (VMAT2) for positron emission tomography (PET) imaging [7]. However, ideal probes for accurate and non-invasive imaging for pancreatic β-cells have not yet been developed.

Glucagon-like peptide 1 (GLP-1) is the incretin peptide released from the intestine in response to nutrient ingestion to augment glucose-induced insulin secretion from pancreatic β-cells through binding to the GLP-1 receptor (GLP-1R) [8,9]. Since GLP-1R is expressed highly in islets, especially on β-cells in pancreas, the ligands of GLP-1R might well be ideal probes for pancreatic β-cell imaging. Because native GLP-1 is degraded rapidly by dipeptidyl peptidase-IV (DPP-IV) distributed throughout the body, DPP-IV-resistant agonistic or antagonistic ligands of GLP-1R [10,11] are preferable to GLP-1 for use as an imaging probe.

In the present study, specific imaging of pancreatic β-cells targeting GLP-1R was evaluated using its antagonist, exendin(9-39), radiolabeled with [^{125}I]-Bolton-Hunter reagent at lysine residues.

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Materials and methods

Radiolabeling of exendin(9-39). [^{125}I]-Bolton-Hunter-labeled exendin(9-39) ([^{125}I]-BH exendin(9-39)) was purchased from Perkin-Elmer (Waltham, MA).

Animals. Six-week-old male ddY mice were obtained from Shimizu Co. (Kyoto, Japan). Transgenic mice expressing green fluorescent protein (GFP) under control of the mouse insulin I gene promoter (MIP) (MIP-GFP mice) were maintained on a C57BL/6 background [12]. Animal care and procedures were approved by the Animal Care Committee of Kyoto University.

Binding assay. The displacing effect of exendin(9-39) on GLP-1R binding was assessed using dispersed islet cells as described previously [13]. Pancreatic islets were isolated from male ddY mice by a collagenase digestion technique [14]. Isolated islets were dispersed using 0.05% trypsin/0.53 mM EDTA (Invitrogen, Carlsbad, CA) and PBS. Islet cells were incubated with [^{125}I]BH-exendin(9-39) (0.1 μCi) in 1 ml of buffer containing 20 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 1 mg/ml bacitracin, and 1 mg/ml BSA for 1 h at room temperature in the presence of varying concentrations of non-radioactive exendin(9-39). Binding was terminated by rapid filtration through Whatman GF/C filters (24 mm) followed by washing three times with 5 ml of ice-cold PBS. The radioactivity of filters was measured in a γ-counter. Results were expressed as the percent radioactivity of bound [^{125}I]BH-exendin(9-39) that remained after addition of non-radioactive compound.

Biodistribution experiments. Biodistribution studies of [^{125}I]BH-exendin(9-39) were performed in male ddY mice. [^{125}I]BH-exendin(9-39) (1 μCi) was administered by tail vein injection. At 15, 30, 60, and 120 min after administration, the mice were sacrificed by exsanguination under anesthesia. Selected organs and blood

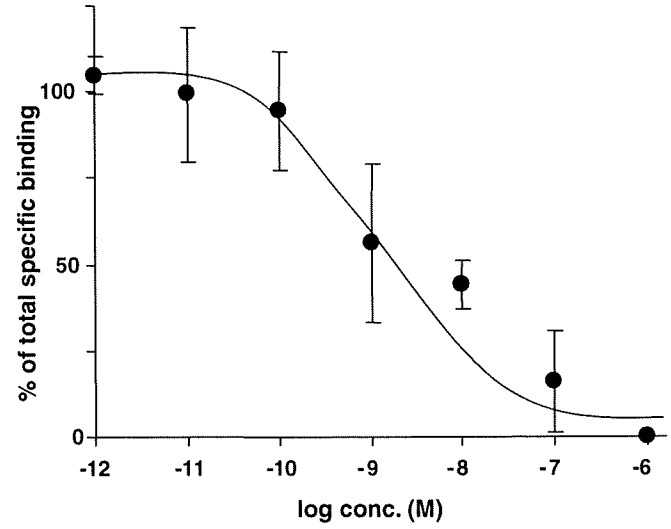

Fig. 1. Binding assay analysis of [^{125}I]BH-exendin(9-39) using mouse pancreatic islet cells. Competitive inhibition of [^{125}I]BH-exendin(9-39) binding by non-radioactive exendin(9-39) is shown. Values are expressed as means ± SD of the percent radioactivity of bound [^{125}I]BH-exendin(9-39) that remained after addition of indicated concentrations of non-radioactive exendin(9-39) ($n = 4$).

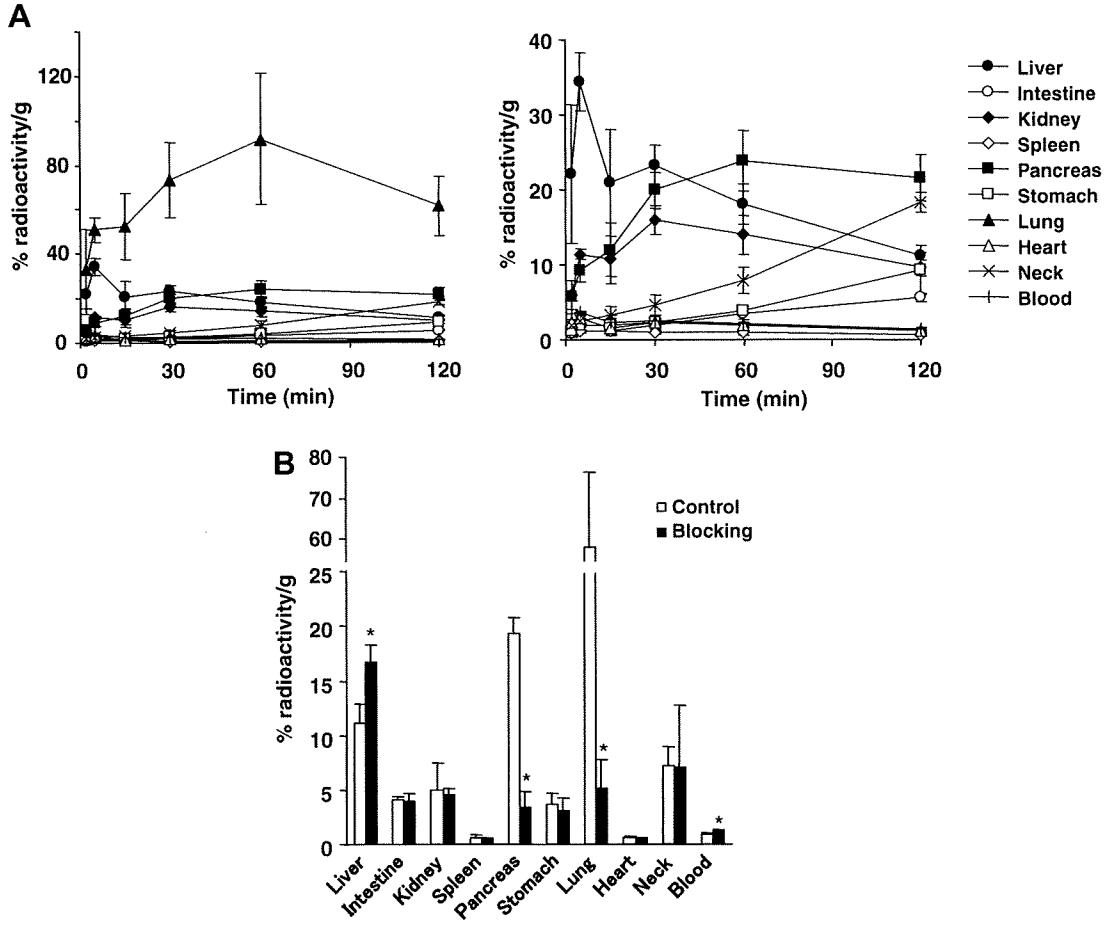

Fig. 2. Tissue distribution of [^{125}I]BH-exendin(9-39) in mice. (A) Time course of tissue distribution of [^{125}I]BH-exendin(9-39). Right graph shows tissue distribution without lung. (B) Blocking of tissue distribution at 120 min after [^{125}I]BH-exendin(9-39) injection by pre-administration of excess non-radioactive exendin(9-39). Values are expressed as means ± SD of the percent radioactivity of injected [^{125}I]BH-exendin(9-39) per gram of organ weight ($n = 5$). $^{*}P < 0.001$ vs. control.

were harvested and weighed, and the radioactivities were measured with a γ-counter. In a blocking study, excess non-radioactive exendin(9-39) (50 μg) in 100 μl of saline was administered 30 min before the [^{125}I]BH-exendin(9-39) injection. Results were expressed as percent radioactivity of injected [^{125}I]BH-exendin(9-39) per gram of organ weight.

Two-dimensional imaging analysis. After intravenous administration of [^{125}I]BH-exendin(9-39) to male MIP-GFP mice, the pancreas was harvested and cut in several pieces. Each piece was put on slide glass and pressed with a cover glass. Signals of fluorescence and radioactivity (autoradiography) of sections of pancreas were evaluated with an image analyzer (Typhoon 9410; GE Healthcare, Buckinghamshire, UK). The fluorescent and radioactive intensity of each section was analyzed with ImageQuant TL software with resolution of 25 and 10 μm per pixel, respectively (GE Healthcare).

Statistical analysis. Data are expressed as means ± SD. Statistical significance of difference was evaluated by unpaired alternate Welch *t* test. $P < 0.05$ was considered significant.

Results and discussion

We first examined binding specificity of exendin(9-39) to pancreatic β-cell membrane *in vitro*. Binding assay analysis using mouse pancreatic islet cells showed competitive inhibition of [^{125}I]BH-exendin(9-39) binding by non-radioactive exendin(9-39) with a logIC_{50} of −8.84 ± 0.18, similarly to the findings in a previous report [15], indicating that exendin(9-39) binds to those cells specifically (Fig. 1).

To examine selectivity of exendin(9-39) to pancreas *in vivo*, we performed biodistribution studies in mice. Radioactivities of selected organs were measured 15, 30, 60, and 120 min after intravenous administration of [^{125}I]BH-exendin(9-39) (1 μCi). The radioactivity of lung was highest at each time point (Fig. 2A, left panel). Radioactivity of pancreas increased with time and was highest at 60 and 120 min among organs examined excepting lung, and rapid and high binding by liver was observed (Fig. 2A, right panel). To determine whether the binding was specific, we performed blocking study. Pre-administration of excess non-radioactive exendin(9-39) (50 μg) significantly blocked the radioactivities of pancreas and lung to 17.8% and 8.8% of control, respectively, 120 min after [^{125}I]BH-exendin(9-39) injection (Fig. 2B), demonstrating that [^{125}I]BH-exendin(9-39) specifically binds to its receptor in these organs. The binding in other organs such as liver was not blocked by excess non-radioactive exendin(9-39).

To confirm high binding of exendin(9-39) in β-cells, we performed [^{125}I]BH-exendin(9-39) injection in MIP-GFP mice specifically expressing GFP in pancreatic β-cells and imaging analysis of sections of the pancreas removed 60 or 120 min after [^{125}I]BH-exendin(9-39) injection. As shown in Fig. 3A, fluorescent GFP signals were observed in the pancreatic sections of MIP-GFP mice with an image analyzer. Localization of the detected radioactive signals corresponded well to that of the GFP signals, indicating specific high binding of exendin(9-39) in pancreatic β-cells. The intensity of the fluorescent signals of each section also correlated with that of the radioactive signals (Fig. 3B).

Studies for detecting β-cell mass have been performed using probes targeting various β-cell-specific molecules, among which GLP-1R appears promising [7]. *In vivo* imaging of GLP-1R-positive tissues using diethylenetriaminepentaacetic acid (DTPA)-conjugated exendin-4, the GLP-1R agonist, was recently reported [16]. The biodistribution examinations showed its specific binding not only to pancreas and lung but also to stomach. Although pancreas and lung as well as pituitary and adrenals were detected in single photon emission computed tomography (SPECT) imaging, it was not determined whether the probe was confined to β-cells due to the low resolution of the imaging apparatus. In the present study, we found that exendin(9-39), an antagonistic ligand of GLP-1R, has high specificity not only to pancreas but also to β-cells in pancreas, suggesting that β-cell mass can be evaluated. High binding of the probe in lung, as previously reported [16], does not affect analysis of islets because lung is an extra-abdominal organ, for which imaging such as SPECT is required.

A better understanding of the relationship between β-cell mass, β-cell function, and glucose homeostasis by precise measurement of β-cell mass should provide important information on not only for early diagnosis and treatment but also for development of new therapies for intervention strategies. Several tests are presently available for evaluation of β-cell function [17]. In contrast,

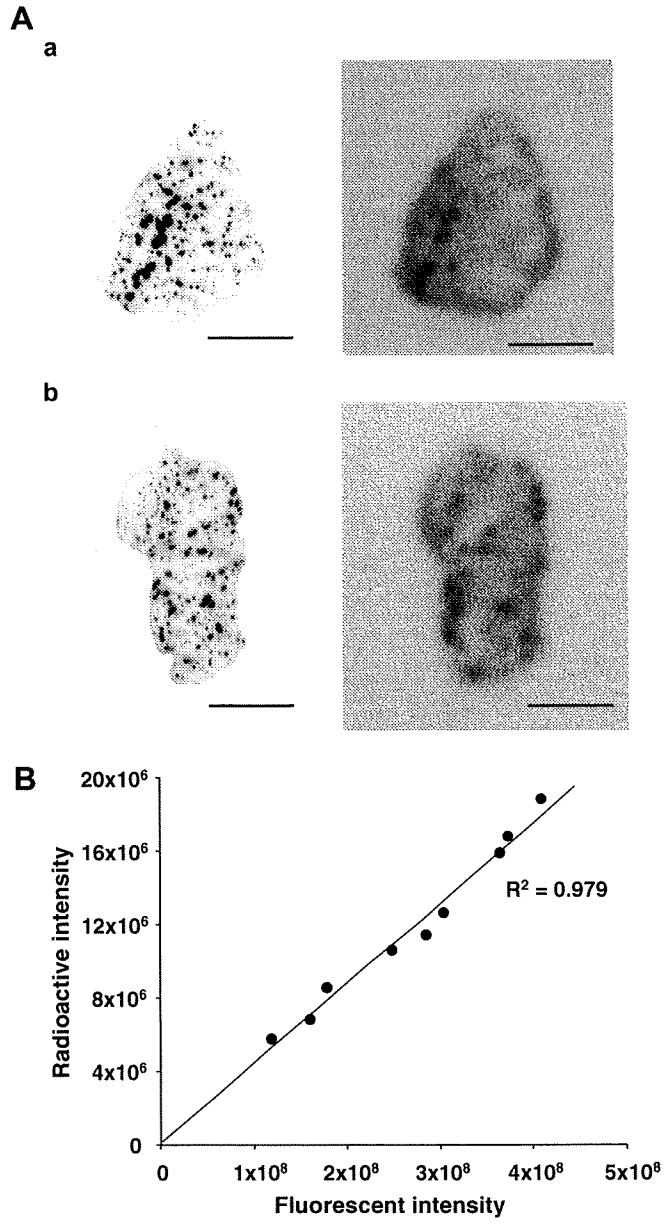

Fig. 3. Imaging analysis of pancreas sections of [^{125}I]BH-exendin(9-39)-injected MIP-GFP mice. (A) Representative fluorescent signals (left panels) and radioactive signals (right panels) of pancreas sections at 60 min (a) and 120 min (b) after [^{125}I]BH-exendin(9-39) injection. Bars represent 1 cm. (B) Correlation of the fluorescent and radioactive intensity. The signal intensity in whole area of each of the nine sections of pancreas harvested 120 min after [^{125}I]BH-exendin(9-39) injection was analyzed with ImageQuant TL software.

measurement of β-cell mass is presently possible only by autopsy. It has recently been reported that antagonistic probes as well as agonistic probes are useful for molecular imaging by targeting peptide receptors [18]. In the present study, we demonstrate for the first time that the GLP-1R antagonist exendin(9-39) is a potential probe for the imaging of pancreatic β-cells.

Acknowledgments

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References

[1] W.V. Tamborlane, W. Bonfig, E. Boland, Recent advances in treatment of youth with type 1 diabetes: better care through technology, Diabet. Med. 18 (2001) 864–870.
[2] L. Groop, Pathogenesis of type 2 diabetes: the relative contribution of insulin resistance and impaired insulin secretion, Int. J. Clin. Pract. Suppl. (2000) 3–13.
[3] H. Sakuraba, H. Mizukami, N. Yagihashi, R. Wada, C. Hanyu, S. Yagihashi, Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese type II diabetic patients, Diabetologia 45 (2002) 85–96.
[4] A.E. Butler, J. Janson, S. Bonner-Weir, R. Ritzel, R.A. Rizza, P.C. Butler, Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes, Diabetes 52 (2003) 102–110.
[5] K.H. Yoon, S.H. Ko, J.H. Cho, J.M. Lee, Y.B. Ahn, K.H. Song, S.J. Yoo, M.I. Kang, B.Y. Cha, K.W. Lee, H.Y. Son, S.K. Kang, H.S. Kim, I.K. Lee, S. Bonner-Weir, Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea, J. Clin. Endocrinol. Metab. 88 (2003) 2300–2308.
[6] J. Rahier, Y. Guiot, R.M. Goebbels, C. Sempoux, J.C. Henquin, Pancreatic beta-cell mass in European subjects with type 2 diabetes, Diabetes Obes. Metab. 10 (Suppl. 4) (2008) 32–42.
[7] S. Schneider, Efforts to develop methods for in vivo evaluation of the native beta-cell mass, Diabetes Obes. Metab. 10 (Suppl. 4) (2008) 109–118.
[8] L.L. Baggio, D.J. Drucker, Biology of incretins: GLP-1 and GIP, Gastroenterology 132 (2007) 2131–2157.
[9] J.J. Holst, The physiology of glucagon-like peptide 1, Physiol. Rev. 87 (2007) 1409–1439.
[10] J. Eng, W.A. Kleinman, L. Singh, G. Singh, J.P. Raufman, Isolation and characterization of exendin-4, an exendin-3 analogue, from *Heloderma suspectum* venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas, J. Biol. Chem. 267 (1992) 7402–7405.
[11] R. Goke, H.C. Fehmann, T. Linn, H. Schmidt, M. Krause, J. Eng, B. Goke, Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells, J. Biol. Chem. 268 (1993) 19650–19655.
[12] M. Hara, X. Wang, T. Kawamura, V.P. Bindokas, R.F. Dizon, S.Y. Alcoser, M.A. Magnuson, G.I. Bell, Transgenic mice with green fluorescent protein-labeled pancreatic beta-cells, Am. J. Physiol. Endocrinol. Metab. 284 (2003) E177–E183.
[13] E. Mukai, H. Ishida, S. Kato, Y. Tsuura, S. Fujimoto, A. Ishida-Takahashi, M. Horie, K. Tsuda, Y. Seino, Metabolic inhibition impairs ATP-sensitive K+ channel block by sulfonylurea in pancreatic beta-cells, Am. J. Physiol. 274 (1998) E38–E44.
[14] R. Sutton, M. Peters, P. McShane, D.W. Gray, P.J. Morris, Isolation of rat pancreatic islets by ductal injection of collagenase, Transplantation 42 (1986) 689–691.
[15] S. Al-Sabah, D. Donnelly, A model for receptor-peptide binding at the glucagon-like peptide-1 (GLP-1) receptor through the analysis of truncated ligands and receptors, Br. J. Pharmacol. 140 (2003) 339–346.
[16] M. Gotthardt, G. Lalyko, J. van Eerd-Vismale, B. Keil, T. Schurrat, M. Hower, P. Laverman, T.M. Behr, O.C. Boerman, B. Goke, M. Behe, A new technique for in vivo imaging of specific GLP-1 binding sites: first results in small rodents, Regul. Pept. 137 (2006) 162–167.
[17] S.E. Kahn, D.B. Carr, M.V. Faulenbach, K.M. Utzschneider, An examination of beta-cell function measures and their potential use for estimating beta-cell mass, Diabetes Obes. Metab. 10 (Suppl. 4) (2008) 63–76.
[18] M. Schottelius, H.J. Wester, Molecular imaging targeting peptide receptors, Methods 48 (2009) 161–177.

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Effects of long-term dipeptidyl peptidase-IV inhibition on body composition and glucose tolerance in high fat diet-fed mice

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ABSTRACT

Aim: Glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are major incretins associated with body weight regulation. Dipeptidyl peptidase-IV (DPP-IV) inhibitor increases plasma active GLP-1 and GIP. However, the magnitude of the effects of enhanced GLP-1 and GIP signaling by long-term DPP-IV inhibition on body weight and insulin secretion has not been determined. In this study, we compared the effects of long-term DPP-IV inhibition on body composition and insulin secretion of high fat diet (HFD)-fed wild-type (WT) and GLP-1R knockout (*GLP-1R*$^{-/-}$) mice.
Main methods: HFD-fed WT and *GLP-1R*$^{-/-}$ mice were treated with or without DPP-IV inhibitor by drinking water. Food and water intake and body weight were measured during 8 weeks of study. CT-based body composition analysis, Oral glucose tolerance test (OGTT), batch incubation study for insulin secretion and quantitative RT-PCR for expression of incretin receptors in isolated islets were performed at the end of study.
Key findings: DPP-IV inhibitor had no effect on food and water intake and body weight, but increased body fat mass in *GLP-1R*$^{-/-}$ mice. DPP-IV inhibitor-treated WT and *GLP-1R*$^{-/-}$ mice both showed increased insulin secretion in OGTT. In isolated islets of DPP-IV inhibitor-treated WT and *GLP-1R*$^{-/-}$ mice, glucose-induced insulin secretion was increased and insulin secretion in response to GLP-1 or GIP was preserved, without downregulation of incretin receptor expression.
Significance: Long-term DPP-IV inhibition may maintain body composition through counteracting effects of GLP-1 and GIP while improving glucose tolerance by increasing glucose-induced insulin secretion through the synergistic effects of GLP-1 and GIP.

Introduction

Oral glucose administration leads to much greater insulin release than the equivalent intravenous glucose challenge. Gut hormonal substances released in response to glucose include the incretins glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide /glucose-dependent insulinotropic peptide (GIP), which are responsible for ~50% of postprandial insulin release. GLP-1 and GIP potentiate glucose-induced insulin secretion from pancreatic β-cells by binding their respective receptors and subsequently increasing the intracellular cAMP concentration. In addition to their action on the enteroinsular axis, GLP-1 inhibits glucagon secretion (Komatsu et al. 1989), delays gastric emptying (Willms et al. 1996), decreases body weight through suppression of appetite (Turton et al. 1996), and suppresses β-cell apoptosis (Toyoda et al. 2008), while GIP enhances energy storage in adipocytes (Miyawaki et al. 2002) and calcium accumulation in bone (Tsukiyama et al. 2006). Thus, the incretins are associated with various systems of metabolic homeostasis, including that of both glucose and body weight.

However, the effects of GLP-1 and GIP are limited by their short half-life of a few minutes, which is primarily due to the action of dipeptidyl peptidase-IV (DPP-IV). DPP-IV is an enzyme distributed throughout the body including plasma and the endothelial lining of several organs, and cleaves two amino acids of biologically active peptides including GLP-1 and GIP by recognizing proline or alanine in the second N-terminal amino acid. The resulting N-terminal-truncated forms of GLP-1 and GIP are devoid of bioactivity. Since DPP-IV-deficient rodents show improved glucose tolerance and increased insulin secretion with elevated plasma active GLP-1 levels after oral glucose loading (Marguet et al. 2000; Nagakura et al. 2001), DPP-IV inhibitor and DPP-IV-resistant GLP-1

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receptor agonist are potential targets for the treatment of type 2 diabetes mellitus as a new class of antidiabetic agent. GLP-1 receptor agonist both increases insulin secretion and improves glucose tolerance and decreases body weight in rodents and humans (Szayna et al. 2000; Buse et al. 2004). DPP-IV inhibitor also increases insulin secretion and improves glucose tolerance, but its effect on body weight is controversial (Pospisilik et al. 2002; Lamont and Drucker 2008; Reimer et al. 2002; Ahrén et al. 2002). It is reported that DPP-IV inhibitor do not increase insulin secretion after glucose loading in GLP-1 receptor (GLP-1R)/GIP receptor (GIPR) double knockout (DIRKO) mice, indicating that both GLP-1 and GIP are critically involved in the insulinotropic action of long-term DPP-IV inhibition (Flock et al. 2007). However, the magnitude of the effects of enhanced GLP-1 and GIP signaling by long-term DPP-IV inhibition on body weight and insulin secretion has not been determined.

In the present study, we investigated the long-term effects of DPP-IV inhibition on body composition and insulin secretion using high fat diet (HFD)-fed wild-type (WT) and GLP-1R knockout (*GLP-1R*$^{-/-}$) mice.

Materials and methods

Animals

Mice (C57BL/6 background) were housed under a light/dark cycle of 12 h with free access to food and water. As ingestion of a meal rich in fat is a strong stimulus of incretin signaling (Harada et al. 2008), male WT and *GLP-1R*$^{-/-}$ mice were fed a high fat diet (45% fat, 20% protein and 35% carbohydrate by energy) from 7 weeks of age. Groups of treated HFD-fed WT and *GLP-1R*$^{-/-}$ mice received DPP-IV inhibitor in drinking water (0.5% W/V), while groups of untreated HFD-fed WT and *GLP-1R*$^{-/-}$ mice received drinking water without DPP-IV inhibitor. All the *GLP-1R*$^{-/-}$ mice were genotyped by Southern blot analysis. The DPP-IV inhibitor, provided by Taisho Pharmaceutical Co., Ltd., showed an inhibitory action on DPP-IV enzymatic activity against substrate H-Gly-Pro-7-amino-4-methyl coumarin (Gly-Pro-AMC) with IC_{50} (half maximal inhibitory concentration) of 0.0046 uM (Fukushima et al. 2008), while its IC_{50} on DPP-8 and DPP-9 were only 1.34 uM and 0.527 uM, respectively (unpublished data). Throughout the 8 weeks of study, water and food intake and body weight were measured once every 3 days. All mice care and procedures were approved by the Animal Care Committee of Kyoto University.

CT-based body composition analysis

The WT and *GLP-1R*$^{-/-}$ mice treated with or without DPP-IV inhibitor for 8 weeks were anesthetized and scanned along the body axis using LaTheta (LCT-100M) experimental animal CT system (Aloka, Tokyo, Japan). Contiguous 1-mm slice images of the whole abdominal cavity were used for quantitative assessment using LaTheta software (version 1.00). Weights of total fat mass, which comprises visceral fat mass and subcutaneous fat mass, and lean mass were quantitatively evaluated.

Oral glucose tolerance test (OGTT)

The WT and *GLP-1R*$^{-/-}$ mice treated with or without DPP-IV inhibitor for 8 weeks were fasted for 16 h and administered glucose (2 g/kg weight body) orally. Blood was collected from the orbital sinus of the mice at the indicated times (0, 15, 30, 60 and 120 min after glucose loading). Blood glucose levels were measured by the enzyme-electrode method. Plasma insulin levels were measured using an ELISA kit (Shibayagi, Gunma, Japan).

Measurement of plasma active GLP-1 levels and DPP-IV activity

For measurement of active GLP-1 levels, blood collected at 15 min after oral glucose loading was mixed with 2% EDTA· 4Na and 1% DPP-IV inhibitor (Linco Research, St Charles, MO). Active GLP-1 levels in plasma obtained by centrifugation (2000× g, 10 min, 4 °C) were measured using an active GLP-1 (7–36) ELISA kit (Linco Research).

Plasma DPP-IV activity was measured using a published method (Fukushima et al. 2008). In brief, 12.5 μl of plasma in duplicate was incubated with 37.5 μl of substrate cocktail (66.7 μM Gly-Pro-AMC, 25 mM HEPES, 140 mM NaCl, 26.6 mM $MgCl_2$, and 1% (w/v) BSA, pH 7.8) in the dark at room temperature for 5 min. The reaction was stopped by addition of 50 μl of 25% (v/v) acetic acid. Fluorescence was measured using a spectrofluorometer at excitation 360 nm/emission 465 nm. A standard curve was drawn using free AMC in standard buffer (25 mM HEPES, 140 mM NaCl, 20 mM $MgCl_2$, 1% (w/v) BSA, pH 7.8). DPP-IV activity (mU) is shown as the AMC (μM) generated in 1 ml plasma for 1 min of reaction time.

Measurement of insulin secretion in isolated islets

Islets were isolated from mice and preincubated at 37 °C for 30 min in 20 ml of Krebs-Ringer bicarbonate buffer (KRBB; 120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 20 mM $NaHCO_3$) supplemented with 10 mM HEPES and 0.2% (w/v) BSA and gassed with a mixture of 95% O_2 and 5% CO_2 (KRBB medium) containing 2.8 mM glucose. 10 size-matched islets collected in each tube were incubated at 37 °C for 30 min in 700 μl of KRBB medium containing 2.8 mM or 11.1 mM glucose with or without incretin peptides (100 nM human GLP-1 or 100 nM human GIP (Peptide Institute, Inc. Osaka, Japan)). Islets were then pelleted by centrifugation (9000× g, 2 min, 4 °C) and aliquots of the buffer were sampled. The amount of immunoreactive insulin was determined by radio-immunoassay (RIA). To determine insulin content, 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.

Measurement of mRNA expression of GLP-1R and GIPR in isolated islets

Measurement of mRNA expression of GLP-1R and GIPR was performed by quantitative RT-PCR as described previously (Harada et al. 2008). Briefly, total RNA was extracted from isolated islets with RNeasy mini kit (Qiagen, Valencia, CA) and treated with DNase (Qiagen). First strand cDNA was synthesized by SuperScript™ II Reverse Transcriptase system (Invitrogens, Grand Island, NY) according to manufacturer's instructions. SYBER Green PCR Master Mix (Applied Biosystems) was prepared for the PCR run. The PCR included 2 min at 50 °C and 10 min at 90 °C, followed by 50 cycles at 95 °C for 15 s and at 60 °C for 1 minute. The sequences of GLP-1R primers were 5′-CAACCGGACCTTTGATGACTA-3′ and 5′-GCTGTGCAGAACCGGTACAC-3′; the sequences of GIPR primers were 5′-CCTCCACTGGTCCCTACAC-3′ and 5′-GATAAACACCCTCCACCAGTAG-3′; the sequences of GAPDH primers were 5′-AAATGGTGAAGGTCGGTGTG-3′ and 5′-TCGTTGATGGCAACAATCTC-3′.

Statistical analyses

Data are expressed as means ± SE. Statistical analyses were performed by ANOVA and unpaired student's *t* test. *P* values < 0.05 were considered significant.

Results

Body weight and body composition of DPP-IV inhibitor-treated HFD-fed mice

Water intake, food intake, and body weight of HFD-fed WT and *GLP-1R*$^{-/-}$ mice with or without DPP-IV inhibitor administration were measured. In WT mice, water and food intake in DPP-IV

inhibitor-treated and untreated mice were similar during the 8 weeks of the study (Fig. 1A). In *GLP-1R*$^{-/-}$ mice, water and food intake in DPP-IV inhibitor-treated and untreated mice also were similar (Fig. 1A). A significant difference in body weight between DPP-IV inhibitor-untreated WT and *GLP-1R*$^{-/-}$ mice appeared from the 36th day (30.9 ± 1.3 g vs. 27.0 ± 0.6 g, $P<0.05$) (Fig. 1B). Body weight of WT mice and *GLP-1R*$^{-/-}$ mice was unaffected by DPP-IV inhibitor treatment during the 8 weeks of the study. To measure the effect of DPP-IV inhibitor on body composition, CT-based analysis was performed (Fig. 1C). In WT mice, there was no significant difference in body fat ratio between DPP-IV inhibitor-treated and untreated mice. However, the body fat ratio of DPP-IV inhibitor-treated *GLP-1R*$^{-/-}$ mice was significantly increased compared with that of untreated *GLP-1R*$^{-/-}$ mice (44.13 ± 1.55 vs. 32.60 ± 3.50, $P<0.05$).

OGTT of DPP-IV inhibitor-treated mice

In OGTT, blood glucose levels at 30 and 60 min were significantly lower in DPP-IV inhibitor-treated WT and *GLP-1R*$^{-/-}$ mice compared to those in untreated WT and *GLP-1R*$^{-/-}$ mice, respectively (Fig. 2A). In WT mice, the plasma insulin level of DPP-IV inhibitor-treated mice was 2.3 times higher at 15 min than that of untreated control mice ($P<0.05$), while in *GLP-1R*$^{-/-}$ mice, the plasma insulin levels of DPP-IV inhibitor-treated mice were 1.6 and 1.4 times higher at 15 and 30 min than those of untreated control mice, respectively ($P<0.05$) (Fig. 2B). In addition, the plasma insulin level of DPP-IV inhibitor-treated WT mice was 1.6 times higher at 15 min compared with that of DPP-IV inhibitor-treated *GLP-1R*$^{-/-}$ mice ($P<0.05$) (Fig. 2B).

We also measured plasma DPP-IV activity and active GLP-1 levels in WT and *GLP-1R*$^{-/-}$ mice at 15 min by OGTT. 75–80% of plasma DPP-IV activity in both untreated WT and *GLP-1R*$^{-/-}$ mice was inhibited by DPP-IV inhibitor treatment (Fig. 2C). Plasma levels of active GLP-1 were significantly elevated in DPP-IV inhibitor-treated WT and *GLP-1R*$^{-/-}$ mice compared to those in the respective untreated mice (Fig. 2D).

Insulin secretion and incretin receptor expression of islets isolated from DPP-IV inhibitor-treated mice

To determine insulin secretion in response to glucose and GLP-1 and GIP, batch incubation experiments were performed using islets isolated from WT and *GLP-1R*$^{-/-}$ mice after 8 weeks of treatment (Fig. 3A). In islets of WT mice, insulin secretion in response to 2.8 mM glucose was similar in DPP-IV inhibitor-treated and untreated mice. However, insulin secretion in response to 11.1 mM glucose, 11.1 mM glucose with GLP-1, and 11.1 mM glucose with GIP was significantly higher in DPP-IV inhibitor-treated mice than those in untreated mice. In addition, both GLP-1 and GIP augmented insulin secretion in the presence of 11.1 mM glucose in both DPP-IV inhibitor-treated and untreated mice. In islets of *GLP-1R*$^{-/-}$ mice, as in those of WT mice, insulin secretion in response to 2.8 mM glucose was similar in DPP-IV inhibitor-treated and untreated mice, and insulin secretion in response to 11.1 mM glucose, 11.1 mM glucose with GLP-1, and 11.1 mM glucose with GIP was significantly higher in DPP-IV inhibitor-treated mice than those in untreated mice. However, in *GLP-1R*$^{-/-}$ mice, potentiation of insulin secretion by incretin in the presence of 11.1 mM glucose was observed only by GIP and not by GLP-1 in both DPP-IV inhibitor-treated and untreated mice. Insulin content was similar among all groups of mice (data not shown).

To determine the effect of DPP-IV inhibitor treatment on the mRNA expression of GLP-1R and GIPR in islets, we performed quantitative RT-PCR after 8 weeks of study (Fig. 3B). The mRNA expression levels of GLP-1R and GIPR in DPP-IV inhibitor-treated and untreated WT mice were similar, as were total mRNA expression levels of GIPR in DPP-IV inhibitor-treated and untreated *GLP-1R*$^{-/-}$ mice.

Discussion

In the present study, we evaluated body composition and glucose control in the absence of the GLP-1 signaling using *GLP-1R*$^{-/-}$ mice treated with DPP-IV inhibitor for 8 weeks to clarify GLP-1 and GIP action under long-term DPP-IV inhibition.

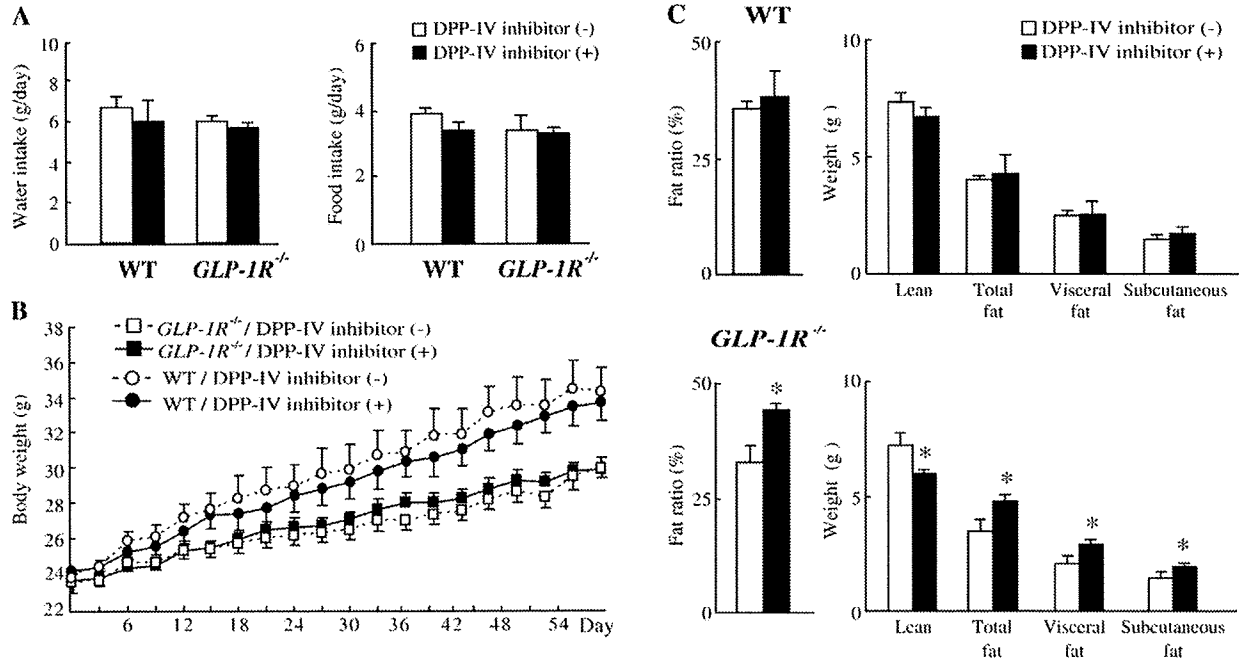

Fig. 1. Water and food intake, body weight and CT-based body composition analysis. (A) Water intake (left) and food intake (right) of WT and *GLP-1R*$^{-/-}$ mice treated with (filled) or without (open) DPP-IV inhibitor at the last week of study (average of 1 day). (B) Body weight change of WT (circle) and *GLP-1R*$^{-/-}$ (square) mice treated with (filled) or without (open) DPP-IV inhibitor. (C) CT-based body composition analysis of WT (upper) and *GLP-1R*$^{-/-}$ (lower) mice treated with (filled) or without (open) DPP-IV inhibitor. Fat ratio calculated as: total fat/(lean + total fat) × 100. Values are means ± SE. *$P<0.05$ vs. untreated mice ($n = 5$–6).

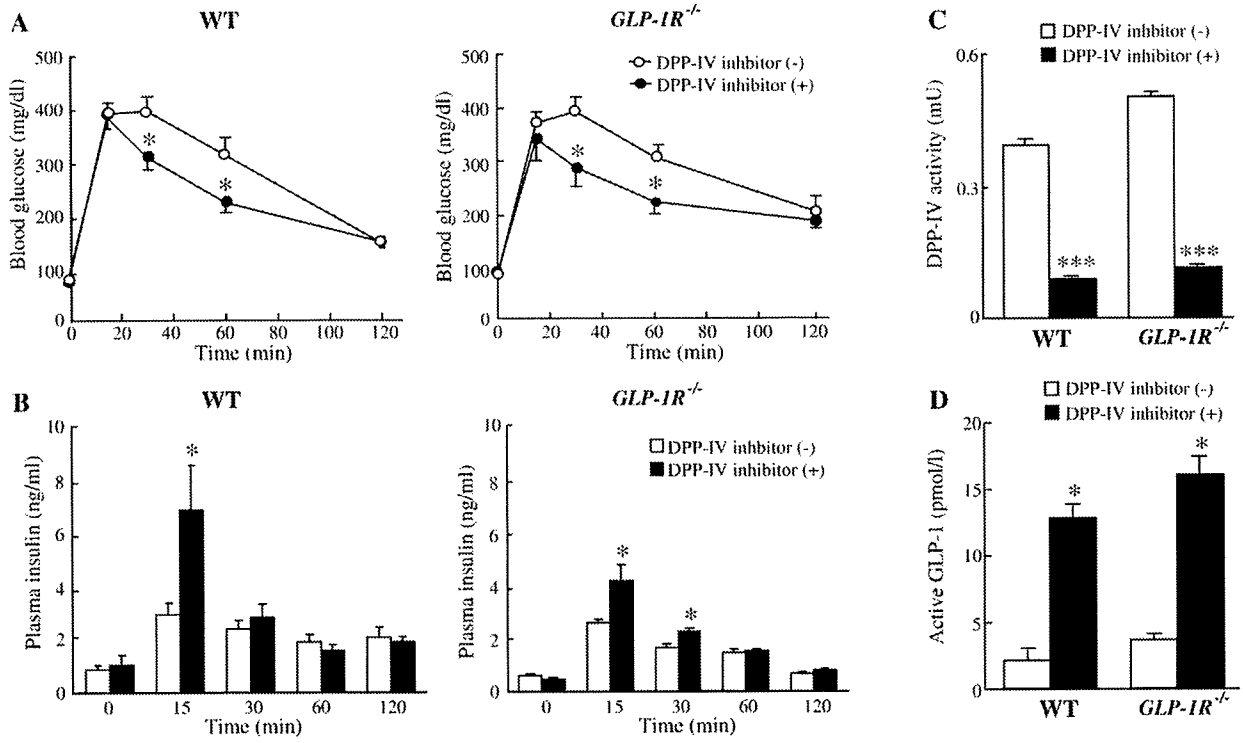

Fig. 2. OGTT. Blood glucose levels (A) and plasma insulin levels (B) of WT (left) and *GLP-1R*$^{-/-}$ (right) mice treated with (filled) or without (open) DPP-IV inhibitor after 8 weeks of study. Plasma DPP-IV activity (C) and plasma levels of active GLP-1 (D) at 15 min for WT and *GLP-1R*$^{-/-}$ mice treated with (filled) or without (open) DPP-IV inhibitor. Values are means ± SE. **P*<0.05, ****P*<0.001 vs. untreated mice (n = 5–6).

HFD-fed DPP-IV-deficient rodents exhibit reduced food intake and resistance to development of obesity with elevated active GLP-1 levels (Yasuda et al. 2002; Conarello et al. 2003), and DPP-IV inhibitor has been shown to reduce body weight in some previous studies using rodent models (Pospisilik et al. 2002; Lamont and Drucker 2008). In the present study, no alteration in body weight was found after 8 weeks of DPP-IV inhibitor treatment either in HFD-fed WT or *GLP-1R*$^{-/-}$ mice, although 75–80% of plasma DPP-IV activity was inhibited and plasma active GLP-1 levels were significantly elevated after oral glucose loading. However, CT-based body composition analysis revealed that DPP-IV inhibitor treatment increased body fat mass in *GLP-1R*$^{-/-}$ mice but not in WT mice. DPP-IV is well known to be involved in inactivation of both GLP-1 and GIP, and plasma active GIP levels are elevated by treatment of DPP-IV inhibitor (Deacon et al. 2001). The receptor for GIP, differently from that for GLP-1, is expressed in adipocytes, and GIP directly facilitates energy accumulation in adipose tissue (Miyawaki et al. 2002; Naitoh et al. 2008). Our results suggest that fat accumulation is potentiated by fat-augmenting factors including GIP in the absence of the GLP-1 signaling under long-term DPP-IV inhibition. Thus, the lack of change in body weight and fat mass in DPP-IV inhibitor-treated WT mice may be due to the counteracting effects of enhanced GLP-1 and GIP signaling. In addition, *GLP-1R*$^{-/-}$ mice showed less body weight gain compared to that of WT mice, consistent with the previous report on *GLP-1R*$^{-/-}$

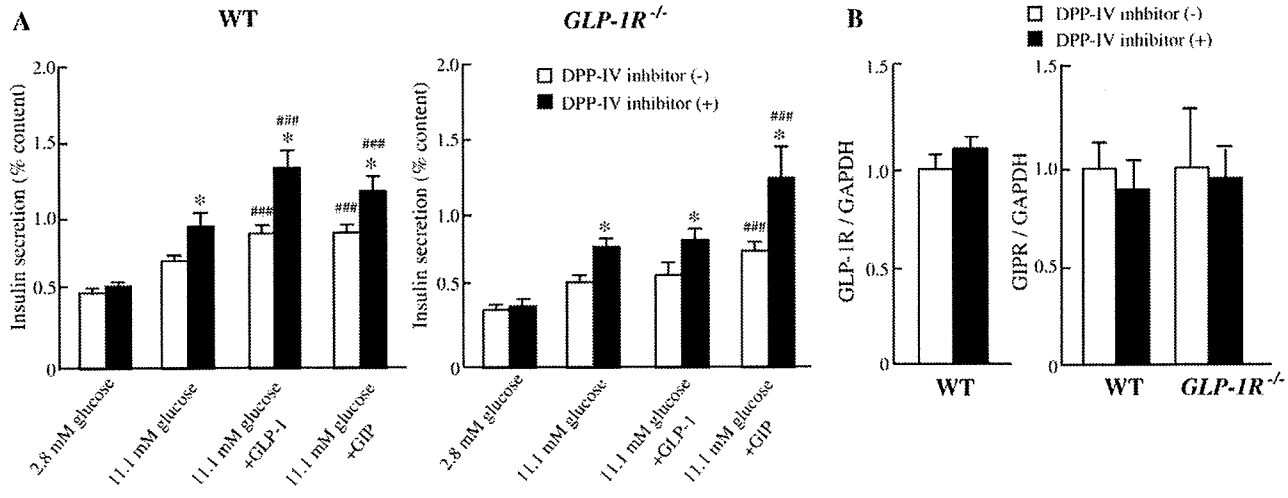

Fig. 3. Functional study of isolated islets. (A) Insulin secretion from islets isolated from WT (left) and *GLP-1R*$^{-/-}$ (right) mice treated with (filled) or without (open) DPP-IV inhibitor after 8 weeks of study. Values are means ± SE. **P*<0.05 vs. untreated mice. ###*P*<0.05 vs. 11.1 mM glucose. (B) The mRNA expression of GLP-1R and GIPR in islets isolated from WT and *GLP-1R*$^{-/-}$ mice treated with (filled) or without (open) DPP-IV inhibitor. GLP-1R and GIPR mRNA levels were corrected for GAPDH mRNA levels, respectively. Data of DPP-IV inhibitor-treated mice is shown relative to untreated mice. Values are means ± SE. (n = 5–8).

mice showing reduced body weight gain compared to that of WT mice, possibly due to enhanced locomotor activity and increased energy expenditure (Hansotia et al. 2007).

GLP-1 and GIP both are clearly involved in the effects of long-term DPP-IV inhibition on improved glucose tolerance, as DPP-IV inhibitor fails to increase insulin secretion and decrease plasma glucose after oral glucose loading in DIRKO mice (Flock et al. 2007). Our comparison in the present study of *GLP-1R*$^{-/-}$ mice and WT mice enables us to estimate the magnitude of the effects of enhanced GLP-1 and GIP by long-term DPP-IV inhibition on insulin secretion separately. Improved glucose tolerance and increased plasma insulin with elevated active GLP-1 levels were found in both DPP-IV inhibitor-treated WT and *GLP-1R*$^{-/-}$ mice by OGTT, indicating that GIP contributes to the insulinotropic effects of long-term DPP-IV inhibition in *GLP-1R*$^{-/-}$ mice. Moreover, blood insulin at 15 min in DPP-IV inhibitor-treated *GLP-1R*$^{-/-}$ mice was about half of that in DPP-IV inhibitor-treated WT mice. These results confirm that GLP-1 and GIP are important mediators of the insulinotropic effects of long-term DPP-IV inhibition. In addition, insulin secretion from islets in response to 11.1 mM glucose was increased in DPP-IV inhibitor-treated WT and *GLP-1R*$^{-/-}$ mice, indicating that glucose sensitivity of insulin secretion is augmented by long-term DPP-IV inhibitor administration unrelated to the GLP-1 signaling. However, the mechanism is not known. A recent report found that the glucose sensitivity of insulin secretion in isolated islets of mice improved after GLP-1 receptor agonist treatment due to augmented cAMP-induced activation of protein kinase A (PKA) through the GLP-1 receptor (Winzell and Ahrén 2008). It also was reported that activated PKA due to GLP-1 signaling increased expression of transcription factor pancreatic-duodenum homeobox-1 (PDX-1), translocation of PDX-1 from cytoplasm to nucleus, and phosphorylation of glucose transporter type 2 (GLUT2) in β-cells (Wang et al. 2001; Thorens et al. 1996). Thus, the increased glucose sensitivity of insulin secretion in islets unrelated to the GLP-1 signaling may be the result of augmented GIP signaling due long-term DPP-IV inhibition through similar mechanisms. Further study is required to clarify the augmentation of glucose sensitivity of islets after long-term DPP-IV inhibitor administration.

In addition to the plasma active incretin level, the expression of incretin receptors in islets also influences their insulinotropic effect (Lynn et al. 2001; Xu et al. 2007). Indeed, it has been reported that continuous GLP-1 stimulation results in desensitization of GLP-1R, which can subsequently reduce insulin secretion in response to GLP-1 in insulin-secreting cell lines (Widmann et al. 1996; Green et al. 2005). However, the expression of GLP-1R and GIPR in islets did not change in DPP-IV inhibitor-treated mice in the present study. Furthermore, insulin secretion in response to incretins was maintained in the islets of DPP-IV inhibitor-treated mice, demonstrating that sensitivity of the incretin receptors did not decrease even after 8 weeks of continuous incretin stimulation. These results suggest that the action of DPP-IV inhibitor in glucose control is preserved during long-term DPP-IV inhibitor administration.

Conclusion

Long-term DPP-IV inhibition does not alter body composition, possibly due to the counteracting effects of enhanced GLP-1 and GIP, but does improve glucose tolerance through the synergistic insulinotropic effects of enhanced GLP-1 and GIP, as well as by improved glucose responsiveness in pancreatic islets.

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References

Ahrén B, Simonsson E, Larsson H, Landin-Olsson M, Torgeirsson H, Jansson PA, Sandqvist M, Båvenholm P, Efendic S, Eriksson JW, Dickinson S, Holmes D. Inhibition of dipeptidyl peptidase IV improves metabolic control over a 4-week study period in type 2 diabetes. Diabetes Care 25 (5), 869–875, 2002.

Buse JB, Henry RR, Han J, Kim DD, Fineman MS, Baron AD. Exenatide-113 Clinical Study Group. Effects of Exenatide (Exendin-4) on glycemic control over 30 weeks in sulfonylurea-treated patients with type 2 diabetes. Diabetes Care 27 (11), 2628–2635, 2004.

Conarello SL, Li Z, Ronan J, Roy RS, Zhu L, Jiang G, Liu F, Woods J, Zycband E, Moller DE, Thornberry NA, Zhang BB. Mice lacking dipeptidyl peptidase IV are protected against obesity and insulin resistance. Proceedings of the National Academy of Sciences of the United States of America 100 (11), 6825–6830, 2003.

Deacon CF, Danielsen P, Klarskov L, Olesen M, Holst JJ. Dipeptidyl peptidase IV inhibition reduces the degradation and clearance of GIP and potentiates its insulinotropic and antihyperglycemic effects in anesthetized pigs. Diabetes 50 (7), 1588–1597, 2001.

Flock G, Baggio LL, Longuet C, Drucker DJ. Incretin receptors for glucagon-like peptide 1 and glucose-dependent insulinotropic polypeptide are essential for the sustained metabolic actions of Vildagliptin in mice. Diabetes 56 (12), 3006–3013, 2007.

Fukushima H, Hiratate A, Takahashi M, Mikami A, Saito-Hori M, Munetomo E, Kitano K, Chonan S, Saito H, Suzuki A, Takaoka Y, Yamamoto K. Synthesis and structure–activity relationships of potent 4-fluoro-2-cyanopyrrolidine dipeptidyl peptidase IV inhibitors. Bioorganic & Medicinal Chemistry 16 (7), 4093–4106, 2008.

Green BD, Liu HK, McCluskey JT, Duffy NA, O'Harte FP, McClenaghan NH, Flatt PR. Function of a long-term, GLP-1-treated, insulin-secreting cell line is improved by preventing DPP IV-mediated degradation of GLP-1. Diabetes, Obesity & Metabolism 7 (5), 563–569, 2005.

Hansotia T, Maida A, Flock G, Yamada Y, Tsukiyama K, Seino Y, Drucker DJ. Extrapancreatic incretin receptors modulate glucose homeostasis, body weight, and energy expenditure. The Journal of Clinical Investigation 117 (1), 143–152, 2007.

Harada N, Yamada Y, Tsukiyama K, Yamada C, Nakamura Y, Mukai E, Hamasaki A, Liu X, Toyoda K, Seino Y, Inagaki N. A novel gastric inhibitory polypeptide (GIP) receptor splice variant influences GIP sensitivity of pancreatic {beta}-cells in obese mice. American Journal of Physiology. Endocrinology and Metabolism 294 (1), E61–E68, 2008.

Komatsu R, Matsuyama T, Namba M, Watanabe N, Itoh H, Kono N, Tarui S. Glucagonostatic and insulinotropic action of glucagon-like peptide-1-(7-36) amide. Diabetes 38 (7), 902–905, 1989.

Lamont BJ, Drucker DJ. Differential antidiabetic efficacy of incretin agonists versus DPP-4 inhibition in high fat-fed mice. Diabetes 57 (1), 190–198, 2008.

Lynn FC, Pamir N, Ng EH, McIntosh CH, Kieffer TJ, Pederson RA. Defective glucose-dependent insulinotropic polypeptide receptor expression in diabetic fatty zucker rats. Diabetes 50 (5), 1004–1011, 2001.

Marguet D, Baggio L, Kobayashi T, Bernard AM, Pierres M, Nielsen PF, Ribel U, Watanabe T, Drucker DJ, Wagtmann N. Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26. Proceedings of the National Academy of Sciences of the United States of America 97 (12), 6874–6879, 2000.

Miyawaki K, Yamada Y, Ban N, Ihara Y, Tsukiyama K, Zhou H, Fujimoto S, Oku A, Tsuda K, Toyokuni S, Hiai H, Mizunoya W, Fushiki T, Holst JJ, Makino M, Tashita A, Kobara Y, Tsubamoto Y, Jinnouchi T, Jomori T, Seino Y. Inhibition of gastric inhibitory polypeptide signaling prevents obesity. Nature Medicine 8 (7), 738–742, 2002.

Nagakura T, Yasuda N, Yamazaki K, Ikuta H, Yoshikawa S, Asano O, Tanaka I. Improved glucose tolerance via enhanced glucose-dependent insulin secretion in dipeptidyl peptidase IV-deficient Fischer rats. Biochemical and Biophysical Research Communications 284 (2), 501–506, 2001.

Naitoh R, Miyawaki K, Harada N, Mizunoya W, Toyoda K, Fushiki T, Yamada Y, Seino Y, Inagaki N. Inhibition of GIP signaling modulates adiponectin levels under high-fat diet in mice. Biochemical and Biophysical Research Communications 376 (1), 21–25, 2008.

Pospisilik JA, Stafford SG, Demuth HU, Brownsey R, Parkhouse W, Finegood DT, McIntosh CH, Pederson RA. Long-term treatment with the dipeptidyl peptidase IV inhibitor P32/98 causes sustained improvements in glucose tolerance, insulin sensitivity, hyperinsulinemia, and β-cell glucose responsiveness in VDF (fa/fa) zucker rats. Diabetes 51 (4), 943–950, 2002.

Reimer MK, Holst JJ, Ahrén B. Long-term inhibition of dipeptidyl peptidase IV improves glucose tolerance and preserves islet function in mice. European Journal of Endocrinology/European Federation of Endocrine Societies 146 (5), 717–727, 2002.

Szayna M, Doyle ME, Betkey JA, Holloway HW, Spencer RG, Greig NH, Egan JM. Exendin-4 decelerates food intake, weight gain, and fat deposition in Zucker rats. Endocrinology 141 (6), 1936–1941, 2000.

Thorens B, Dériaz N, Bosco D, DeVos A, Pipeleers D, Schuit F, Meda P, Porret A. Protein kinase A-dependent phosphorylation of GLUT2 in pancreatic beta cells. The Journal of Biological Chemistry 271 (14), 8075–8081, 1996.

Toyoda K, Okitsu T, Yamane S, Uonaga T, Liu X, Harada N, Uemoto S, Seino Y, Inagaki N. GLP-1 receptor signaling protects pancreatic beta cells in intraportal islet transplant by inhibiting apoptosis. Biochemical and Biophysical Research Communications 367 (4), 793–798, 2008.

Tsukiyama K, Yamada Y, Yamada C, Harada N, Kawasaki Y, Ogura M, Bessho K, Li M, Amizuka N, Sato M, Udagawa N, Takahashi N, Tanaka K, Oiso Y, Seino Y. Gastric

inhibitory polypeptide as an endogenous factor promoting new bone formation after food ingestion. Molecular Endocrinology 20 (7), 1644–1651, 2006.
Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379 (6560), 69–72, 1996.
Wang X, Zhou J, Doyle ME, Egan JM. Glucagon-like peptide-1 causes pancreatic duodenal homeobox-1 protein translocation from the cytoplasm to the nucleus of pancreatic beta-cells by a cyclic adenosine monophosphate/protein kinase A-dependent mechanism. Endocrinology 142 (5), 1820–1827, 2001.
Widmann C, Dolci W, Thorens B. Desensitization and phosphorylation of the glucagon-like peptide-I (GLP-1) receptor by GLP-1 and 4-Phorbol 12-Myristate 13-Acetate. Molecular Endocrinology 10 (1), 62–75, 1996.
Willms B, Werner J, Holst JJ, Orskov C, Creutzfeldt W, Nauck MA. Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: Effects of exogenous glucagon-like peptide-1 (GLP-1)-(7–36) amide in type 2 (noninsulin-dependent) diabetic patients. The Journal of Clinical Endocrinology and Metabolism 81 (1), 327–332, 1996.
Winzell MS, Ahrén B. Durable islet effects on insulin secretion and protein kinase A expression following exendin-4 treatment of high-fat diet-fed mice. Journal of Molecular Endocrinology 40 (2), 93–100, 2008.
Xu G, Kaneto H, Laybutt DR, Duvivier-Kali VF, Trivedi N, Suzuma K, King GL, Weir GC, Bonner-Weir S. Downregulation of GLP-1 and GIP receptor expression by hyperglycemia: Possible contribution to impaired incretin effects in diabetes. Diabetes 56 (6), 1551–1558, 2007.
Yasuda N, Nagakura T, Yamazaki K, Inoue T, Tanaka I. Improvement of high fat-diet-induced insulin resistance in dipeptidyl peptidase IV-deficient Fischer rats. Life Sciences 71 (2), 227–238, 2002.

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Factors responsible for age-related elevation in fasting plasma glucose: a cross-sectional study in Japanese men

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Abstract

To evaluate the factors associated with age-related increase in fasting plasma glucose (FPG) in Japanese men with normal fasting glucose, we measured FPG, fasting immunoreactive insulin, glycated hemoglobin, total cholesterol, triglyceride, and high-density lipoprotein cholesterol levels in health check examinees. Subjects with FPG less than 6.1 mmol/L together with glycated hemoglobin less than 5.6% were enrolled in the study. The homeostasis model assessment of insulin resistance (HOMA-IR) and HOMA-β were used as the indices of insulin sensitivity and insulin secretion, respectively. Fasting plasma glucose increased significantly with age ($r = 0.30$, $P < .0001$), and HOMA-β decreased significantly with age ($r = 0.24$, $P < .0001$). The HOMA-IR had no significant relation with age ($r = 0.06$, not significant), whereas body mass index and serum triglyceride were associated with HOMA-IR ($r = 0.49$, $P < .0001$ and $r = 0.33$, $P < .0001$, respectively). Thus, in Japanese male subjects with normal fasting glucose, it is suggested that the FPG increment with age is associated with decreased β-cell function rather than with insulin resistance. Further analyses were performed by comparing 3 groups: low FPG (FPG <5.0 mmol/L), high FPG (5.0 ≤ FPG < 5.6 mmol/L), and mild impairment of fasting glycemia (mild IFG) (5.6 ≤ FPG < 6.1 mmol/L). The insulin levels in mild IFG and high FPG were significantly higher than in low FPG ($P < .001$), but those in mild IFG were similar to those in high FPG. Analysis of the 3 subgroups revealed that, whereas insulin sensitivity was impaired more in high FPG, there was little compensatory increase in insulin in mild IFG, suggesting that β-cell function is already deteriorated when the FPG level is greater than 5.6 mmol/L.

1. Introduction

Type 2 diabetes mellitus is characterized by both decreasing insulin secretion and insulin sensitivity, partly due to genetic factors [1-3]. Although diabetes is a worldwide health problem [4], it is clear that there are ethnic differences in the pathophysiology of the decreasing glucose tolerance characteristic of its development [5]. Factors responsible for glucose intolerance occur from a prediabetic state: impaired glucose regulation according to the World Health Organization classification. Impaired glucose regulation comprises 2 subgroups: impaired fasting glycemia (IFG) characterized by increasingly impaired fasting plasma glucose (FPG) with 2-hour plasma glucose (2h-PG) within normal limits and impaired glucose tolerance (IGT) characterized by increasingly impaired 2h-PG [6,7]. We previously reported that insulin secretory capacity and insulin sensitivity are both decreased in Japanese subjects with IFG [8-10]. Although β-cell function and insulin sensitivity may well begin to deteriorate earlier, there are few studies of the normal glucose tolerance (NGT) population. Fasting plasma glucose is known to increase with age [11], and both insulin secretory capacity and insulin

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sensitivity are reported to decrease with age [12-14]. We have reported that some subgroups of Japanese NGT subjects show especially decreased β-cell function [15]. However, it is unclear whether deteriorated insulin secretion or insulin sensitivity is the primary factor in the increase in FPG during the period of development from NGT to IFG in Japanese.

In addition, the American Diabetes Association (ADA) lowered the cutoff value of IFG from 6.1 to 5.6 mmol/L [16]. Subjects with FPG from 5.6 to 6.1 mmol/L and with normal postprandial glucose level are categorized as having IFG in the ADA criteria, although they are categorized as having NGT in the criteria of the World Health Organization and the Japanese Diabetes Association. Thus, analysis of these subjects with mild IFG (mild impairment of fasting glucose) in view of insulin secretion and insulin sensitivity is crucial to elucidate the characteristic of subjects with borderline glucose dysregulation. To investigate the pathogenesis of prediabetes in Japanese, we compared insulin secretory capacity and insulin sensitivity in health check examinees exhibiting normal fasting glucose (NFG).

2. Subjects and methods

2.1. Subjects

Among health check examinees between 1993 and 2004 at Kyoto University Hospital, Kansai-Denryoku Hospital, and Kyoto Preventive Medical Center, 657 male subjects with FPG <6.1 mmol/L and glycated hemoglobin (HbA_{1c}) <5.6% were enrolled in the study (Table 1). Subjects with known history or signs of diabetes, previous gastrointestinal operation, liver disease, renal failure, endocrine disease, malignancy, hypertension, frequent heavy exercise, or history of medications before the study were excluded.

2.2. Measurements

Physical measurement (body height, body weight) and laboratory measurements (urine test, FPG, fasting immunoreactive insulin [F-IRI], HbA_{1c}, total cholesterol [TC], triglyceride [TG], and high-density lipoprotein cholesterol [HDL-C] level) were taken. The study was designed in compliance with the ethics regulations of the Helsinki Declaration. Blood samples were collected after overnight fasting for 16 hours [8]. Plasma glucose levels were measured by glucose oxidase method using the Hitachi Automatic Clinical Analyzer 7170 (Hitachi, Tokyo, Japan). Serum insulin levels were measured by radioimmunoassay (RIA beads II; Dainabot, Tokyo, Japan), which shows low cross-reaction with C-peptide of less than 0.005% and proinsulin less than 0.5% [8]. Glycated hemoglobin levels were measured by high-performance liquid chromatography methods. Serum TC, TG, and HDL-C levels were measured as reported previously [17]. To evaluate insulin resistance, we used the homeostasis model assessment of insulin resistance index (HOMA-IR) calculated by the formula FPG (in millimoles per liter) × IRI (in microunits per milliliter)/22.5. The HOMA-IR is a reliable measure of insulin resistance, correlating well with values obtained by glucose clamp and minimal model studies [18-20]. To calculate pancreatic β-cell function (HOMA β-cell), we used the formula 20 × IRI (in microunits per milliliter)/[FPG (in millimoles per liter) − 3.5] [18].

Table 1
Clinical characteristics of the subjects with NFG

	Data
n	657
Age (y)	44.9 ± 11.2
BMI (kg/m^2)	23.6 ± 2.8
HbA_{1c} (%)	4.8 ± 0.3
FPG (mmol/L)	5.1 ± 0.4
F-IRI (μU/mL)	5.2 ± 2.9
TC (mmol/L)	5.19 ± 0.88
TG (mmol/L)	1.45 ± 1.01
HDL-C (mmol/L)	1.45 ± 0.35

Data are mean ± SD.

2.3. Statistical analysis

Clinical data are expressed as mean ± SD. Analyses were performed using the STATVIEW 5 system (StatView, Berkeley, CA). Multiple regression analysis was used to compare age and FPG, HOMA-β, HOMA-IR, and body mass index (BMI). The same analysis was performed between HOMA-IR and BMI and TG. The NFG group was divided into low and high FPG and mild IFG, and the metabolic profiles were compared using analysis of variance. The data are expressed as mean ± SE. $P < .05$ is considered significant.

3. Results

3.1. Characteristics of the study population

As shown in Table 1, the mean age of the subjects is 44.9 ± 11.2 years and the mean BMI is 23.6 ± 2.8 kg/m^2. Among them, the number of subjects with BMI more than 30 are 22 (3.4%), concomitant with the representative epidemiologic studies in Japanese [21-23].

3.2. Correlation between age and FPG, HOMA-β, and HOMA-IR

Fig. 1A shows a positive relationship of FPG with age ($r = 0.30$, $P < .0001$; FPG [in millimoles per liter] = 0.011 × age + 4.6). Fig. 1B shows that HOMA-β has a negative correlation with age ($r = 0.24$, $P < .0001$), whereas there is no significant correlation between HOMA-IR and age ($r = 0.06$, not significant).

3.3. Correlation between HOMA-IR and BMI and serum TG levels

Fig. 2A, B shows that BMI and serum TG levels are associated with HOMA-IR ($r = 0.49$, $P < .0001$ and $r = 0.33$,

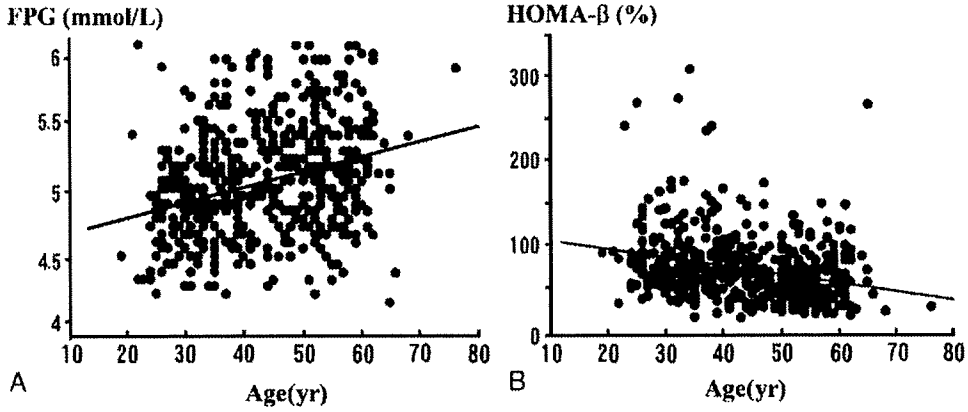

Fig. 1. Distribution of FPG (A) and HOMA-β (B) cell by age. The FPG increases with age (r = 0.30, P < .0001). The HOMA-β cell is negatively correlated with age (r = 0.24, P < .0001).

P < .0001, respectively). Multiple regression analysis shows that both BMI and TG are independently associated with HOMA-IR (standardized β = 0.41 and 0.15, respectively). Body mass index was the strongest determinant of HOMA-IR, and BMI did not increase with age significantly in Japanese men (r = 0.07, not significant).

3.4. Analysis of 3 subgroups of NFG subjects

To evaluate the factors involved in increasing FPG in Japanese NFG and the ADA recommendation of lowering the threshold of upper limit of normal FPG from 6.1 to 5.6 mmol/L [16], we divided our NFG subjects into 3 subgroups: low FPG (FPG <5.0 mmol/L), high FPG (5.0 ≤ FPG < 5.6 mmol/L), and mild impairment of fasting glucose (mild IFG) (5.6 ≤ FPG < 6.1 mmol/L); and age, BMI, TG, and insulin secretion and sensitivity were compared. As shown in Table 2, high FPG and mild IFG have higher age and BMI than low FPG (both P < .0001). Insulin in high FPG and mild IFG is increased compared with that in low FPG (P < .001); insulin in mild IFG is similar to that in high FPG. The HOMA-IR in high FPG and mild IFG is increased compared with that in low FPG (P < .0001). The HOMA-β in high FPG and mild IFG is decreased compared with that in low FPG (P < .0001); the HOMA-β in mild IFG is decreased compared with that in high FPG (P < .001).

4. Discussion

In this study, we analyzed the factors responsible for age-related elevation of FPG in Japanese men with NFG. Fasting plasma glucose was found to increase with age primarily because of reduced β-cell function rather than increased insulin resistance. In addition, we have elucidated that there was no compensatory increase in insulin secretion in mild IFG (FPG 5.6-6.1 mmol/L).

Our study subjects were composed only of men because the number of female subjects was 158, which is not comparable with male subjects. Some reports showed a difference between men and women in the elevation of FPG [24-26], and another showed similar results between men and women in the elevation of FPG [27]. We analyzed the results from our 158 female subjects, and we could not find

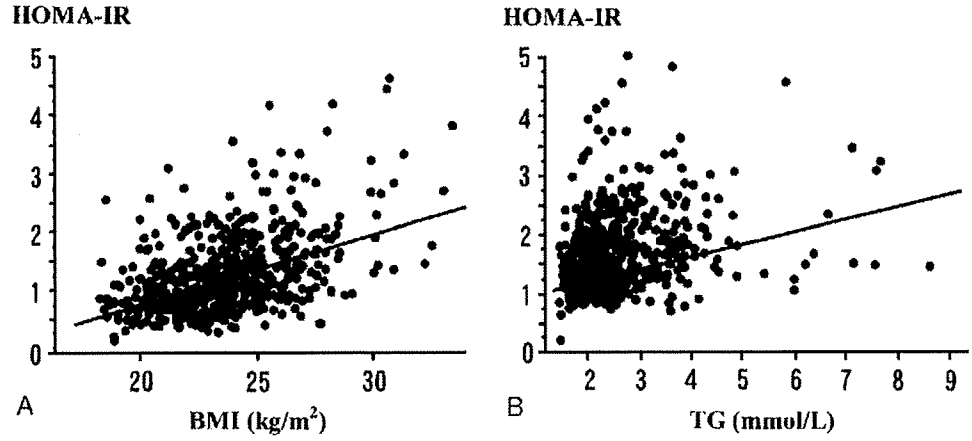

Fig. 2. Distribution of HOMA-IR by BMI (A) and TG (B). Both BMI and TG are associated with HOMA-IR (BMI: r = 0.49, P < .0001; TG: r = 0.33, P < .0001).

Table 2
Comparison of 3 FPG subgroups of NFG subjects

	Low FPG (FPG <5.0 mmol/L)	High FPG (5.0 ≤ FPG < 5.6 mmol/L)	Mild IFG (5.6 ≤ FPG < 6.1 mmol/L)
n	268	288	101
Age (y)	42.0 ± 0.7	45.7 ± 0.6[a]	49.8 ± 1.0[a, b]
BMI (kg/m^2)	23.0 ± 0.2	23.9 ± 0.1[a]	24.3 ± 0.3[a]
TC (mmol/L)	5.07 ± 0.05	5.23 ± 0.05[c]	5.35 ± 0.08[d]
TG (mmol/L)	1.30 ± 0.05	1.55 ± 0.06[d]	1.56 ± 0.09[c]
HDL-C (mmol/L)	1.45 ± 0.02	1.44 ± 0.02	1.45 ± 0.03
F-IRI (μU/mL)	4.6 ± 0.2	5.7 ± 0.2[a]	5.6 ± 0.3[e]
HOMA-IR	0.96 ± 0.04	1.31 ± 0.04[a]	1.44 ± 0.07[a]
HOMA-β (%)	78.5 ± 3.1	65.2 ± 1.9[a]	49.2 ± 2.4[a, b]

Data are mean ± SE.
[a] $P < .0001$ vs low FPG.
[b] $P < .001$ vs high FPG.
[c] $P < .05$ vs low FPG.
[d] $P < .005$ vs low FPG.
[e] $P < .0005$ vs low FPG.

remarkable differences with male subjects (data not shown). Further studies are necessary to elucidate the sex difference of the factors responsible for elevation of FPG. Although some reports showed an increase in insulin resistance in subjects older than 70 years, our male subjects were younger than 70 years. Insulin resistance in subjects older than 70 years was reported mainly because of the change in abdominal adiposity [28,29]; and in representative epidemiologic studies such as the Funagata study and the Hisayama study, the mean age of developing glucose intolerance is around 50 years in Japanese [21-23]. For these reasons, our subjects being around the age of 50 years was enough for our purpose in this study of elucidating the factors responsible for FPG elevation from normal to borderline glucose dysregulation.

Fasting plasma glucose increased by 0.011 mmol/L per year, in accord with previous reports [30]. The HOMA-β decreased by 0.85% per year, clearly indicating reduced basal insulin secretion. Although previous studies in whites and in other populations have found that insulin resistance is closely associated with age-related FPG elevation [12,31], HOMA-IR did not increase with age significantly in our subjects. To characterize the insulin resistance of our study population, we performed both simple and multiple regression analyses between HOMA-IR and the other measured factors. The BMI and serum TG levels were strongly associated with HOMA-IR ($P < .0001$), in accord with our previous results in Japanese diabetic patients [32]. Although BMI was the strongest determinant of HOMA-IR, it did not increase with age; the mean BMI of 23.6 kg/m^2 is in accord with Japanese statistical data [21-23] and is much lower than in whites [33,34]. The BMI of Asians in other studies is also reported to be lower, suggesting a common metabolic profile [35]. The leaner Japanese subjects in this study might therefore be expected to be less influenced by insulin resistance in comparison with whites.

Impaired fasting glycemia is a prediabetic state characterized by FPG elevation without increased 2h-PG. We previously reported that insulin secretory capacity and insulin sensitivity are both already decreased in IFG [8-10], suggesting the clinical importance of early deterioration of β-cell function and insulin sensitivity in developing prediabetes. In addition, we regarded the PG level of 5.6 mmol/L as an important FPG threshold value according to ADA recommendation [16]. Therefore, we compared insulin secretion and insulin sensitivity in 3 subgroups of NFG subjects: low FPG (FPG <5.0 mmol/L), high FPG (5.0 ≤ FPG < 5.6 mmol/L), and mild IFG (5.6 ≤ FPG < 6.1 mmol/L). Insulin secretion in mild IFG was not increased compared with that in high FPG, indicating impaired compensatory insulin secretion against increasing insulin resistance. Some reports have found that early-phase insulin secretion and insulin sensitivity are both decreased in NGT at a higher range of FPG (FPG >5.1-5.3 mmol/L) [36-38]. Fortunately, we could analyze 56 subjects during the 8-year follow-up period using oral glucose tolerance test results [39]. The subjects who developed from NFG to IFG showed decreasing insulin sensitivity and insulin secretory capacity, and those who developed from NFG to IGT showed decreased early insulin secretory response. These follow-up data were compatible with our previous data of IFG and IGT [5,8,10,39]. Taken together, these data indicate that insulin secretory capacity is already decreased in NGT at the higher range of FPG and that a lack of compensatory insulin secretion appears at greater than 5.6 mmol/L in FPG.

We find in Japanese NFG subjects that age-related FPG elevation is mainly due to decreased β-cell function rather than to increasing insulin resistance as in white subjects. In addition, analysis of 3 degrees of increasing FPG indicates that failure of compensatory insulin secretion is responsible for the elevation in FPG in these subjects. Thus, these data could be helpful in reconsideration of the threshold FPG for prediabetes to be recommended by the ADA [16]. However, decreasing the upper threshold of FPG entails increasing the IFG population, a costly social health problem [40]. Further studies are required to clarify the ethnic differences in the development of diabetes and diabetic complications and the value of clinical interventions in newly diagnosed IFG patients.

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References

[1] Porte Jr D. Banting lecture 1990. Beta-cells in type II diabetes mellitus. Diabetes 1991;40:166-80.

[2] Lillioja S, Mott DM, Spraul M, et al. Insulin resistance and insulin secretory dysfunction as precursors of non–insulin-dependent diabetes mellitus. Prospective studies of Pima Indians. N Engl J Med 1993;329: 1988-92.

[3] DeFronzo RA. Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM. Diabetes 1988;37:667-87.

[4] Mandavilli A, Cyranoski D. Asia's big problem. Nat Med 2004;10: 325-7.

[5] Fukushima M, Suzuki H, Seino Y. Insulin secretion capacity in the development from normal glucose tolerance to type 2 diabetes. Diabetes Res Clin Pract 2004;66:S37-43.

[6] Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 1998;15:539-53.

[7] Suzuki H, Fukushima M, Usami M, et al. Factors responsible for development from normal glucose tolerance to isolated postchallenge hyperglycemia. Diabetes Care 2003;26:1211-5.

[8] Fukushima M, Usami M, Ikeda M, et al. Insulin secretion and insulin sensitivity at different stages of glucose tolerance: a cross-sectional study of Japanese type 2 diabetes. Metabolism 2004;53:831-5.

[9] Nishi Y, Fukushima M, Suzuki H, et al. Insulin secretion and insulin sensitivity in Japanese subjects with impaired fasting glucose and isolated fasting hyperglycemia. Diabetes Res Clin Pract 2005;70: 46-52.

[10] Izuka M, Fukushima M, Taniguchi A, et al. Factors responsible for glucose intolerance in Japanese subjects with impaired fasting glucose. Horm Metab Res 2007;39:41-5.

[11] Wiener K, Roberts NB. Age does not influence levels of HbA1c in normal subject. QJM 1999;92:169-73.

[12] Chang AM, Halter JB. Aging and insulin secretion. Am J Physiol Endocrinol Metab 2003;284:E7-E12.

[13] Paolisso G, Tagliamonte MR, Rizzo MR, et al. Advancing age and insulin resistance: new facts about an ancient history. Eur J Clin Invest 1999;29:758-69.

[14] Coon PJ, Rogus EM, Drinkwater D, et al. Role of body fat distribution in the decline in insulin sensitivity and glucose tolerance with age. J Clin Endocrinol Metab 1992;75:1125-32.

[15] Kuroe A, Fukushima M, Usami M, et al. Impaired beta-cell function and insulin sensitivity in Japanese subjects with normal glucose tolerance. Diabetes Res Clin Pract 2003;59:71-7.

[16] Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care 2003;26:S5-S20.

[17] Taniguchi A, Fukushima M, Sakai M, et al. Remnant-like particle cholesterol, triglycerides, and insulin resistance in nonobese Japanese type 2 diabetic patients. Diabetes Care 2000;23:1766-9.

[18] Bonora E, Targher G, Alberiche M, et al. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. Diabetes Care 2000;23:57-63.

[19] Matthews DR, Hosker JP, Rudenski AS, et al. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; 28:412-9.

[20] Fukushima M, Taniguchi A, Sakai M, et al. Homeostasis model assessment as a clinical index of insulin resistance. Comparison with the minimal model analysis. Diabetes Care 1999;22:1911-2.

[21] Ohmura T, Ueda K, Kiyohara Y, et al. The association of the insulin resistance syndrome with impaired glucose tolerance and NIDDM in the Japanese general population: the Hisayama study. Diabetologia 1994;37:897-904.

[22] Tominaga M, Eguchi H, Manaka H, et al. Impaired glucose tolerance is a risk factor for cardiovascular disease, but not impaired fasting glucose. The Funagata diabetes study. Diabetes Care 1999;22:920-4.

[23] Ministry of Health, Labour and Welfare Statistical database: Statistics and Information Department, second edition, chapter 1 "Public health "

[24] Williams JW, Zimmet PZ, Shaw JE, et al. Gender differences in the prevalence of impaired fasting glycaemia and impaired glucose tolerance in Mauritius. Does sex matter? Diabet Med 2003;20:915-20.

[25] Schianca GP, Castello L, Rapetti R, et al. Insulin sensitivity: gender-related differences in subjects with normal glucose tolerance. Nutr Metab Cardiovasc Dis 2006;16:339-44.

[26] Rutter MK, Parise H, Benjamin EJ, et al. Impact of glucose intolerance and insulin resistance on cardiac structure and function: sex-related differences in the Framingham Heart Study. Circulation 2003;107: 448-54.

[27] Yates AP, Laing I. Age-related increase in haemoglobin A1c and fasting plasma glucose is accompanied by a decrease in beta cell function without change in insulin sensitivity: evidence from a cross-sectional study of hospital personnel. Diabet Med 2002;19:254-8.

[28] DeNino WF, Tchernof A, Dionne IJ, et al. Contribution of abdominal adiposity to age-related differences in insulin sensitivity and plasma lipids in healthy nonobese women. Diabetes Care 2001;24:925-32.

[29] Bryhni B, Jenssen TG, Olafsen K, et al. Age or waist as determinant of insulin action? Metabolism 2003;52:850-7.

[30] Bando Y, Ushiogi Y, Okafuji K, et al. The relationship of fasting plasma glucose values and other variables to 2-h postload plasma glucose in Japanese subjects. Diabetes Care 2001;24:1156-60.

[31] Utzschneider KM, Carr DB, Hull RL, et al. Impact of intra-abdominal fat and age on insulin sensitivity and beta-cell function. Diabetes 2004; 53:2867-72.

[32] Taniguchi A, Fukushima M, Sakai M, et al. The role of the body mass index and triglyceride levels in identifying insulin-sensitive and insulin-resistant variants in Japanese non–insulin-dependent diabetic patients. Metabolism 2000;49:1001-5.

[33] Kuczmarski MF, Kuczmarski RJ, Najjar M. Effects of age on validity of self-reported height, weight, and body mass index: findings from the Third National Health and Nutrition Examination Survey, 1988-1994. J Am Diet Assoc 2001;101:28-34.

[34] Flegal KM, Carroll MD, Ogden CL, et al. Prevalence and trends in obesity among US adults, 1999-2000. JAMA 2002;288:1723-7.

[35] Qiao Q, Nakagami T, Tuomilehto J, et al. Comparison of the fasting and the 2-h glucose criteria for diabetes in different Asian cohorts. Diabetologia 2000;43:1470-5.

[36] Piche ME, Arcand-Bosse JF, Despres JP, et al. What is a normal glucose value? Differences in indexes of plasma glucose homeostasis in subjects with normal fasting glucose. Diabetes Care 2004;27:2470-7.

[37] Sato Y, Komatsu M, Katakura M, et al. Diminution of early insulin response to glucose in subjects with normal but minimally elevated fasting plasma glucose. Evidence for early beta-cell dysfunction. Diabet Med 2002;19:566-71.

[38] Godsland IF, Jeffs JA, Johnston DG. Loss of beta cell function as fasting glucose increases in the non-diabetic range. Diabetologia 2004; 47:1157-66.

[39] Mitsui R, Fukushima M, Nishi Y. Factors responsible for deteriorating glucose tolerance in newly diagnosed type 2 diabetes in Japanese men. Metabolism 2006;55:53-8.

[40] Genuth S, Alberti KG, Bennett P, et al. Follow-up report on the diagnosis of diabetes mellitus. Diabetes Care 2003;26:3160-7.

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Factors responsible for elevation of 1-h postchallenge plasma glucose levels in Japanese men

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ABSTRACT

The 1-h postchallenge plasma glucose (1-h PG) level is considered to be a good index of the development of glucose intolerance and type 2 diabetes as well as of diabetic complications. In some cases, in Japanese, 1-h PG is elevated despite normal fasting glucose during oral glucose tolerance test (OGTT), but the factors responsible remain unclear. In the present study, subjects with normal glucose tolerance (NGT), isolated impaired fasting glucose (IFG), and isolated impaired glucose tolerance (IGT) were divided into subgroups at 1-h PG of 10.0 mM, and the four indices of insulin secretion and insulin sensitivity were compared. In all three categories, the insulinogenic index in subjects with elevated 1-h PG was remarkably lower than in those without elevated 1-h PG. In addition, the insulinogenic index was the strongest factor in elevated 1-h PG according to the multiple regression analysis. Interestingly, one third of the NGT subjects enrolled in this study had elevated 1-h PG. These subjects showed significantly elevated area under the curve of glucose (G-AUC) compared to NGT subjects without 1-h PG elevation. Thus, elevated 1-h PG in Japanese subjects indicates mildly impaired glucose tolerance due to decreased early-phase insulin secretion.

1. Introduction

Type 2 diabetes is characterized by both decreased insulin secretion and reduced insulin sensitivity [1–3]. Some patients with glucose intolerance leading to type 2 diabetes show elevated postchallenge plasma glucose without elevated fasting glucose during oral glucose tolerance test (OGTT) [4–6]. Although elevated 1-h postchallenge plasma glucose involves a different regulatory mechanism than 2-h postchallenge plasma glucose (2-h PG), 1-h postchallenge plasma glucose (1-h PG) is also as reliable an index of glucose tolerance as 2-h PG generally [7,8]. However, the relevance of 1-h PG and 2-h PG for diabetes screening is controversial [9,10]. It has been reported that subjects with 1-h PG higher than 10.0 mmol/l show higher risk of developing diabetes than subjects with lower 1-h PG [11]. In addition, 1-h PG higher than 11.2 mmol/l was found to be an independent risk factor for mortality in cardiovascular disease [12–14]. It was recommended in a

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number of studies that subjects having normal fasting plasma glucose at OGTT together with high 1-h PG are followed as carefully as IGT subjects in cases of higher frequency of elevated HbA1c, hypertension, family history of diabetes, or peripheral vascular involvement [15]. In addition, 1-h PG is used in diagnosis of gestational diabetes mellitus (GDM) and risk of macrosomia and other perinatal complications [16,17].

In the present study, the insulin secretion and insulin sensitivity indices of Japanese subjects undergoing OGTT in three WHO categories, normal glucose tolerance (NGT), isolated impaired fasting glucose (IFG) and isolated impaired glucose tolerance (IGT), subdivided at 1-h PG of 10.0 mmol/l were evaluated and compared.

2. Subjects and methods

2.1. Subjects

We recruited subjects undergoing OGTT because of positive urine glucose test, >5.0% HbA1c level, >5.6 mmol/l fasting plasma glucose level, and family history of diabetes at initial examination for medical check-up at Kyoto University Hospital, Ikeda Hospital, Kansai Electric Power Hospital, Kansai Health Management Center, and Kyoto Preventive Medical Center from 1993 to 2005. Subjects in the three categories of glucose tolerance, NGT ($n = 179$: fasting plasma glucose (FPG) level < 6.1 mmol/l and 2-h PG level < 7.8 mmol/l), isolated IFG ($n = 44$: FPG level of 6.1–7.0 mmol/l and 2-h PG < 7.8 mmol/l), and isolated IGT ($n = 103$: FPG level < 6.1 mmol/l and 2-h PG level of 7.8–11.1 mmol/l) according to the diagnostic criteria of World Health Organization in 1998 [18] were enrolled in the study. All subjects were men with no signs of hypertension, hepatic or renal dysfunction, endocrine or malignant disease, engaging in heavy exercise, history of gastrectomy, or history of medication known to affect glucose metabolism. The study was designed in compliance with the ethics regulations of the Helsinki Declaration. After the subjects fasted overnight for 10–16 h, standard OGTT with 75 g glucose was administered according to the National Diabetes Data Group recommendations [16].

The three WHO categories of glucose tolerance were divided into subgroups at 1-h PG of 10.0 mmol/l in this study: NGT with higher 1-h plasma glucose (NGT-HG: NGT criteria and 1-h PG ≥10.0 mmol/l), NGT with lower 1-h plasma glucose (NGT-LG: NGT criteria and 1-h PG <10.0 mmol/l), isolated IFG with higher 1-h plasma glucose (IFG-HG: IFG criteria and 1-h PG ≥10.0 mmol/l), isolated IFG with lower 1-h plasma glucose (IFG-LG: IFG criteria and 1-h PG <10.0 mmol/l), isolated IGT with higher 1-h plasma glucose (IGT-HG: IGT criteria and 1-h PG ≥10.0 mmol/l), and isolated IGT with lower 1-h plasma glucose (IGT-LG: IGT criteria and 1-h PG <10.0 mmol/l).

2.2. Laboratory examination

Blood samples were collected at 0, 30, 60, and 120 min after OGTT, and plasma glucose and serum insulin levels were measured for all subjects. Plasma glucose and serum insulin levels at 90 min were measured for 75 NGT subjects. Blood samples for measurements of HbA1c, total cholesterol, HDL cholesterol, and triglycerides were drawn after an overnight fast.

The plasma glucose level was measured by glucose oxidase method using the Hitachi Automatic Clinical Analyzer 7170 (Hitachi, Tokyo, Japan). Serum insulin was measured by two-site radioimmunoassay (Insulin Riabead II, Dainabot, Tokyo, Japan) as reported previously [19]. Serum total cholesterol and triglycerides levels were measured as reported previously [20].

2.3. Measurement

Basal insulin secretion and sensitivity were evaluated by HOMA β-cell and HOMA-IR [21,22], respectively. Early-phase insulin secretion and systemic insulin sensitivity during OGTT were evaluated by insulinogenic index [23] and ISI composite [24,25]. The calculations were as follows:

$$\text{HOMA } \beta\text{-cell} = \frac{20 \times \text{fasting serum insulin level (FI) (mU/l)}}{\text{fasting plasma glucose level (FPG) (mmol/l)} - 3.5}$$

$$\text{HOMA-IR} = \frac{\text{FI (mU/l)} \times \text{FPG (mmol/l)}}{22.5}$$

$$\text{Insulinogenic index} = \frac{\text{30-min insulin} - \text{FI (pmol/l)}}{\text{30-min plasma glucose} - \text{FPG (mmol/l)}}$$

$$\text{ISI composite} = \frac{10{,}000}{[\text{FPG (mg/dl)}\,\text{FI (mU/ml)} \times \text{mean OGTT glucose (mg/dl)} \times \text{mean OGTT serum insulin (mU/ml)}]^{0.5}}$$

2.4. Statistical analysis

All analyses were performed using STATVIEW 5 system (Stat View, Berkeley, CA). Differences between two groups were assessed by unpaired t-test in terms of age, BMI, plasma glucose level, serum insulin level, HbA1c, triglyceride, total cholesterol, insulinogenic index, ISI composite, HOMA-IR, and HOMA β-cell. We used simple regression analysis and multiple regression analysis for comparison of the relationship between 1-h PG and the indices of insulin secretion and sensitivity. Probability (p) values less than 0.05 were considered statistically significant. Data are presented as mean ± S.E.

3. Results

Table 1 shows the clinical and metabolic characteristics of the six subgroups. NGT-HG had higher average age, BMI, FPG, 2-h PG and HbA1c than NGT-LG. IFG-HG had higher BMI than IFG-LG. IGT-HG had higher BMI, FPG, 2-h PG, 1-h insulin and HbA1c than IGT-LG. There were no significant differences in insulin (fasting and 2-h), triglycerides, total cholesterol and HDL-cholesterol levels between the two subgroups of NGT, isolated IFG, and isolated IGT.

The insulin secretion indices of insulinogenic index and HOMA β-cell indices in the three WHO categories are shown in