and retinal detachment that resemble human diabetic ocular complications [4]. In the present study, we studied whether they develop gastroenteropathy with diarrhea by morphological and functional analysis.

2. Materials and methods

2.1. Animals

Male SDT rats were obtained from Research Laboratories of Torii Pharmaceutical Co. Ltd. Age-matched male Sprague Dawley (SD) rats (Crj: CD(SD)IGS, Charles River Japan, Kanagawa, Japan) were used as control animals. All rats were housed in a pathogen free, air-controlled room (25 ± 2 °C and 50% humidity) with 12-h light/12-h dark cycle. The studies were performed in accordance with the Declaration of Helsinki. The diagnostic criteria for diabetes were the presence of glucosuria and hyperglycemia characterized by non-fasting blood glucose concentration exceeding 200 mg/dl. The diabetic rats were divided into two groups, untreated SDT rats ($n = 8$) and insulin-treated SDT (SDT-INS) rats ($n = 8$). The plasma glucose concentration was measured and doses of ultralente insulin (2–12 U/day: Shimizu Pharmaceutical, Shizuoka, Japan) was determined using a sliding scale from 20 weeks of age.

2.2. Intestinal motility of small intestine

Transit distance through the stomach and small intestine was measured by a non-absorbed-marker (10% charcoal suspension in 5% gum Arabic), as previously described [7–9]. Briefly, food was withheld for 16 h before the experiment, with free access to drinking water, and the rats received the suspension orally by gavage using a straight blunt-ended feeding needle. After 20 min, the animals were killed by cervical dislocation, and the entire gastrointestinal tract was removed. The distance from the pylorus to the front of the charcoal bolus and the ileocecal junction was measured. The rate of transit was determined as [(distance to charcoal front)/(length of small intestine)] × 100 (%) [9].

2.3. Weight of bowel segment

The animals were anesthetized with pentobarbital administrated intramuscularly. Ten-centimeter segments of jejunum, beginning 5 cm distal to the ligament of Treitz and 10-cm segments of ileum, ending 5 cm proximal to the ileocecal valve (measured as accurately as possible by ruler; the intestine in these conditions is in a relaxed, non-contracted state), were removed and weighed. The full length of the villi from the tip to the bottom was removed by scraping firmly with a glass slide and weighed.

2.4. Morphometric measurements

The circumference of the small intestine was determined by measuring the width at three points of the intestine, which was flattened and stapled onto cardboard. The sheets of intestine were then fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. Mucosal thickness (villus tip to crypt bottom) was measured on at least three representative villus-crypt units per section; only well-oriented sections were used. All measurements were made by the same researcher on coded sections [10].

2.5. Estimation of blood glucose levels and fecal water content

Blood glucose levels were measured using Novo-assist (LIFE SCAN Inc., Milpitas, CA) to sample from the tail vein. Feces were sampled immediately after defecation.

Fecal water content was calculated as [(fecal net weight − fecal dry weight after being freeze-dried)/fecal net weight] × 100 (%). Body weight and blood glucose levels were measured every week from 14 to 28 weeks of age. The percentage of fecal water content was measured at 14 and 28 weeks of age.

2.6. Fecal fat content

Feces were lyophilized and ground. Fat content in feces was determined by extraction with diethyl ether after acid hydrolysis (4 M HCl) for 30 min [11].

2.7. Statistical analyses

Significant difference was determined using one-factor ANOVA followed by t-test. P values of <0.05 were considered statistically significant. All values are expressed as mean ± standard error of the mean (S.E.M., n = number).

2.8. Ethical considerations

All studies were performed in the laboratories of the Department of Diabetes and Clinical Nutrition, Kyoto University, in accordance with the Declaration of Helsinki.

3. Results

3.1. Body weight and blood glucose

Body weights of the control SD rats and the SDT and SDT-INS rats are compared in Fig. 1A. The average body weight of SD, SDT, and SDT-INS rats was similar at 18 weeks of age (525 ± 3, 534 ± 5, and 535 ± 10 g, respectively). The body weights of both the SDT and SDT-INS rats then became significantly less than controls. At 28 weeks of age, the body weight of the SDT rats was significantly lower than both SD (447 ± 33 g versus 657 ± 8 g, $P < 0.01$) and SDT-INS rats (447 ± 3 g versus 607 ± 9 g, $P < 0.01$). Although the body weight of the SDT-INS rats increased after 20 weeks of age, it was

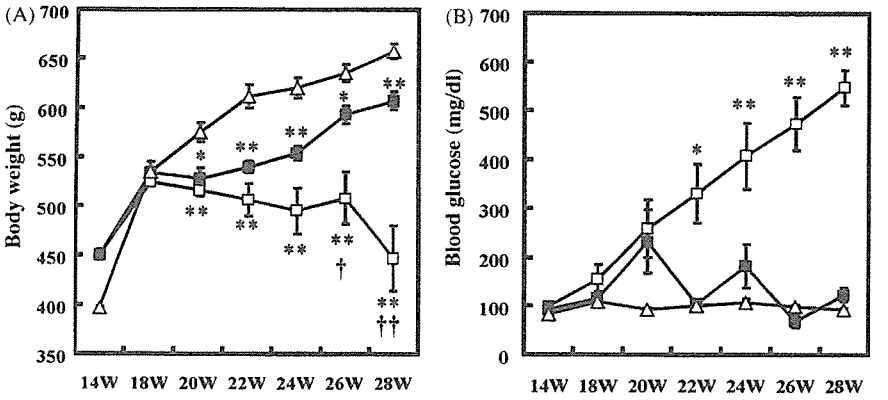

Fig. 1. Body weight and blood glucose levels in SD rats, SDT rats, and SDT-INS rats from 14 to 28 weeks of age. (A) Body weight; (B) blood glucose level. (△) SD rats; (□) SDT rats; (■) SDT-INS rats. All values are expressed as means ± S.E.M. ($n = 8$ for each group). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the value of SD rats; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the value of SDT-INS rats.

nevertheless significantly lower than in SD rats from 20 to 28 weeks of age (607 ± 9 g versus 657 ± 7 g at 28 weeks, $P < 0.01$). Food intake in SDT rats and SD rats at 28 weeks was 32 ± 3 and 23 ± 1 g/day, respectively ($P < 0.05$).

Non-fasting blood glucose levels of the control SD and the SDT and SDT-INS rats are compared in Fig. 1B. The blood glucose levels of SDT and SDT-INS rats were similar at 20 weeks of age (258 ± 59 and 231 ± 65 mg/dl, respectively), while the blood glucose level of the control SD rats was significantly lower (93 ± 2 mg/dl). The blood glucose levels of the SDT rats increased linearly with advancing age (547 ± 36 mg/dl at 28 weeks), while those of the SDT-INS rats remained under 200 mg/dl after 20 weeks. The control SD rats maintained normoglycemia for the duration of the study. The non-fasting blood glucose level of the SDT rats at 28 weeks (547 ± 36 mg/dl) was significantly higher than in both SD rats (93 ± 3 mg/dl) and SDT-INS rats (123 ± 13 mg/dl) ($P < 0.01$).

3.2. Morphological changes and weight changes

Laparotomy reveals a larger intestine in SDT rats compared to control SD rats at 28 weeks (Fig. 2).

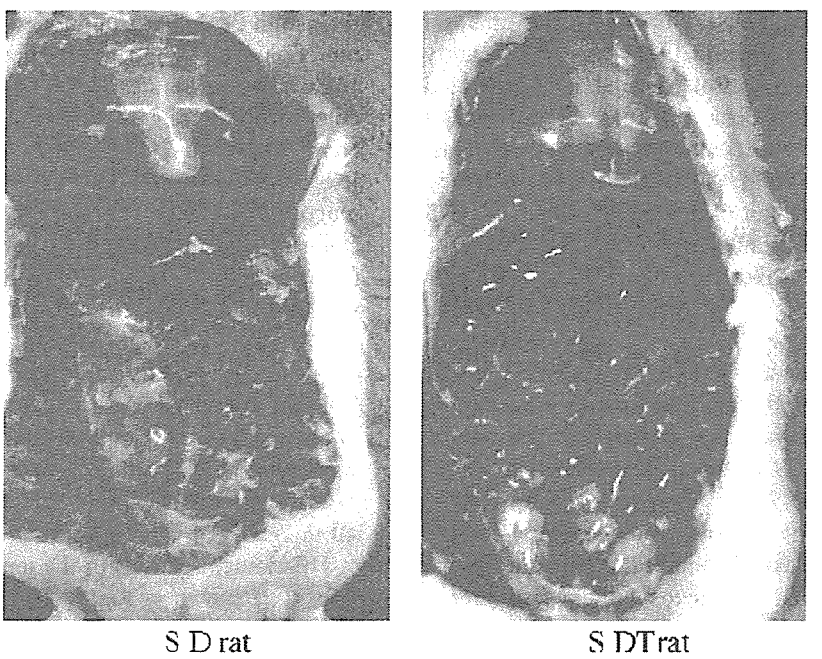

Fig. 2. Laparotomy shows that the intestine of the SDT rats was enlarged at 28 weeks of age compared to control SD rats.

Table 1
Morphological differences and weight differences in SD, SDT, and SDT-INS rats at 28 weeks of age in jejunum (A), ileum (B), and proximal colon (C)

	SD	SDT	SDT-INS
(A) Jejunum			
Circumference (mm)	7.5 ± 0.3	15.0 ± 1.4**	9.6 ± 0.8†
Mucosal thickness (μm)	594 ± 18	963 ± 15**	829 ± 12**,††
Mucosal weight (mg/10 cm)	619 ± 63	1617 ± 87**	772 ± 132††
Mucosal weight (% of intestinal weight)	73 ± 3	80 ± 1*	68 ± 3†
Intestinal weight (mg/10 cm)	846 ± 73	2017 ± 87**	1112 ± 160††
(B) Ileum			
Circumference (mm)	7.8 ± 0.5	14.5 ± 0.6**	11.8 ± 0.7**,†
Mucosal thickness (μm)	544 ± 25	867 ± 23**	693 ± 38*,†
Mucosal weight (mg/10 cm)	558 ± 91	1483 ± 91**	854 ± 114††
Mucosal weight (% of intestinal weight)	65 ± 4	74 ± 4	66 ± 5
Intestinal weight (mg/10 cm)	848 ± 88	2017 ± 117**	1284 ± 121*,††
(C) Proximal colon			
Circumference (mm)	11.0 ± 0.4	13.2 ± 0.6*	12.8 ± 0.6*
Mucosal thickness (μm)	153 ± 32	373 ± 13**	293 ± 3*,†
Mucosal weight (mg/10 cm)	729 ± 29	1003 ± 71*	804 ± 61
Mucosal weight (% of colon weight)	70 ± 4	67 ± 6	55 ± 4*
Colon weight (mg/10 cm)	1041 ± 62	1517 ± 87**	1460 ± 81**

SDT rats had the largest circumference, mucosal thickness, mucosal weight, and intestinal weight in jejunum and ileum and colon. Insulin treatment ameliorated all of the deteriorated morphological parameters of jejunum and ileum in SDT rats. Values are expressed as means ± S.E.M. ($n = 8$ for each group). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the value of SD rats; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the value of SDT rats.

The morphological changes and weight changes of control SD and SDT and SDT-INS rats are summarized in Table 1.

Jejunum (Table 1(A)): The circumference of jejunum in SDT rats was significantly greater than in both control SD rats and SDT-INS rats. The mucosal thickness of jejunum of SDT rats was significantly greater than in both control SD and insulin-treated SDT-INS rats. The mucosal thickness of jejunum of SDT-INS rats was significantly greater than in SD rats. The mucosal weight of jejunum in SDT rats was significantly higher than in control SD and SDT-INS rats. The intestinal weight of jejunum of untreated SDT rats was significantly higher than in both SD and SDT-INS rats.

Insulin treatment ameliorated all of the deteriorated morphological parameters of jejunum.

Ileum (Table 1(B)): The circumference of the ileum in SDT rats was significantly greater than in both control SD and SDT-INS rats. The circumference of ileum of SDT-INS rats was significantly greater than in SD rats. The mucosal thickness of ileum in SDT rats was significantly greater than in both control SD and SDT-INS rats. The mucosal thickness of ileum of SDT-INS rats was significantly greater than in SD rats. The mucosal weight of ileum in the untreated SDT rats was significantly higher than in both control and SDT-INS rats. The intestinal weight of the SDT rats also was significantly higher than in both control SD and SDT-INS rats. The intestinal weight of ileum in SDT-INS rats was significantly greater than in SD rats.

Insulin treatment ameliorated all of the deteriorated morphological parameters of ileum.

Proximal colon (Table 1(C)): The circumference of colon in SDT rats was significantly greater than in control SD rats. The circumference of colon in SDT-INS rats was significantly greater than in SD rats. The mucosal thickness of colon in SDT rats was significantly greater than in both control SD and SDT-INS rats. The mucosal thickness of colon of SDT-INS rats was significantly greater than in SD rats.

The mucosal weight of colon in the untreated SDT rats was significantly higher than in control SD rats. Colon weight of SDT rats was significantly higher than in control SD rats. Colon weight of SDT-INS rats was significantly higher than in control SD rats.

Insulin treatment ameliorated deterioration in mucosal thickness of colon.

HE-stained sections of the jejunum and ileum at 28 weeks are shown in Fig. 3. Villi of both jejunum and ileum were significantly longer in SDT rats than in SD rats and SDT-INS rats. Mucosal inflammation was not seen in SDT and SD rats.

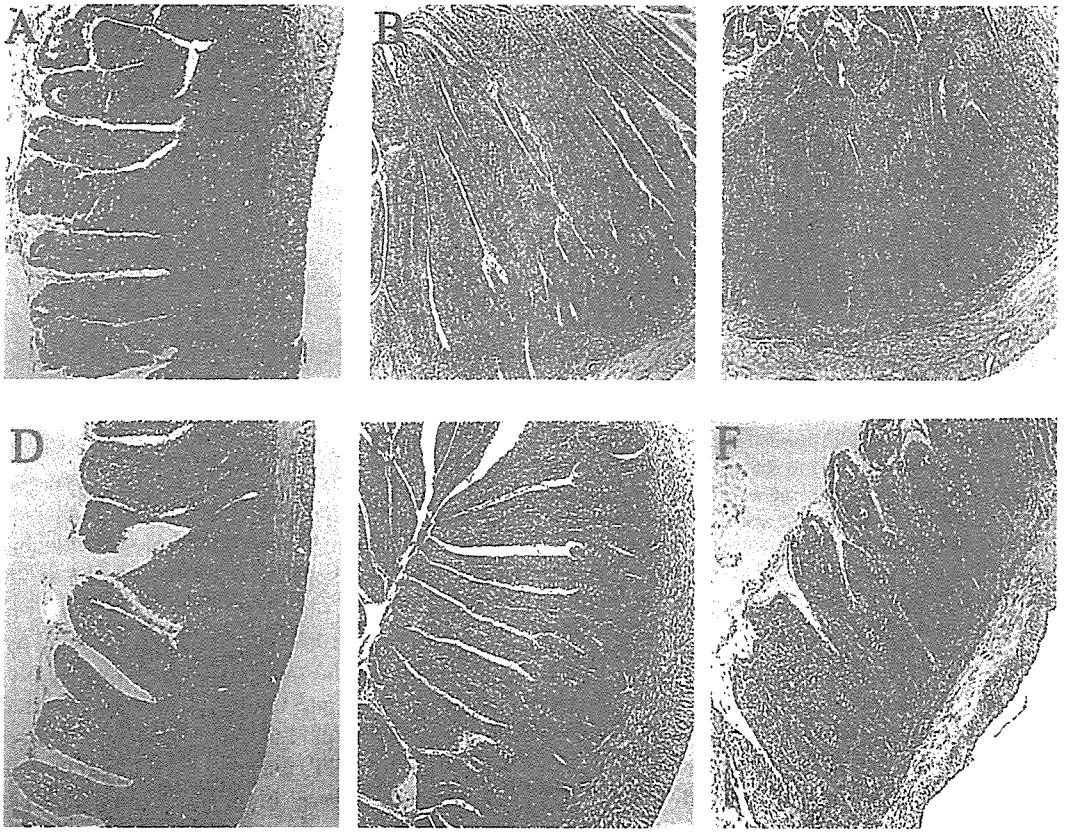

Fig. 3. Morphology of small intestine using HE-stained sections. Villi at 28 weeks of age were significantly longer in SDT rats than in SD rats and in SDT-INS rats. Jejunum in SD rats (A), SDT rats (B), and SDT-INS rats (C). Ileum in SD rats (D), SDT rats (E), and SDT-INS rats (F) (original magnification 100×).

3.3. Fecal water content percentage

At 14 weeks of age, the ratio of fecal water to fecal solid of SD (53.8 ± 1.8%), SDT (49.8 ± 1.3%), and SDT-INS rats (51.8 ± 1.4%) showed no significant difference (Fig. 4). At 28 weeks, the fecal water content of the SDT rats (73.6 ± 3.3%) was significantly higher than in both SD rats (51.5 ± 2.5%) and SDT-INS rats (54.4 ± 3.2%) ($P < 0.01$).

To exclude the possibilty of a pancreatogenic cause of the impaired stool, we measured fecal fat content. At 28 weeks, the fecal fat content (g/100 g feces) of SD (11.1 ± 1.6), SDT (11.3 ± 2.3) and SDT-INS rats (11.6 ± 2.0) was similar.

3.4. Intestinal motility of small intestine

The intestinal transit rate was evaluated by comparing the length of small intestine traversed by the charcoal suspension. Intestinal transit distance in untreated SDT rats was significantly greater than in control SD rats (54.1 ± 2.6% versus 43.0 ± 1.2%, $P < 0.05$) and treated SDT-INS rats, while that in SD and SDT-INS rats was similar (Fig. 5).

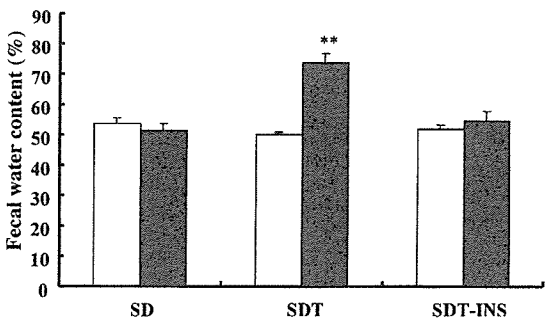

Fig. 4. Fecal water content in SD, SDT, and SDT-INS rats at 14 and 28 weeks. The ratio of fecal water to fecal solids in SDT rats was significantly higher than in SDT-INS and SD rats at 28 weeks of age. There was no significant difference among the three groups at 14 weeks (□, 14 weeks; ■, 28 weeks). Data are shown as means with S.E.M. ($n = 8$ for each group). $^{**}P < 0.01$ compared with the value of SD rats.

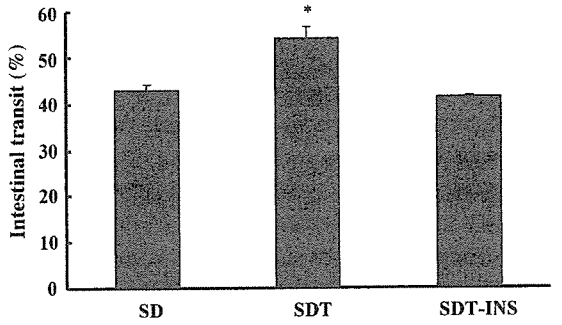

Fig. 5. Intestinal motility after oral administration of non-absorbed marker (10% charcoal suspension in 5% gum Arabic). Twenty minutes after administration by gavage, the animals were killed and the entire gastrointestinal tract removed. The distance from the pylorus to the front of the charcoal bolus and to the ileocecal junction was measured. The rate of transit was determined [(distance to charcoal front)/(length of small intestine)] × 100 and expressed as percent. Intestinal transit distance in the SDT rats was significantly greater than that in SDT-INS and SD rats at 28 weeks. Data are shown as means with S.E.M. ($n = 8$ for each group). $^{*}P < 0.05$ compared with the value of SD rats.

4. Discussion

STZ rats [12], BB rats [13] and non-obese diabetic (NOD) mice [14] have been reported to develop diarrhea, but non-infectious diarrhea in the spontaneously diabetic Torii rat has not been investigated. This report is the first characterization of type 2 diabetes in rats exhibiting diarrhea. In this study, SDT rats developed loose stool characteristic of diarrhea together with rapid gastrointestinal transit at 28 weeks of age.

Patients with diabetic gastroenteropathy show a wide range of symptoms including dysphagia, nausea, vomiting, abdominal pain, diarrhea, constipation, and fecal incontinence [15–18]. The frequency of these gastrointestinal manifestations in diabetic patients ranges between 25% and 76% [15–18]. In some cases, the symptoms severely impair glycemic control [19], which in turn increases the risk of hyper/hypoglycemia and the severity of consequent complications of chronic diabetes. In extreme cases, the symptoms cause malnutrition.

Diarrhea occurs in approximately 10% of diabetic patients [20], most of whom are young to middle-aged, poorly controlled, requiring insulin, and having peripheral or autonomic neuropathy [21]. However, these patients usually show minimal or no fat malabsorption [22].

SDT rats exhibited more rapid intestinal transit, indicating that the diarrhea is partly attributable to impaired gastrointestinal motility, which is regulated mainly by the enteric autonomic nerve. Recently, several groups have reported impairment of NO nerve in the alimentary tract of diabetic rats. Yoneda et al. reported enchanced colonic peristalsis due to impaired nitrergic enteric neurons in OLETF rats [23]. Martinez-Cuesta et al. has reported impaired nitrergic-mediated relaxation of rat isolated duodenum in STZ-diabetic rats [24]. In addition, recent evidence strongly suggests that interstitial cells of Cajal are central in the control of gastrointestinal motility [25,26]. He et al. reported loss of interstitial cells of Cajal in jejunum and inhibitory innervation in insulin-dependent diabetes in human [27]. Nakahara et al. reported loss of interstitial cells of Cajal, which express c-kit receptor tyrosine kinase, in the colon of patients with diabetes [28]. Further investigation is required to determine if such abnormalities are found in SDT rats.

SDT rats show markedly higher hyperglycemia than other animal models of human type 2 diabetes [1–3]. In this study, non-fasting blood glucose levels in the SDT rats reached approximately 600 mg/dl at 28 weeks. In addition, STZ-diabetic rats with diarrhea were reported to a show non-fasting blood glucose level over 500 mg/dl [29]. On the other hand, non-fasting blood glucose levels in GK and OLETF rats were reported to be less than 300 mg/dl. Such chronic and extreme hyperglycemia in SDT rats may well damage the enteric autonomic nerve, but the pathogenesis remains to be elucidated. In the present study, while daily insulin therapy was insufficient to maintain normoglycemia, partial glycemic control seems to have inhibited the progression of impaired intestinal motility in the treated SDT rats, supporting the possibility that the diabetic gastroenteropathy in SDT rats is due to hyperglycemia. Thus, while the cause of the impaired intestinal motility and the consequent diarrhea in the SDT rats is unclear, it may well be due to the extreme hyperglycemia.

BB rats, a model of type 1 diabetes, have been reported to exhibit diarrhea. A striking ultrastructual change in the vagus nerve, the presence of axonal glycogenosomes in unmyelinated fibers, was reported in diabetic BB rats by Yagihashi et al. [30].

Atrophic myelinated axons also were seen. Schmidt et al. reported degeneration of unmyelinated axons in the colonic submucosa and muscularis in STZ diabetic rats [29]. Further investigation is required to determine if such abnormalities are found in the ultrastructure of the vagus nerve or colonic nerve plexus in SDT rats.

Several researchers have shown that insulin therapy is effective in diabetic neuropathy [31,32], but this is the first report that insulin treatment ameliorates the progression of gastroenteropathy in a spontaneous rat model of diabetes. Further study required to evaluate the efficacy of insulin in the treatment of diabetic gastroenteropathy. Morphological examination revealed intestinal epithelial

hyperplasia in SDT rats consistent with previous investigations of other animal models of both type1 and type2 diabetes [33–37].

Such hyperplasia may be due to decreased glucose utilization and hyperphagia according to those reports [37–39]. Indeed, the food intake of SDT rats was also greater than that of SD control rats in the present study. Insulin treatment clearly ameliorated intestinal epithelial hyperplasia in SDT rats. Mayhew et al. also has reported that insulin therapy improved intestinal morphology in STZ-diabetic rats [40].

The SDT rat is thus a potentially useful animal model for further investigation of diabetic gastroenteropathy.

Acknowledgements

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Adult pancreatic islets require differential pax6 gene dosage

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Abstract

Pax6, a paired homeodomain transcription factor, plays crucial roles in morphogenesis of eye, central nervous system, and pancreatic islets. Recently, heterozygosity for pax6 mutation has been reported in some individuals with glucose intolerance and aniridia. To investigate the role of pax6 for pancreatic islet function, we examined the pancreatic phenotype of small eye rat strain ($rSey^2$) with a point mutation in the pax6 locus resulting in truncated PAX6 proteins. Analyses of the insulin secretory profile of heterozygous $rSey^2/+$ revealed that insulin secretion is significantly increased in response to membrane-depolarizing stimuli such as arginine, tolbutamide, and KCl. The processes of insulin granule exocytosis were suggested to be enhanced in $rSey^2/+$. On the other hand, pancreatic insulin and glucagon content and islet architecture in $rSey^2/+$ showed no significant differences compared to wild-type. These findings indicate differential requirements for pax6 gene dosage in displaying function and maintaining architecture of adult pancreatic islets.

Keywords: Pax6; Pancreatic islets; Insulin secretion; Arginine; Small eye; Pancreas

Transcription factors playing a role in pancreatic development have been shown to orchestrate the process of cell differentiation and transition by regulating the expression of numbers of genes [1,2]. To form the pancreas and organize pancreatic islets, multiple transcription factors play roles at precise steps in the developmental program. Various models in which these transcription factors are inactivated have revealed defects of pancreatic development or pancreatic islet morphogenesis.

The paired homeobox (Pax) family of transcription factors is involved in embryonic development of many organs including eyes, brain, kidney, thyroid gland, immune system, and the pancreas [3,4]. Two of its members, Pax4 and Pax6, play important roles in pancreatic endocrine cell differentiation [5,6]. In addition, Pax6 is essential for the development of eye and central nervous system and regulates the expression of various functional molecules in these tissues [7]. During the mouse embryogenesis, PAX6 protein can be detected already around E9.0 in the pancreatic endoderm, and its expression is maintained throughout pancreas development in all endocrine cells [8]. Analyses of pax6 mutant animals (Fig. 1) have revealed that differentiation of endocrine cells and the forming of proper islet architecture are severely affected in the fetal pancreas in the homozygous state. Pax6 knockout mice lack glucagon-producing α-cells and do not form proper islet structure [6]. In Sey^{NEU} mice, in which the PAX6 protein is

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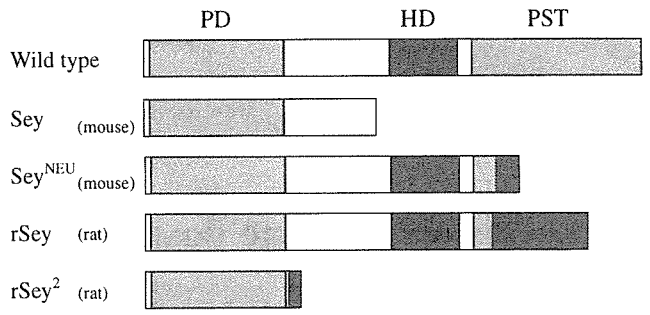

Fig. 1. Schematic diagram of coding region of pax6 gene and mutants. Wild-type PAX6 has a paired domain (PD), homeodomain (HD), and proline/serine/threonine rich transactivation domain (PST). Sey^{NEU} and rSey have a mutation, which results in a PAX6 protein that has a PD and HD but lacks the functional PST domain. Sey and $rSey^2$ (this study) have a mutation resulting in a PAX6 that has a PD but lacks the HD and PST domain completely.

truncated directly after the homeodomain, all four endocrine cell types are decreased in number [9]. Thus, Pax6 is involved in pancreatic development, particularly in endocrine cell differentiation and pancreatic islet organogenesis.

Transcription factors are involved not only in regulating pancreas development but also in pancreatic endocrine cell function. Many mutation models of transcription factors have shown that these mutations influence β-cell molecular events and the insulin secretory profile by altering the gene expression.

However, the role of PAX6 in adult islet function is little known except for the observations *in vitro* that PAX6 increases insulin, somatostatin, and glucagon gene transcription by binding with their promoters [9,10], and is involved in the regulation of enzymes and transcription factors [11,12]. The homozygous mice of pax6 knockout, Sey^{NEU} lack eyes and even model mice with conditional inactivation of pax6 in the endocrine pancreas [13] die shortly after birth, limiting the analyses of PAX6 function in the postnatal pancreatic islet function.

To investigate the mechanisms by which alterations in PAX6 affect islet function, we examined pancreatic islet function and architecture in small eye rat strain ($rSey^2$) with point mutation in the pax6 locus resulting in truncated PAX6 proteins [14] and found that in contrast to showing normal insulin secretion in response to glucose, $rSey^2/+$ showed surprisingly increased insulin release in response to membrane depolarizing stimuli and that $rSey^2/+$ had normal pancreatic islet architecture, indicating different requirement for pax6 gene dosage in the function and the morphology of the pancreatic islets.

Materials and methods

Animals. Mutant rats with small eyes ($rSey^2$) [14] were used in this study. Studies for the adult rats were performed in heterozygous ($rSey^2/+$) and their age-matched wild-type littermates. Homozygous rat embryos were obtained by inter-crossing male and female heterozygotes. Animal care and procedures were approved by the Animal Care Committee of Kyoto University.

Measurement of blood glucose, insulin, and glucagon levels. Blood glucose levels were measured by enzyme-electrode method. Plasma insulin levels were measured using ELISA kit (Shibayagi, Gunma, Japan). Plasma glucagon levels were measured using ELISA kit (Yanaihara Institute Inc., Shizuoka, Japan). Different groups of age-matched 20- to 24-week-old male rats were used for intraperitoneal glucose tolerance test. After an overnight fast, plasma insulin, glucagon, and glucose levels were measured and D-glucose (2 g/kg body weight) was loaded. In the insulin tolerance test, human insulin (1 U/kg) was injected subcutaneously in the fed condition. Blood samples were taken from the tail vein at indicated times.

Quantification of pancreatic peptide content. Protein was extracted from the dissected pancreas using acid extraction. Protein content was measured by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The amount of immunoreactive insulin was determined by RIA, using rat insulin as described [15]. The amount of immunoreactive glucagon was determined by using RIA kit (Linco Research, St. Charles, MO).

Immunohistochemistry. The pancreata of rats were removed under pentobarbital anesthesia (40 mg/kg body weight) and fixed in Bouin's solution. Pancreatic specimens were embedded in paraffin and sectioned at 3.5 μm. The avidin–biotin complex method with alkaline phosphatase or with peroxidase was used as previously described [16] with a slight modification. After deparaffinization, the following were sequentially applied: normal goat or rabbit serum (diluted to 1:75, Dako, Kyoto, Japan), primary antibodies, biotin-labeled goat anti-rabbit or rabbit anti-goat IgG serum (diluted to 1:300, Dako), and avidin–biotin–alkaline phosphatase complex or avidin–biotin–peroxidase complex (diluted to 1:100, Vector Laboratories, Burlingame, CA), followed by hematoxylin nuclear counterstaining. Staining was visualized in black and red by alkaline phosphatase substrate (Vector Laboratories) and in brown by peroxidase substrate. For PAX6 analysis, paraffin sections of pancreata were deparaffinized and autoclaved for 10 min at 121 °C in 10 mM citrate buffer (pH 6.0). The following primary antibodies were used: rabbit anti-insulin polyclonal antibody (diluted to 1:350, Dako), rabbit anti-glucagon serum (diluted to 1:500, OAL-123, Otsuka Assay Laboratory, Tokushima, Japan), rabbit anti-somatostatin polyclonal antibody (diluted to 1:200, Dako), rabbit anti-pancreatic polypeptide polyclonal antibody (diluted to 1:200, Dako), goat anti-GLUT2 polyclonal antibody (diluted to 1:50, C-19, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-PAX6 polyclonal antibody (diluted to 1:20, H-295, Santa Cruz Biotechnology).

Isolated pancreatic perfusion. The pancreas was isolated as previously described [17]. All perfusions were accomplished with Krebs–Ringer Bicarbonate Buffer (KRBB) containing 0.25% bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO) and 4.6% dextran (mean molecular weight 70,000; Pharmacia, Uppsala, Sweden). The perfusate was gassed with 95% O_2–5% CO_2 to maintain pH 7.4 at 37 °C. The flow rate was kept constant at 1.9 ml/min. After 20 min of equilibration, the perfusate was collected at 1-min intervals by cannula inserted into the portal vein. The collected effluent was frozen immediately with 1000 U aprotinin (Bayer, Leverkusen, German). The amount of immunoreactive insulin and immunoreactive glucagon was determined by RIA as described above.

Measurement of insulin release from isolated rat pancreatic islets. Isolated islets were cultured for 18 h in RPMI 1640 medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Insulin release from intact islets was monitored using batch incubation system described previously [18] with slight modifications. Cultured islets were preincubated at 37 °C for 30 min in KRBB supplemented with 2.8 mM glucose, 0.2% BSA, and 10 mM Hepes, adjusted to pH 7.4. Groups of 10 islets were then batch-incubated for 30 min in 0.4 ml of KRBB with test materials. The amount of immunoreactive insulin was determined by RIA as described above.

Measurement of intracellular Ca^{2+}. For intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurement, cultured islets were loaded with fura-PE3 during 2 h of preincubation in the presence of 2 μM fura-PE3AM (Calbiochem, La Jolla, LA) as previously described [18]. Islets were placed at 36 ± 1 °C, superfused with KRBB containing 2.8 mM glucose and 10 mM Hepes adjusted to pH 7.4 for 30 min, and subsequently exposed to the medium containing a high concentration of K^+. The islets were excited successively

at 340 and 380 nm, and fluorescence emitted at 510 nm was captured by a charge-coupled device (CCD) camera (Micro Max 5-MHz System; Roper Industries, Trenton, NJ). Fluorescence signals at 340-nm (F340) and 380-nm (F380) were detected every 20 s. Results are expressed as the ratios (F340/F380).

Measurement of insulin release from electrically permeabilized islets. Cultured islets were preincubated with KRBB with 2.8 mM glucose and 0.2% BSA for 30 min. The islets were washed twice in cold potassium aspartate buffer (KA buffer) containing 140 mM KA, 7 mM $MgSO_4$, 2.5 mM EGTA, 30 mM Hepes (pH 7.0), and 0.5% BSA, with $CaCl_2$ added to a Ca^{2+} concentration of 30 nM. The islets were then permeabilized by high voltage discharge (four exposures, each of 450 μs duration, to an electrical field of 4.0 kV/cm) in KA buffer and washed once with the same buffer. Groups of electrically permeabilized islets were then batch-incubated for 30 min at 37 °C in 0.4 ml KA buffer with various concentrations of Ca^{2+} and ATP. At the end of the incubation period, permeabilized islets were pelleted by centrifugation (15000*g*, 180 s), and aliquots of the buffer were sampled. The amount of immunoreactive insulin was determined by RIA as described above.

Statistical analysis. Results are expressed as means ± SE. Statistical significance was evaluated by unpaired Student's *t*-test. $P < 0.05$ was considered significant.

Results and discussion

One intact allele in the pax6 gene is sufficient for maintenance of adult pancreatic islet architecture

In fetal pancreas in the homozygous state ($rSey^2/rSey^2$), insulin-positive cells are remarkably reduced and the alignment of the endocrine cells is not preserved (Fig. 2A). Especially, few or no glucagon-positive cells were found in the pancreas, and glucagon content could not be detected in RIA study of pancreas extract (data not shown). Recently, it has been reported that PAX6 is important especially for the endocrine cells to obtain final differentiation, rather than to proliferate [13]. Pax6 knockout mice have been shown to completely lack glucagon-producing cells in fetal pancreas [6]. In contrast, in the homozygous (Sey^{NEU}/Sey^{NEU}) mice, in which the PAX6 protein has a paired domain and homeodomain but lacks the transactivation domain (Fig. 1), the number of α-cells is reduced but is still present in the late fetal stage [9]. In the homozygous Sey mice [19] and $rSey^2$ rats (this study), in which the PAX6 protein has a paired domain but lacks a homeodomain and transactivation domain (Fig. 1), few or no glucagon-positive cells were detected in the later fetal stage in the homozygous state. These findings suggest that homeodomain is especially important in the formation of the pancreatic α-cells. In contrast to the homozygous fetal pancreatic islets, immunohistochemical evaluation of pancreata from adult heterozygote rats ($rSey^2/+$) revealed normal islet morphology with insulin-positive β-cells located in the center of the islet (Fig. 2B and C), and glucagon-positive α-cells (Fig. 2B and C), somatostatin-positive δ-cells (data not shown), and pancreatic polypeptide-positive PP-cells (data not shown) located in the periphery of the islets. Pancreatic insulin and glucagon contents in $rSey^2/+$ were the same as in wild-type (data not shown). Because of a recent report showing that expression of the glucose transporter GLUT2 was down-regulated in the pancreas of conditional inactivation of pax6 model mice [13], we examined GLUT2 expression in $rSey^2/+$ pancreatic islets. Adult $rSey^2/+$

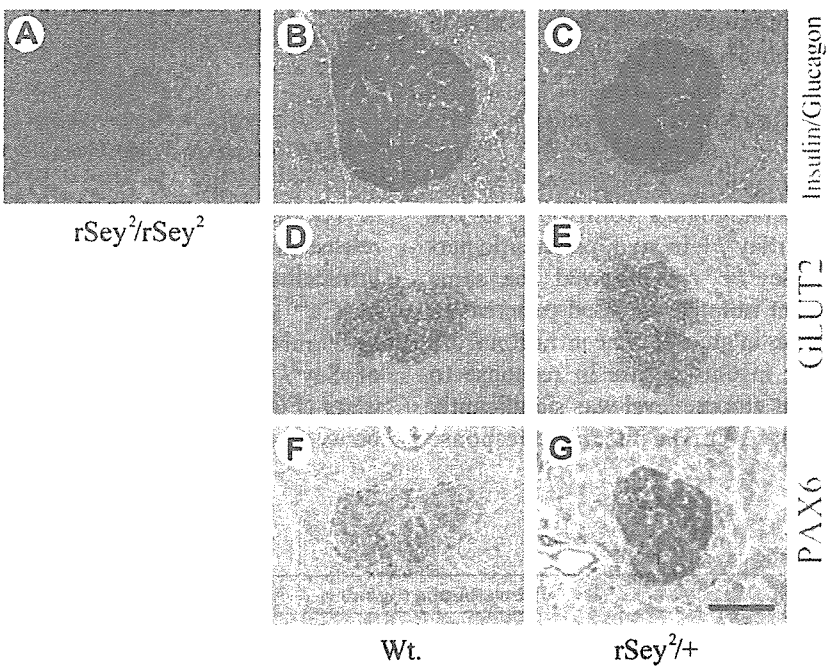

Fig. 2. One intact allele in the pax6 gene is sufficient for maintenance of adult pancreatic islet architecture. Immunohistochemical staining for islet protein was performed on paraffin-embedded sections of the pancreas. The sections were double-labeled for insulin (red) and glucagon (black) in the fetal (20.5E) homozygous state ($rSey^2/rSey^2$) (A) and adult wild-type (B), $rSey^2/+$ (C) rat pancreas or labeled for GLUT2 (D,E) and PAX6 (F,G) in the adult wild-type (D,F) and $rSey^2/+$ (E,G) rat pancreas. Bar 100 μm.

showed normal GLUT2 expression in the pancreatic endocrine cells (Fig. 2D and E). Thus, one allele of the wild-type pax6 gene is essential and sufficient to maintain morphologically normal pancreatic islets in adult. Anti-PAX6 antibody, which recognizes the C-terminus of PAX6 protein, was used to identify wild-type PAX6 protein derived from the wild-type pax6 allele, and rSey^2/+ was found to have similar PAX6 protein expression pattern in the nuclei of pancreatic islets (Fig. 2F and G).

Glucose-induced insulin secretion is preserved in heterozygous small eye rat strain (rSey^2/+)

Both male and female rSey^2/+ had similar body weight as wild-type rats (Fig. S1(A)). Pancreatic weight measured in males was similar in rSey^2/+ and wild-type (data not shown). Male rSey^2/+ showed normal fasting blood glucose levels, but had significantly higher fasting plasma insulin levels (Table 1). In the fed state, rSey^2/+ showed significantly lower blood glucose levels, and the plasma insulin level was similar in the two groups (Table 1). As these findings indicate relative hyperinsulinemia in rSey^2/+, we assessed the glucose-lowering effect of insulin by insulin tolerance test (ITT). rSey^2/+ and wild-type showed similar insulin sensitivity (Fig. S1(B)), indicating that the hyperinsulinemia is not derived from insulin resistance. In intraperitoneal glucose tolerance test (IPGTT), plasma glucose elevation elicited by glucose load in rSey^2/+ was similar to that of wild-type rats (Fig. S1(C)). Insulin secretion during IPGTT was also similar (Fig. S1(D)). Thus, it is possible that insulin secretion in response to secretagogues other than glucose is enhanced, resulting in hyperinsulinemia in rSey^2/+ rats. Glucagon is not a candidate as plasma glucagon levels were similar in the fasted and fed state (Table 1).

Insulin secretion induced by arginine is augmented in rSey^2/+ perfused pancreas

To determine which secretagogues contribute to the enhanced insulin release of rSey^2/+ in vivo, pancreatic perfusion was performed. rSey^2/+ rats showed the same biphasic insulin release from isolated perfused pancreas in response to stepwise increases in glucose concentration from 5.5 to 16.7 mM. However, insulin release in response to 10 mM arginine at the basal glucose level was significantly increased in rSey^2/+ rats (Fig. 3A). The integrated response to 10 mM arginine in the presence of 5.5 mM glucose was 1062 ± 285 ng of insulin in wild-type ($n = 5$) versus 2068 ± 131 ng of insulin in rSey^2/+ rats ($n = 5$) ($P < 0.05$) (Fig. 3B). On the other hand, rSey^2/+ rats showed similar glucagon release from the perfused pancreas in response to 10 mM arginine in the presence of 5.5 mM glucose (data not shown). This finding demonstrates that increased insulin response to 10 mM arginine in rSey^2/+ is not due to simultaneous enhancement of glucagon release.

Insulin secretory response to membrane-depolarizing stimuli in rSey^2/+ pancreatic islets

As one of the mechanisms by which arginine potentiates insulin release is direct depolarization of the pancreatic β-cell membrane, we examined insulin secretion evoked by membrane-depolarizing insulin secretagogues other than arginine. In batch incubation experiments, insulin release from isolated pancreatic islets in response to glucose stimulation was similar in rSey^2/+ (16.7 mM glucose: rSey^2/+ 0.82 ± 0.065 ng/islet/30 min ($n = 4$)) and wild-type rats (0.97 ± 0.087 ng/islet/30 min ($n = 4$)) ($P = 0.22$). However, insulin secretory responses to 10 mM arginine, 30 mM K^+, or 100 μM tolbutamide were significantly increased in rSey^2/+ pancreatic islets (10 mM arginine in the presence of 5.5 mM glucose: rSey^2/+ 0.55 ± 0.028 ng/islet/30 min ($n = 5$) vs. wild-type 0.21 ± 0.017 ng/islet/30 min ($n = 4$), $P < 0.001$; 30 mM K^+ in the presence of 2.8 mM glucose: rSey^2/+ 0.58 ± 0.035 ng/islet/30 min ($n = 5$) vs. wild-type 0.39 ± 0.040 ng/islet/30 min ($n = 4$), $P < 0.01$; 100 μM tolubutamide in the presence of 2.8 mM glucose: rSey^2/+ 0.45 ± 0.034 ng/islet/30 min ($n = 6$) vs. wild-type 0.31 ± 0.022 ng/islet/30 min ($n = 6$), $P < 0.01$) (Fig. 3C).

$[Ca^{2+}]_i$ elevation in pancreatic islets induced by 30 mM K^+-induced membrane depolarization in rSey^2/+ rats

To determine if the increase in K^+-induced insulin release in rSey^2/+ isolated islets is associated with increased intracellular Ca^{2+}, fura-PE3 was used to measure changes in $[Ca^{2+}]_i$. Five minutes before and 15 min after exposure to 30 mM K^+ in the presence of 2.8 mM glucose, $[Ca^{2+}]_i$ of rSey^2/+ islets was somewhat lower than that of wild-type islets (Fig. 4A and B). However, there was no difference between the depolarization-stimulated increment ratio in

Table 1
Blood glucose and plasma pancreatic hormone levels

	Blood glucose (mg/dl)		Plasma insulin (ng/ml)		Plasma glucagon (ng/ml)	
	Fed	Fasted	Fed	Fasted	Fed	Fasted
Wt	91 ± 2.1 ($n = 14$)	76 ± 1.0 ($n = 17$)	2.4 ± 0.36 ($n = 12$)	0.30 ± 0.09 ($n = 8$)	0.80 ± 0.03 ($n = 9$)	1.2 ± 0.44 ($n = 9$)
rSey^2/+	$80 \pm 1.8^{**}$ ($n = 11$)	72 ± 1.3 ($n = 15$)	1.9 ± 0.23 ($n = 11$)	$0.91 \pm 0.15^{*}$ ($n = 6$)	0.73 ± 0.05 ($n = 9$)	1.1 ± 0.22 ($n = 9$)

Values indicated as means ± SE.
* $P < 0.01$.
** $P < 0.001$ for rSey^2/+ vs. Wt.

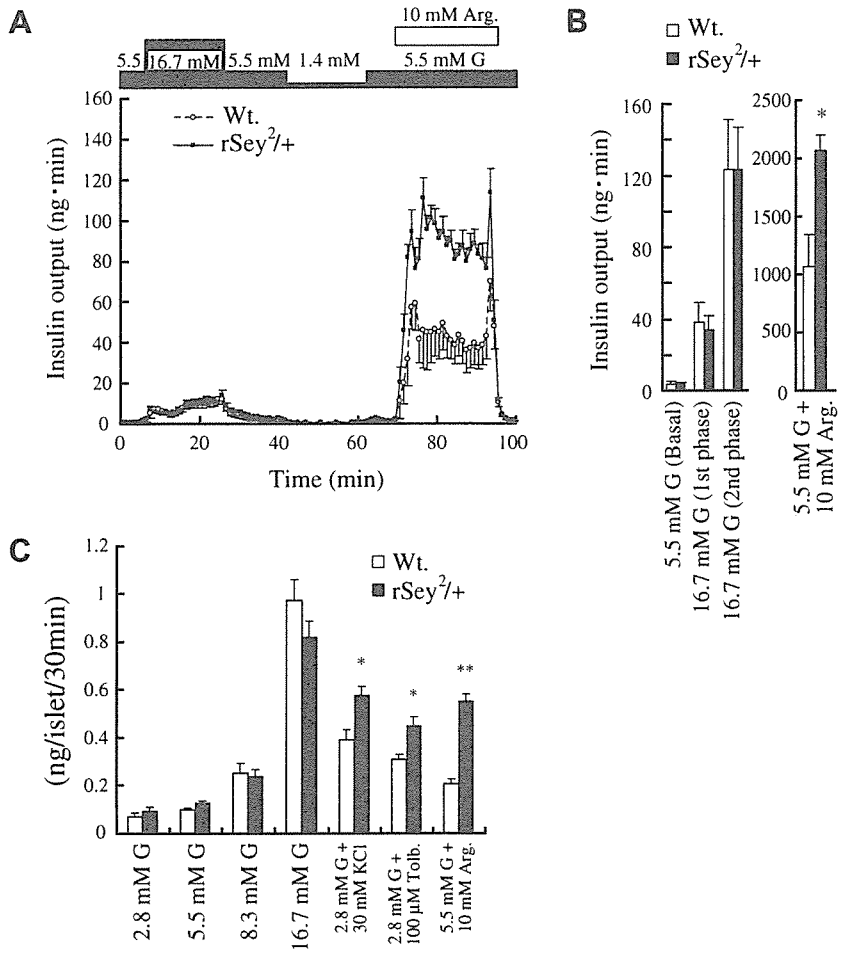

Fig. 3. (A,B) Insulin secretory responses from isolated perfused pancreas. Insulin release in response to glucose and arginine from the pancreas of rSey^2/+ (■) and wild type littermates (○). Values are expressed as mean ± SE. (B) AUC of insulin release at 5.5 mM glucose (basal level, 1–6 min), first (6–13 min) and second (13–26 min) phase insulin release at 16.7 mM glucose, and insulin release in response to 5.5 mM glucose and 10 mM arginine (70–96 min) from the perfused pancreas of wild type (open bars, $n = 5$) and rSey^2/+ (filled bars, $n = 5$). Values are expressed as mean ± SE. * $P < 0.05$, for rSey^2/+ vs. wild type. G: glucose, Arg.: arginine. (C) Depolarization-induced insulin release from isolated islets. Insulin release from batch-incubated islets of wild type (open bars) and rSey^2/+ (filled bars) was examined in response to the indicated concentrations of glucose with or without membrane depolarizing insulin secretagogues. Values are expressed as mean ± SE of 4–7 determinations from several experiments. * $P < 0.01$, ** $P < 0.001$ for rSey^2/+ vs. wild type. G: glucose, Tolb.: tolbutamide, Arg.: arginine.

rSey^2/+ islets and wild-type islets (rSey^2/+ 1.076 ± 0.0033 vs. wild-type 1.073 ± 0.0044, $P = 0.54$) (Fig. 4B).

Ca^{2+} efficacy in insulin release under low ATP condition in rSey^2/+ pancreatic islets

To determine if intracellular Ca^{2+} efficacy is altered in rSey^2/+ islets, we measured insulin release from pancreatic β-cell at $[Ca^{2+}]_i$ clamped by extracellular medium. As shown in Fig. 4C and D, raising the Ca^{2+} concentration from 30 nM to 10 μM elicited an increase in insulin release from electrically permeabilized islets. In the presence of 5 mM ATP, insulin release in rSey^2/+ was similar to wild-type at all Ca^{2+} concentrations (Fig. 4C). This result is commensurate with the findings that rSey^2/+ has a similar insulin secretory response to glucose as wild-type. However, in the presence of 1 mM ATP, insulin release in rSey^2/+ islets at Ca^{2+} concentrations from 30 to 1000 nM was greater than in wild-type islets (at 30 nM Ca^{2+}: rSey^2/+ 0.44 ± 0.031 vs. wild-type 0.30 ± 0.032 ($n = 8$), $P < 0.01$; at 100 nM Ca^{2+}: rSey^2/+ 0.44 ± 0.039 vs. wild-type 0.32 ± 0.035 ($n = 8$), $P < 0.05$; at 300 nM Ca^{2+}: rSey^2/+ 0.66 ± 0.040 vs. wild-type 0.45 ± 0.052 ($n = 7$), $P < 0.01$; at 1000 nM Ca^{2+}: rSey^2/+ 0.85 ± 0.033 vs. wild-type 0.54 ± 0.074 ng/islet/30 min ($n = 8$), $P < 0.01$) (Fig. 4D). This might well underlie the increased insulin secretion seen in rSey^2/+ β-cells in response to membrane depolarizing stimuli at the basal glucose level.

Thus, our findings show that pax6 gene mutation modifies the insulin secretory profile of adult pancreatic islets and that the disturbance in the insulin secretory mechanism in rSey^2/+ pancreatic islets is in the triggering of insulin granule exocytosis by the rise in $[Ca^{2+}]_i$, although the molecular mechanism remains to be determined.

In this study, rSey^2/+ islets showed increased insulin response to membrane-depolarizing stimuli such as

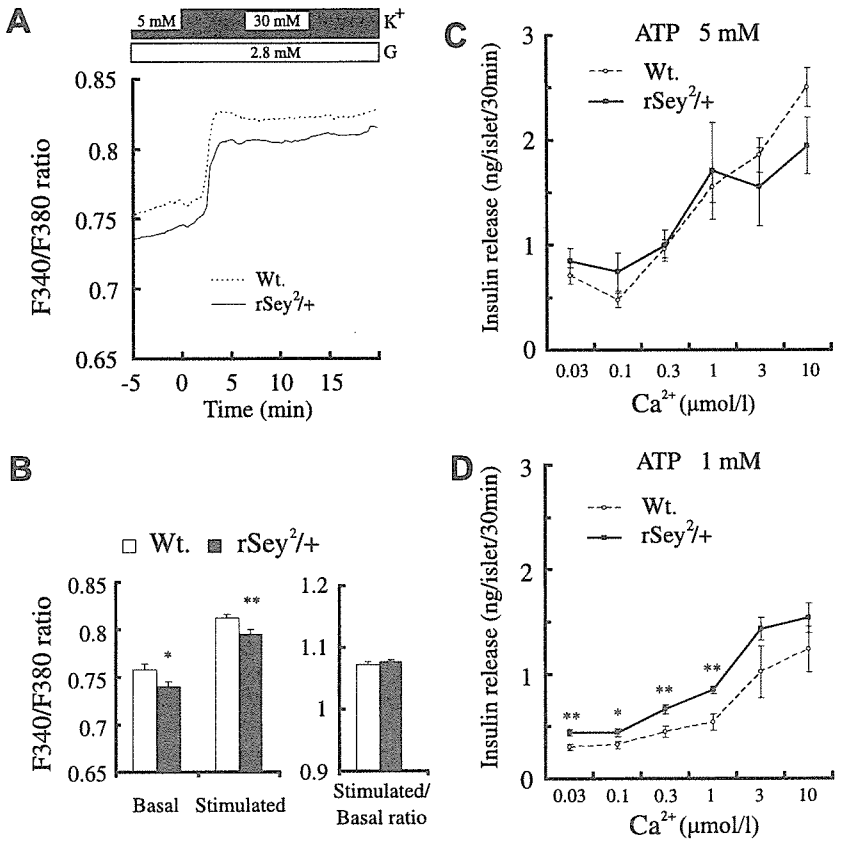

Fig. 4. (A,B) Fluorescence measurement of $[Ca^{2+}]_i$ elevation induced by 30 mM K^+-induced depolarization in the presence of 2.8 mM glucose. (A) Time course of $[Ca^{2+}]_i$ in perfused islets. Values represent mean of 83 (wild-type) and 74 (rSey^2/+) determinations from the several experiments. (B) Left, average values calculated from the data from (A). Basal, average values from −5 to 0 min in the presence of 5 mM K^+ with 2.8 mM glucose. Stimulated, average value from 0 to 15 min in the presence of 30 mM K^+ with 2.8 mM glucose. Right, values represent means ± SE of the ratio of stimulated value to basal value. $^*P < 0.05$, $^{**}P < 0.01$ for rSey^2/+ vs. wild-type. G, glucose. (C,D) Ca^{2+} dose–response of insulin release from electrically permeabilized islets. After preincubation with 2.8 mM glucose for 50 min, islets were electrically permeabilized and incubated with medium containing Ca^{2+} and ATP at the concentration indicated in the figure. Ca^{2+} dose-dependent insulin release from electrically permeabilized islets of wild-type (○) and rSey^2/+ (■) in the presence of 5 mM (C) or 1 mM (D) ATP. Values represent means ± SE of 7–8 determinations in the same experiment for each. $^*P < 0.05$, $^{**}P < 0.01$ for rSey^2/+ vs. wild-type.

arginine. This findings may underlie the relative hyperinsulinemia of rSey^2/+ rats in vivo. In contrast to rSey^2/+, it recently has been reported that rSey/+, another small eye rat strain (Fig. 1), in which the pax6 mutation is located in the transactivation domain [20], has impaired insulin response to glucose but show normal insulin secretory response to arginine [21]. These differences in pancreatic islet function suggest that pax6 plays a key role in regulating the insulin secretory response to various nutrients in pancreatic islets.

Morphological and functional analyses of rSey^2/+ pancreatic islets have important implications regarding gene dosage on pancreatic islet architecture and function. The maintenance of islet morphology in adult pancreas showed low sensitivity to quantity of pax6 gene. In contrast, islet function is necessarily more sensitive to alterations in pax6 gene. While the pax6 gene mutation in rSey^2/+ altered the insulin secretory profile, glucagon secretion was unaffected by the same mutation. These findings indicate that for displaying normal function, insulin-secreting β-cells among the pancreatic islet hormone-secreting cells require higher quantity of pax6 gene than glucagon-secreting α-cells. Outside of the pancreas, it is well known that pax6 is a key regulator of eye formation and that heterozygous pax6 mutations result in eye size reduction due to sensitivity to pax6 gene dosage for eye formation [22,23]. rSey^2/+ eye size also is reduced [14], indicating that the pancreas and the eye of same individual has differing sensitivity to the quantity of pax6 gene. Thus, there are differential dosage requirements for the pax6 gene between organs as well as between morphological and functional characteristics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.11.105.

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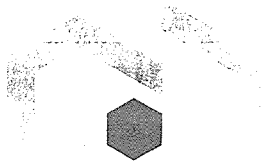

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Factors Responsible for Glucose Intolerance in Japanese Subjects with Impaired Fasting Glucose

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Key words
- impaired fasting glucose
- impaired glucose tolerance
- insulin secretion
- insulin sensitivity
- insulinogenic index

Abstract

Impaired fasting glucose (IFG) represents risk of development of diabetes (DM) and its complications. We investigated insulin secretion and insulin sensitivity in 403 IFG subjects divided into three levels of 2-hour postchallenge glucose (2-h PG) to clarify the factors responsible in the development of glucose intolerance in Japanese IFG. Nearly 60% of the subjects at annual medical check-up with FPG of 6.1–7.0 mmol/l at the first screening were diagnosed by 75 g oral glucose tolerance test (OGTT) to have impaired glucose tolerance (IGT; FPG <7.0 mmol/l and 7.8 mmol/l <2-h PG <11.1 mmol/l) or DM (isolated postchallenge hyperglycemia (IPH); FPG <7.0 mmol/l and 11.1 mmol/l <2-h PG level). The primary factor in the decreased glucose tolerance was a decrease in early-phase insulin, with some contribution of increasing insulin resistance. In addition, IFG/IGT and IFG/IPH subjects showed a compensatory increase in basal insulin secretion sufficient to keep FPG levels within the non-diabetic range. IFG is composed of three different categories in basal, early-phase insulin secretion, and insulin sensitivity.

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Introduction

Diabetes mellitus (DM) and impaired glucose regulation (IGR) are heterogeneous disorders characterized by impaired insulin secretion and decreased insulin sensitivity. Impaired fasting glucose (IFG) represents risk of development of diabetes; impaired glucose tolerance (IGT) represents risk of development of both diabetes and cardiovascular diseases and atherosclerosis [1–3]. The frequency of progression to diabetes differs considerably in the two groups [4–6]. Moreover, in a cohort study in Europe, the rate of development to DM from IFG was twice than that of IGT [5]; a report from the U.S. showed that IFG and IGT exhibited a similar rate of deterioration leading to diabetes [6]. In addition, the contribution of deteriorating insulin secretion and insulin sensitivity in the development of type 2 diabetes differs among ethnic groups [7–10]. We have previously reported that while both impaired insulin secretion and decreased insulin sensitivity contribute in the development from normal glucose tolerance (NGT) via IFG to DM with isolated fasting hyperglycemia (DM/IFH) in Japanese subjects, progression from NGT via IGT to isolated postchallenge hyperglycemia (IPH) is mainly due to impaired early-phase insulin secretion [11–13]. Thus, IFG exhibits different clinical characteristics and different pathophysiology from IGT, however, IFG is a complex group and the pathophysiological characters are not enlightened enough. To elucidate the physiological abnormalities, IFG, according to the diagnostic criteria of the ADA (IFG: 6.1 mmol/l ≤ fasting plasma glucose level (FPG) <7.0 mmol/l) [14], was divided into three subgroups: isolated IFG [2-hour postchallenge glucose(2-hPG) level <7.8(mmol/l]; IFG with IGT (IFG/IGT: 7.8 mmol/l ≤2-h PG level <11.1 mmol/l); and IFG with IPH (IFG/IPH: 11.1 mmol/l ≤2-h PG level). In this study, we have examined insulin secretion and insulin sensitivity in 403 Japanese IFG subjects in three subgroups of 2-h PG level to clarify the factors responsible for developing glucose intolerance in IFG. HOMA β-cell and HOMA-IR indices calculated by homeostasis model assessment were used to determine insulin secretion and insulin sensitivity in the fasting state [15–17]. Insulinogenic index (I.I.) and ISI composite were determined by 75 g oral glucose tolerance test (OGTT) [18–20]. These indices were compared to evaluate the causative factors of glucose intoler-

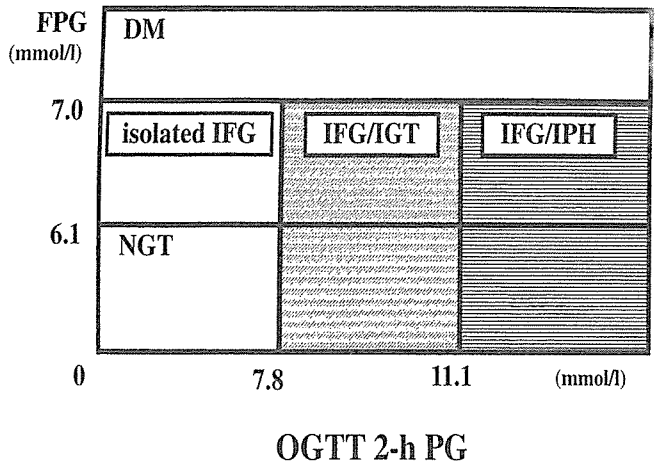

Fig. 1 Classification of glucose intolerance. Degrees of glucose intolerance diagnosed only by FPG according to the diagnostic criteria of the ADA: NGT: normal glucose tolerance; IFG: impaired fasting glucose; and DM. IGT: impaired glucose tolerance. IPH: diabetes mellitus with isolated postchallenge hyperglycemia. IFG subdivided into 3 groups: isolated IFG, IFG/IGT; IFG with IGT, and IFG/IPH; IFG with IPH.

ance between isolated IFG, IFG/IGT, and IFG/IPH in Japanese subjects.

Subjects and Methods

Subjects

To screen the subjects who have abnormal glucose metabolism at initial examination for regular medical check-up, we recruited subjects with abnormal urine glucose test (± or more using the urine dip sticks), fasting plasma glucose level (FPG) >5. 6 mmol/l, HbA_{1C} level >5.0%, or family history of diabetes at Kyoto University Hospital, Ikeda Hospital, Kansai-Denryoku Hospital, Kansai Health Management Center, and Kyoto Preventive Medical Center between 1993 and 2004. Subjects with FPG less than 7.0 mmol/l and more than 6.1 mmol/l were enrolled in this study. No subjects showed signs of hypertension, hepatic, renal, endocrine, or malignant diseases. No subjects had engaged in heavy exercise, past history of gastrectomy, or any medications affecting glucose metabolism before the study. OGTT was carried out within three months of the initial examination. Standard OGTT was administered according to the National Diabetes Data Group recommendations [21], which require the subjects to fast overnight for 10–16 hours. Fasting (0), 0.5, 1, 1.5, and 2-h blood samples were obtained for measurement of plasma glucose and serum insulin after oral administration of 75 g glucose. Blood samples at fasting were used to measure HbA_{1C}, total cholesterol, HDL cholesterol, and triglycerides levels.

Based on the results of 75 g OGTT, the patients were divided into three groups, isolated IFG, IFG/IGT, and IFG/IPH (○ **Fig. 1**), according to 1998 World Health Organization (WHO) diagnostic criteria [22]. Isolated IFG: 6.1 mmol/l ≤ FPG < 7.0 mmol/l; 2-hour plasma glucose (PG) level: < 7.8 mmol/l (n = 158); IFG/IGT: 7.8 mmol/l, ≤ 2-hour PG level < 11.1 mmol/l (n = 164); and IFG/IPH: 11.1 mmol/l ≤ 2-hour PG level (n = 81). The study was designed in compliance with the ethics regulations set out by the Helsinki Declaration.

Measurements

Plasma glucose 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 [11]. Serum total cholesterol, HDL cholesterol, and triglycerides levels were measured as reported previously [23].

To evaluate insulin secretion capacity, the HOMA β-cell and insulinogenic index were used. To estimate insulin sensitivity, the HOMA-IR index and ISI composite were used. HOMA β-cell and HOMA-IR are homeostasis model assessments. HOMA β-cell represents basal insulin secretion [24]. HOMA-IR shows good correlation to measurements of insulin sensitivity obtained by glucose clamp method and minimal model study [11, 15]. Insulinogenic index was used to measure the capacity of early-phase insulin secretion [18, 19] and ISI composite was used to measure systemic insulin sensitivity [20]. The formulas for calculation of the indices are as follows:

HOMA β-cell = 20 × Ins-0 (μU/ml)/[Glu-0 (mmol/l) − 3.5] [24]

Insulinogenic index (I.I.) = [Ins-0.5 − Ins-0 (pmol/l)]/[Glu-0.5 − Glu-0 (mmol/l)] [18, 19]

HOMA-IR = Glu-0 (mmol/l) × Ins-0 (μU/ml)/22.5 [23]

ISI composite = 10000/[Glu-0 (mg/dl) × Ins-0 (μU/ml)] × [mean Glu 0–2 (mg/dl) × mean Ins 0–2 (μU/ml)]$^{0.5}$ [20]

Statistical analysis

All data are mean ± standard error (SE). Statistical analyses were performed using STATVIEW 5 system (Abacus concepts, Berkeley, CA, USA). Age, BMI, Glu 0–2 h, Ins 0–2 h, HbA_{1C}, triglycerides, total cholesterol, HDL cholesterol, HOMA β-cell, insulinogenic index, HOMA-IR, and ISI composite were compared among the isolated IFG, IFG/IGT, and IFG/IPH groups by analysis of variance (ANOVA). For comparison of clinical parameters between two groups, unpaired Student's *t*-test was performed. A p value of < 0.05 was considered statistically significant. Simple regression analysis was used for comparisons of clinical parameters. Stepwise regression analysis was used for comparison of the relationship between 2-h PG and the indices of insulin secretion and sensitivity and *F*-value >4 is considered statistically significant.

Results

Clinical characteristics

The clinical and metabolic characteristics of subjects with isolated IFG, IFG/IGT, and IFG/IPH are shown in **Tab. 1**. There was no significant difference in age and BMI among the three groups. The mean BMI in all three groups was similar to that in Japanese subjects in a population-based study: NGT 23.1(kg/m^2), IGT 24.4, and diabetes 24.3 [11]. The fasting glucose level increased in order of the glucose intolerance as isolated IFG, IFG/IGT and IFG/IPH. Moreover, the fasting insulin level of the IFG/IPH group was significantly higher than in the isolated IFG group ($p = 0.003$). In the IFG/IPH group, HbA_{1C} and triglycerides levels were remarkably higher than in the isolated IFG group (HbA_{1C}; $p < 0.0001$, TG; $p = 0.01$). There was no significant difference in total cholesterol levels and HDL cholesterol levels among the three groups.

Insulin secretion capacity

The insulinogenic index decreased in order of the glucose intolerance as isolated IFG, IFG/IGT, and IFG/IPH (○ **Fig. 2A**). On the other hand, HOMA β-cell, representing basal insulin secretory capacity, increased as glucose intolerance from isolated IFG via IFG/IGT to IFG/IPH), especially in the IFG/IPH group (○ **Fig. 2B**) ($p=0.021$). (○ **Fig. 2**)

Insulin sensitivity

HOMA-IR is shown in ○ **Fig. 3A**. HOMA-IR in the IFG/IPH group was significantly higher than in the isolated IFG groups ($p=0.001$), but the difference in the absolute values among the three group was only about 0.2 (IFG; 1.66, IFG/IGT; 1.84, IFG/IPH; 2.03). ISI composite is shown in ○ **Fig. 3B**. Although ISI composite decreased from isolated IFG, IFG/IGT to IFG/IPH, there was no significant difference among the three groups. (○ **Fig. 3**)

Relationship of insulin secretion and sensitivity to 2-h PG

The relationship between 2-h PG and the indices of insulin secretion and sensitivity were then compared (**Tab. 2**). There was a significant relationship between 2-h PG and the four indices using simple regression analysis; insulinogenic index having the strongest correlation coefficient. Stepwise regression analysis showed that insulinogenic index, HOMA-IR, and HOMA β-cell were independent factors to explain 2-h PG ($p<0.0001$) and that insulinogenic index was the strongest factor in determining 2-h PG among the indices. ISI composite was not an independent factor to explain 2-h PG in stepwise regression analysis. After the adjustment for BMI, insulinogenic index, HOMA-IR, and HOMA β-cell were also independent factors to explain 2-h PG, and ISI composite was not an independent factor for 2-h PG.

Tab. 1 Demographic/metabolic characteristics of subjects with isolated IFG, IFG/IGT and IFG/IPH

	Isolated IFG	IFG/IGT	IFG/IPH
n	158	164	81
Age (years)	54.7 ± 0.7	56.7 ± 0.9	58.3 ± 1.2
BMI (kg/m^2)	23.8 ± 0.3	24.0 ± 0.3	24.3 ± 0.4
FPG (mmol/l)	6.40 ± 0.0	6.5 ± 0.0*	6.5 ± 0.0‡,§
2-h PG (mmol/l)	6.20 ± 0.1	9.3 ± 0.1‡	13.2 ± 0.2‡,§
Fasting insulin (p mol/l)	41.9 ± 1.7	45.9 ± 1.7	49.9 ± 3.6†
HbA_{1c} (%)	5.50 ± 0.0	5.6 ± 0.0	6.0 ± 0.1‡,§
Triglycerides (mmol/l)	1.28 ± 0.10	1.48 ± 0.07	1.69 ± 0.20*
Total cholesterol (mmol/l)	5.40 ± 0.09	5.48 ± 0.09	5.53 ± 0.11
HDL-Cholesterol (mmol/l)	1.62 ± 0.05	1.52 ± 0.04	1.54 ± 0.05

Data are mean ± SE.

$^{*}p<0.05$;

$^{\dagger}p<0.005$;

$^{\ddagger}p<0.001$ vs. isolated IFG,

$^{\S}p<0.05$ vs. IFG/IGT.

Discussion and Conclusions

In the present study, isolated IFG, IFG/IGT, and IFG/IPH comprised 39, 41, and 20% of the subjects, respectively. Nearly 60% of the IFG subjects, judged only by fasting plasma glucose levels, had IGT or DM. As this percentage is higher than in other populations [25–27], Japanese subjects with FPG greater than 6.1 mmol/l at medical check-up after the first screening should better receive OGTT to detect IGT and DM. The indices of insulin secretion (HOMA β-cell) and insulin resistance (HOMA-IR) were evaluated from a fasting sample by homeostasis model assessment [15–17]. The estimations correlated well with the insulin secretion and insulin sensitivity indices of minimal model analysis [16]. Insulinogenic index is a well-known measure of early-phase insulin secretion during OGTT [18,19]. Matsuda and DeFronzo developed the index of insulin sensitivity as an ISI composite, which is a good surrogate measure of whole body insulin sensitivity by glucose clamp study [20]. Comparison of the four indices of glucose tolerance among the three subgroups of Japanese IFG shows that the developing glucose intolerance from isolated IFG, IFG/IGT to IFG/IPH is primarily due to decreased early-phase insulin secretion, with only a small increase in insulin resistance. Stepwise regression analysis in this study showed that the strongest determinant of 2-h PG was insulinogenic index in comparison with the other indices.

In this study, insulinogenic index in isolated IFG, IFG/IGT, and IFG/IPH was 0.26, 0.18, and 0.12, respectively, considerably lower than previously reported in other populations [28,29]. This manifests in remarkably decreased early-phase insulin secretion in IFG. In a study of Caucasians (Botnia study) [30], the I.I. value was reduced by half as glucose intolerance progressed from NGT to IGT and by half again from IGT to DM, however, isolated IFG exhibited the same I.I. value as NGT. Interestingly, HOMA β-cell in IFG/IPH was significantly higher than in isolated IFG despite the decreasing insulinogenic index with glucose intolerance. In

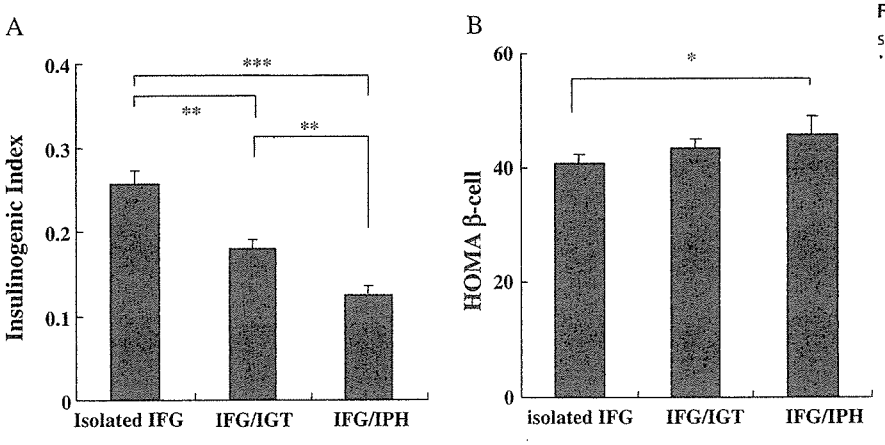

Fig. 2 Indices of insulin secretion capacity in subjects with isolated IFG, IFG/IGT and IFG/IPH. $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.001$.

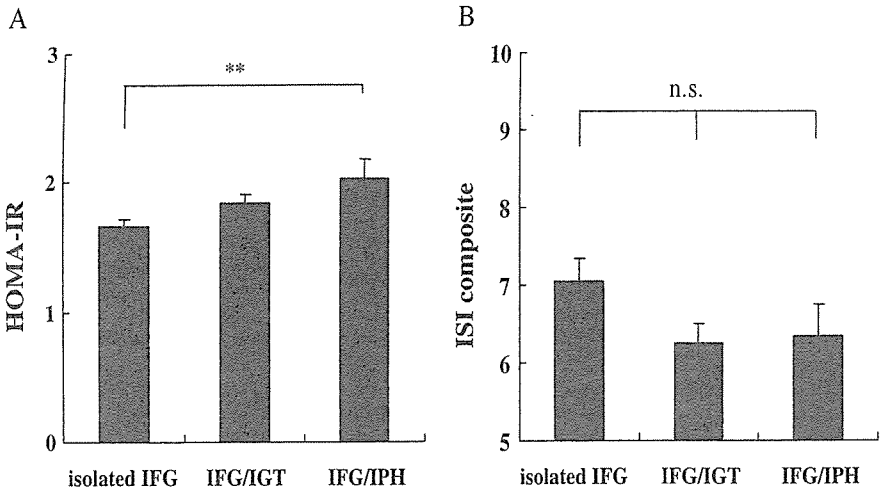

Fig. 3 Insulin sensitivity indices in subjects with isolated IFG, IFG/IGT and IFG/IPH. **p<0.005, n.s.; not significant

Tab. 2 Relationship between 2-h PG and indices of insulin secretion and sensitivity

	Correlation coefficients	p-value	F-value
Insulinogenic index	0.29	<0.0001	45.7
HOMA-IR	0.19	<0.0001	19.7
HOMA β-cell	0.13	0.008	8.8
ISI composite	0.11	0.033	0.8

this study, FPG and FIRI increased along with glucose intolerance from isolated IFG, IFG/IGT to IFG/IPH. HOMA β-cell is calculated as the ratio of FIRI to FPG. Thus, the increase in basal insulin secretion might compensate, keeping fasting glucose within the non-diabetic range in IFG.

In the Botnia study, Tripathy et al. found that the main causative factor in IFG was insulin resistance. IGT was the result of both abnormal insulin secretion due to dysfunction of the compensatory response of the β-cell and insulin resistance [30] and the HOMA-IR in NGT, IFG, and IFG/IGT was 1.73, 2.64, 3.59, respectively. In Japanese NGT, isolated IFG and IFG/IGT, HOMA-IR were 1.21, 1.66, 1.84, respectively, remarkably lower than in the Botnia study.

We have previously reported that HOMA-IR value of >2.5 represents an overt insulin resistant state based on our investigation of Japanese type 2 diabetes patients. HOMA-IR in Japanese IFG is 1.7–2.0, not a remarkable increase in insulin resistance and still within normal range. In contrast, HOMA-IR in IFG/IGT and DM in Caucasians are nearly twice as high as in Japanese. We reported in previous studies that BMI has a strong association with insulin resistance. The BMI of Caucasians with IFG in the Botnia study was 26.7; the BMI in Japanese IFG was 23.8. The mean BMI of Japanese diabetics is 23–25 by epidemiological studies, lower than in other ethnic groups [31–34], further suggesting that the major factor in glucose intolerance in IFG may also differ in various ethnic populations.

We have reported that the major factor responsible for developing glucose intolerance in Japanese diabetics differs from that in other ethnicities according to the indices of insulin secretion and sensitivity obtained by 75 g OGTT and minimal model analysis [8, 35–37]. IGT is a risk factor for the development of diabetes in isolated IFG cases as well, but it is also an important risk factor for atherosclerosis and cardiovascular disease. The risk of development to diabetes of isolated IFG compared to NGT was 20.5 while that of IGT was 5.37 in the Funagata study [4]. Moreover, Bonora et al. found that the diabetic incidence rate for ten years of IGT, isolated IFG, and IFG/IGT was 3.9, 11, and 20.5 to that of NGT, respectively [5], Meigs et al. indicated that 40% of isolated IFG and 39% of IGT had progressed to diabetes [6]. Further cohort studies are required to compare the risk of development of diabetes and diabetic complications in these subgroups [38].

In conclusion, we have shown that the primary factor in the glucose intolerance as isolated IFG, IFG/IGT and IFG/IPH in Japanese IFG is the decreased early-phase insulin, with a considerably lesser contribution of increased insulin resistance. Interestingly, HOMA β-cell, which indicates basal insulin secretory capacity, increased as glucose intolerance increases from isolated IFG to IFG/IPH. Almost 60% of Japanese IFG subjects exhibit FPG of 6.1–7.0 mmol/l and are included in IGT or diabetes at annual medical checkup. Therefore, the subjects with FPG 6.1–7.0 mmol/l are composed of three different categories in basal insulin secretion, early-phase insulin secretion, and insulin resistance.

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