Impaired insulin secretion from the pancreatic islets of hypothyroidal growth-retarded mice

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Abstract

The growth-retarded (*grt*) mouse shows thyroid dysfunction-related hyporesponsiveness to TSH. Thyroid hormone is a critical regulator of metabolism in many cells; thus, derangement of thyroid function affects many organs and systems. Experiments were conducted focusing on the function of the pancreatic islets in *grt* mice. We showed occurrence of a fasting hyperglycemia and a decreased plasma insulin level response to a glucose load in *grt* mice, despite normal insulin molecules being stored in secretory granules of pancreatic islets. We also demonstrated a reduction of insulin secretion in response to glucose administration from islets of *grt* mice *in vitro*, while the insulin release in response to KCl stimulation was comparable to that in normal mice, indicating that the isolated islets from *grt* mice have normal ATP-sensitive K^+ channels and postchannel activity. The mRNA expression levels of glucose transporter 2 and glucokinase in the islets of *grt* mice were similar to those in normal mice. Triiodothyronine administration to *grt* mice improved insulin secretion very slightly. On the other hand, mRNA for tyrosylprotein sulfotransferase 2 (*Tpst2*) was found to be expressed in the pancreatic islets of *grt* mice. Considering that *Tpst2* is the responsible gene of *grt* mice, mutation of which is associated with a poor function of TSH receptor, the findings raise a possibility of involvement of factors including *Tpst2* in the insulin hyposecretion in *grt* mice.
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Introduction

The growth-retarded (*grt*) mouse was first reported as a mutant spontaneously derived from phenotypically normal siblings of the Snell's dwarf mouse (DW/J strain) with a characteristic growth pause followed by delayed onset of pubertal growth in contrast to *dw* mice (Yoshida *et al.* 1994). In *grt* mice, plasma concentrations of thyroxine (T_4) are significantly lower, whereas levels of thyroid-stimulating hormone (TSH) are greatly elevated (Yoshida *et al.* 1994, Tomita *et al.* 1995, Kobayashi *et al.* 2001). The unresponsive nature of TSH receptors to TSH is considered to be attributable to dysfunction of the *grt* thyroid gland (Kobayashi *et al.* 2001, 2005). Recently, the *grt* phenotype has been shown to be caused by a single missense mutation in the tyrosylprotein sulfotransferase 2 (*Tpst2*) gene with a C-to-G transition at nucleotide 798, leading to replacement of a highly conserved histidine with glutamine at codon 266 in the sulfotransferase domain (Sasaki *et al.* 2007). Impaired tyrosine sulfation of TSH receptor molecules by inactivation of *Tpst2* reduces responsiveness to TSH and causes the functional failure of *grt* mice thyroid (Sasaki *et al.* 2007).

Thyroid hormones play a fundamental role in the initiation and maintenance of somatic growth in mammalian species, and affect many aspects of metabolism. It has also been reported that insulin secretion is regulated by thyroid hormone (Cortizo *et al.* 1985, Doong *et al.* 1997), and that there is a relationship between serum thyroid hormone levels and diabetes risk (Iossa *et al.* 2001, Crunkhorn & Patti 2008). Hyperthyroidism reduces glucose tolerance in mammals, and the insulin-secretory capacity of the pancreatic islets is reduced by an excess of thyroid hormones (Lenzen & Bailey 1984). On the other hand, hypothyroidism is associated with deterioration of glucose metabolism, and this effect is accompanied by reduced plasma insulin concentrations but increased plasma glucagon concentrations (Handisurya *et al.* 2008). Thyroid hormone effects on glucose homeostasis and on endocrine function of the pancreas are the result of an interaction of the thyroid hormone with its receptors (Yen 2001). The consequences of hypothyroidism on these metabolic pathways seem to depend on the degree of reduction of circulating thyroid hormones (Mackowiak *et al.* 1999). To date, only a few studies have investigated the effect of thyroid dysfunction and its recovery by thyroid hormone treatment on glucose metabolism, and the results have been controversial (Dessein *et al.* 2004, Stanická *et al.* 2005, Brenta *et al.* 2007, Handisurya *et al.* 2008). It is not clear whether the glucose-induced insulin-secretory capacity is

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reduced in *grt* mice, and whether thyroid hormone replacement is effective for restoring the insulin secretion.

Impairment in glucose sensing contributes to pancreatic β-cell dysfunction. The glucose transporter 2 (GLUT2) and glucokinase (GK) are key molecules with a high K_m for glucose transport and for glucose phosphorylation respectively, which affect various processes of glucose sensing in pancreatic β-cells (Matschinsky 1990). Glucose sensing is the initial event of glucose-stimulated insulin secretion. Therefore, it is necessary to maintain adequate expression levels of GLUT2 and GK to ensure normal β-cell function (Cerf 2007). The expression of GLUT2 and GK is under hormonal control. Triiodothyronine (T_3) can modulate the expression of GLUT2 and GK mRNAs and proteins in pancreatic islets (García-Flores *et al.* 2001) and liver (Kemp *et al.* 1997). However, the effect of hypothyroidism on the expression of glucose sensors in pancreatic islets is unclear.

Experiments were conducted in order to provide further insight into the relationship between thyroid hormones and glucose homeostasis in hypothyroidal *grt* mice.

Materials and Methods

Animals

The mice were maintained under controlled temperature (23±1 °C), relative humidity (50±10%), and lighting (0800–2000 h) conditions at the animal facility at Saitama University (Saitama, Japan). A standard laboratory diet (Labo MR Breeder, Nosan, Yokohama, Japan) and tap water were made available *ad libitum*. Normal phenotype (+/+ or +/*grt*) and *grt* (*grt*/*grt*) male mice were obtained by mating wild (+/+) or heterozygous (+/*grt*) female mice with *grt* male mice. These mice were initially obtained as the offspring born to a pair of phenotypically normal siblings of DW/J strain mice and were distinguished on the basis of their body weights as described elsewhere (Yoshida *et al.* 1994). Animals were studied at 2–12 months of age, and all experimental procedures were performed in accordance with the Institutional Guidelines for Animal Care and Use of Saitama University.

Glucose tolerance test

Intraperitoneal glucose tolerance tests (IGTTs) were carried out on 3-month-old normal and *grt* mice that had been subjected to an overnight (24 h) fast. Glucose (1 mg/g body weight) was administered to mice via i.p. injection. Blood samples were collected by retro-orbital sinus puncture before (0 min) and 30, 60, 90, and 120 min after glucose administration. Blood glucose levels were determined using an automated blood glucose meter (Nipro Free Style Meter; NIPRO, Osaka, Japan). Plasma samples were obtained by centrifugation at 4 °C and were stored at −20 °C until analysis. Plasma insulin concentrations were determined using an ELISA (Morinaga Institute of Biological Science, Yokohama, Japan).

Insulin tolerance test

Bovine insulin (1 mU/g body weight; 26 U/mg; Wako, Osaka, Japan) was administered via i.p. injection to 3-month-old normal and *grt* mice. Blood samples were collected via retro-orbital sinus puncture. Blood glucose measurements were also carried out immediately before (0 min) and 15, 30, and 45 min after insulin injection using an automated blood glucose meter.

Islet isolation

Pancreatic islets were isolated from 6- to 8-month-old normal and *grt* mice, or from 3-month-old *grt* and T_3-treated *grt* mice, by collagenase digestion, using the modified method of Lacy & Kostianovsky (1967) and Gotoh *et al.* (1985). Briefly, after the mouse was anesthetized using pentobarbital (0·063 mg/g body weight; Dainippon Sumitomo Pharma, Osaka, Japan), 0·1% collagenase type IV (Worthington Biochemical, Lakewood, NJ, USA), which was dissolved in Hanks' solution (Nissui Pharmaceutical, Tokyo, Japan), was injected into the pancreas via the bile duct. The pancreas was then removed and incubated for 30 min at 37 °C. The pancreas was then dispersed by injecting the mixture through a syringe and was washed in Hanks' balanced salts solution without calcium chloride (Sigma–Aldrich). Islets were handpicked under a stereomicroscope.

Immunohistochemical staining of pancreatic islets

Isolated islets were immunocytochemically characterized using the peroxidase/anti-peroxidase method as follows. Islets were fixed in 10% neutral buffered formaldehyde for 24 h and then placed in 70% ethanol. The islets were dehydrated in graded ethanol and embedded in paraffin. These sections (10 μm thick) were cut using a microtome (model 840; American Optical Corp., Buffalo, NY, USA) and mounted. Deparaffinized sections were hydrated in a series of graded ethanol solutions and 10 mM PBS (pH 7·4). The sections were incubated in a solution of 3% H_2O_2 (Wako) for 20 min to block endogenous peroxidase activity. After rinsing with PBS, the sections were treated with normal swine serum (1:20; Dako, Glostrup, Denmark) for 60 min at room temperature. The specimens were washed with PBS and were then incubated with guinea pig anti-insulin antibody (ready-to-use primary antibody; Dako; diluted 1:5) overnight at 4 °C to detect β-cells. Secondary antibodies (swine anti-rabbit IgG, 1:100; Dako) were incubated with the specimens for 90 min at room temperature, following which a rabbit peroxidase/anti-peroxidase complex (Dako) was added and incubated for 60 min at room temperature. The sections were rinsed in PBS, and peroxidase activity was visualized using 0·05% diaminobenzidine (Wako) in 0·1 M Tris–HCl (pH 7·4) containing 0·006% H_2O_2. The slides were counterstained with hematoxylin to visualize the nuclei. These sections were dehydrated through a graded series of

ethanol, were cleared using xylene, coverslipped using Canada balsam (Wako), and were studied using conventional light microscopy.

Immunoelectron microscopy of pancreatic β-cells

For immunoelectron microscopy, pancreases of 3-month-old normal and *grt* mice were fixed with a mixture of 4% paraformaldehyde (Merck KGaA) and 0·5% glutaraldehyde (TAAB Laboratories Equipment, Berks, UK) in 0·1 M PBS (pH 7·4) for 48 h at 4 °C. The tissues were postfixed with a mixture of 1% osmium tetroxide (Merck) and 1·5% potassium ferrocyanide (Wako) in 0·1 M phosphate buffer (PB, pH 7·4) for 1 h at 4 °C. The tissues were then dehydrated through a graded ethanol series, placed in LR White resin (London Resin, Basingstoke, UK), embedded in the pure resin, and were then polymerized in gelatin capsules at 55 °C for 48 h. Ultrathin sections were cut using an ultra microtome (MT2-B; E.I. du Pont de Nemours & Company, Wilmington, DE, USA), were mounted on Formvar-coated nickel grids that had been cooled in a freezer (−20 °C), and were then preincubated with 1% BSA (Sigma) in 0·01 M PBS for 1 h. After washing in PBS, the sections were incubated with guinea pig anti-insulin antibody in 1% BSA–PBS (1:5) overnight at 4 °C. The sections were well washed with PBS, reacted with gold-labeled (10 nm) secondary antibody (1:20; British BioCell International, Cardiff, UK) for 2 h, and were then washed with PBS and distilled water. After air drying, the sections were double stained with saturated aqueous uranyl acetate (TAAB Laboratories Equipment) and Sato's lead citrate (Sato & Shamoto 1973), and were examined using a Hitachi H700H electron microscope operated at 100 kV.

Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopic (MALDI-TOF/MS) analysis

Islets were isolated from mice as described above. They were then placed on to a matrix-assisted laser desorption/ionization (MALDI) sample plate. The matrix of α-cyano 4-hydroxycinnamic acid (Bruker Daltonics, Leipzig, Germany) was saturated in a solution of acetonitrile/water 50:50 (v/v) containing 0·1% trifluoroacetic acid (Wako). The fresh matrix solution was added to the sample and dried. MALDI-TOF mass spectra were acquired using a Bruker AutoflexIII MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. External calibration was performed using the Peptide Calibration Standard II (Bruker Daltonics).

Insulin secretion assay

Islets of similar sizes, which were isolated from mice as described above, were grouped separately. Islets were washed in Dulbecco's PBS (DPBS; 137 mM NaCl, 2·7 mM KCl, 1·5 mM KH_2PO_4, and 8·1 mM Na_2HPO_4, pH 7·4) (Sigma), containing 0·9 mM $CaCl_2$ and 0·5 mM $MgCl_2$, and were preincubated in the same buffer for 60 min at 37 °C in humidified 5% CO_2–95% air. Experiments for insulin secretion were carried out in 96-well plates (ten islets per well) for 60 min in DPBS (300 μl) in the absence (control) or presence of 16·1 mM glucose or 28·9 mM KCl. The medium was collected, gently centrifuged, and stored at −20 °C for measurement of insulin. Insulin concentration was determined using an ELISA.

Real-time PCR analysis of GLUT2 and GK mRNA expression

Total RNA was extracted from 750 to 850 islets using the Spin Tissue RNA Mini Kit (Invitek Gmbh, Berlin, Germany) and RNA stabilizer (Wako) according to the manufacturer's protocol. Total RNA (0·4 μg) was then reverse transcribed using a Random Primer (TaKaRa Bio, Otsu, Japan) and the M-MLV reverse transcriptase (Invitrogen). Real-time PCR was performed using the LightCycler ST300 (Roche Diagnostics). To prepare standard samples for each gene transcript, an equal amount of each cDNA sample was combined and then serially diluted with EASY Dilution (TaKaRa Bio). For PCR of standards and single samples of unknowns, 2 μl of RT product were amplified in 20 μl of a reaction mixture containing 200 nM of each primer (Table 1) and 10 μl of 2×SYBR premix Ex Taq (TaKaRa Bio). The gene-specific primer sets, designed using LightCycler Probe Design Software 2.0 (Roche) with reference to Genbank sequences downloaded from the Mouse Genome Informatics (http://www.informatics.jax.org/) website, were designed so as to have a melting temperature of ∼60 °C, a GC content of ∼50%, and to generate amplicon lengths ranging from 150 to 200 bp. The conditions of real-time PCR included an initial step of 10 s at 95 °C to activate Taq polymerase, followed by 40 cycles of 95 °C for 5 s for denaturation and 60 °C for 20 s for annealing and extension, with a temperature transition

Table 1 Sequences of the primers used for real-time PCR of pancreatic islets of mice

Target gene	**Forward primer sequence** (5′–3′)	**Reverse primer sequence** (5′–3′)	**Product length** (bp)	**NCBI reference sequence**
Glut2	GAGAGATCGCTCCAACCAC	CACAGCAGATAGGCCAAGT	152	NM_031197
Gk	GGTGAGCTGGACGAGTT	AAGATTCTCCTCTACCAGCTT	153	NM_010292
Gapdh	CCAAGGCTGTGGGCAA	CCCTCAGATGCCTGCT	189	NM_008084

Table 2 Sequences of the primers used for RT-PCR of pancreatic islets of mice

Target gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Product length (bp)	NCBI reference sequence
Tpst1	TACTCTTGGCGTGTCTGGTG	TGCATGGCAGAATCTAGCAC	391	NM_001130476
Tpst2	ACTGCCTCACCAAGTGGAAC	AAGCCATGTGCTTGGGTTAG	587	NM_009419
Gapdh	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452	NM_008084

rate of 20 °C/s. After real-time PCR, a melting curve analysis was carried out to demonstrate the specificity of the PCR products, and showed that the melting curve for the PCR products targeting each gene transcript had a single peak (data not shown). The mRNA levels of *Glut2* and *Gk* in each sample were normalized by division by the mRNA level of the housekeeping gene *Gapdh* in the same sample.

In vivo T_3 treatment

T_3, dissolved in PBS (pH 7·4), was injected i.p. (0·2 μg/g body weight; Sigma–Aldrich) into 3-week-old *grt* mice once every other day for 9 weeks. Mice injected with the vehicle alone were used as controls.

Reverse transcription-PCR analysis of TPST1 and TPST2 mRNA expression

Total RNA (0·4 μg) from normal and *grt* mice was reverse transcribed as described above. The gene-specific primer sets were designed using the Primer3 (http://primer3.sourceforge.net/) website with reference to Genbank sequences downloaded from the Mouse Genome Informatics website (Table 2). PCR amplifications were performed using TaKaRa Ex Taq (TaKaRa Bio). The PCR cycle conditions consisted of denaturation at 94 °C for 2 min, followed by 40 cycles of 94 °C for 1 min (denaturation), 61 °C (TPST1), 63 °C (TPST2), or 58 °C (GAPDH) for 1 min (annealing), and then 72 °C for 1 min (elongation).

Statistical analysis

Values were expressed as the mean±S.E.M. The statistical significance ($P<0·05$) of differences was determined using Student's *t*-test.

Results

Intraperitoneal glucose tolerance tests

Results of the IGTTs in 3-month-old normal and *grt* mice are shown in Fig. 1. Blood glucose levels under fasting conditions (0 min) were higher in *grt* mice compared with normal mice (114·2±3·1 vs 51·0±5·5 mg/100 ml, $P<0·05$, Fig. 1A). Thirty minutes after glucose administration, blood glucose levels increased 3·8-fold in the normal mice. Thereafter, those levels were slightly decreased. On the other hand, blood glucose levels continued to increase in *grt* mice after glucose administration, and these levels were significantly higher at 90 and 120 min compared with normal mice. Quantitatively, the area under the glucose curve was significantly increased in *grt* mice as compared with that in normal mice (Fig. 1B).

Plasma insulin levels under fasting conditions (0 min) did not differ between normal and *grt* mice. Thirty minutes after glucose administration, plasma insulin levels increased in the normal mice, but not in *grt* mice, which showed significantly lower levels (284·0±58·2 vs 658·0±78·1 pg/ml, $P<0·05$, Fig. 1C). Thereafter, these levels decreased rapidly in normal mice and remained significantly lower in *grt* mice compared with normal mice, indicating that *grt* mice have an impaired glucose tolerance and reduced insulin secretion.

In addition, fasting hyperglycemia that was comparable to that in 3-month-old *grt* mice was observed in 2-, 6-, and 12-month-old *grt* mice (Fig. 1D), suggesting that *grt* mice are in a state of impaired glucose tolerance during their lives.

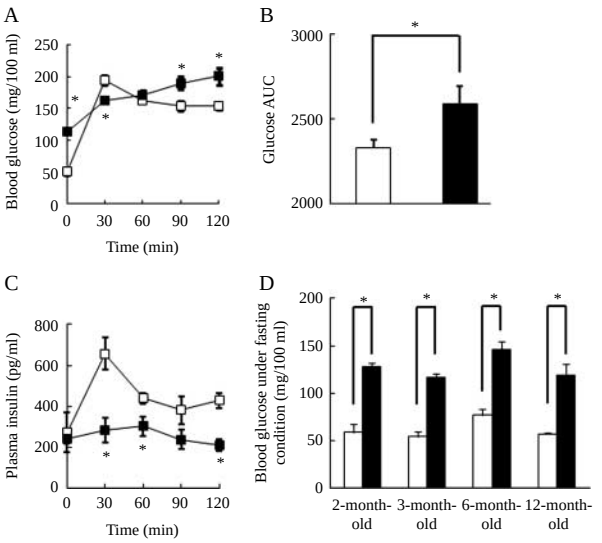

Figure 1 Intraperitoneal glucose tolerance test (IGTT). Blood glucose levels and plasma insulin levels were measured at the indicated time points following i.p. glucose administration (1 mg/g body weight) to normal (□) and *grt* mice (■). Blood samples were collected via retro-orbital sinus puncture. Blood glucose levels were measured using an automated blood glucose meter (A). To clarify the blood glucose levels quantitatively, the glucose AUC was calculated (B). Plasma insulin concentrations were determined using an ELISA (C). The blood glucose levels under fasting conditions were measured in 2- to 12-month-old mice (D). Results are presented as means±S.E.M. ($n=5$–6). *Significantly different from normal mice ($P<0·05$).

Insulin tolerance tests

To study the degree of insulin sensitivity, insulin tolerance tests were performed in 3-month-old normal and *grt* mice using a fixed dose of insulin (1 mU/g body weight i.p). The fall in blood glucose levels was almost the same for normal and *grt* mice, except for at 15 min after insulin administration, illustrating nearly equal insulin sensitivity (Fig. 2). Therefore, impaired insulin secretion in *grt* mice does not seem to be associated with insulin resistance.

Histology of pancreatic islets

Isolated islets from normal and *grt* mice were immunocytochemically stained with anti-insulin antibodies. There were no differences in the population or location of anti-insulin antibody-positive β-cells between islets of normal and *grt* mice (Fig. 3A and B respectively). We further examined the localization of insulin in pancreatic β-cells in detail using immunogold electron microscopy. Anti-insulin antibody-positive gold particles were detected in secretory granules in the islets of both normal and *grt* mice (Fig. 3C and D respectively). These observations indicate that, although *grt* mice as well as normal mice have insulin stored in secretory granules, pancreatic β-cells in *grt* mice may not be able to secrete insulin in a normal manner.

MALDI-TOF/MS

To analyze the molecular weight of insulin in the islets of normal and *grt* mice, we used MALDI-TOF/MS. The molecular weight of insulin in the islets of *grt* mice produced a major peak at an m/z ratio of 5794·1, which is very close to the major peak for insulin from normal islets which have an m/z ratio of 5793·4 (Fig. 4).

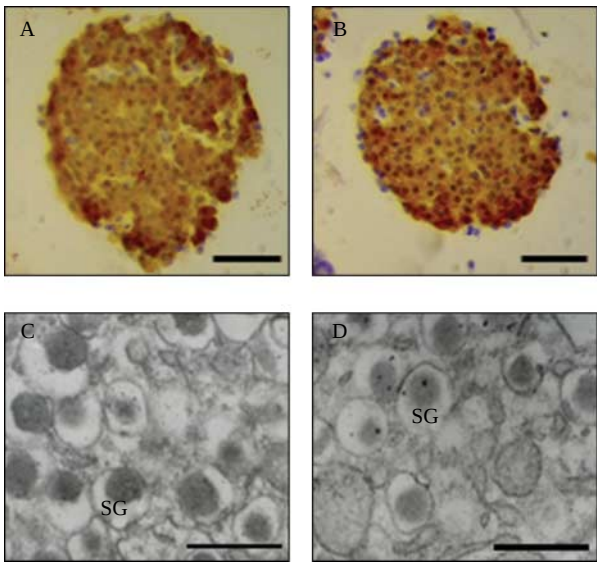

Figure 3 Immunohistochemical and immunoelectron microscopic analysis of insulin in pancreatic islets. Isolated pancreatic islets of normal (A and C) and *grt* (B and D) mice were stained for insulin using guinea pig anti-insulin antibodies. (A) and (B) were immunohistochemically analyzed following the addition of the peroxidase anti-peroxidase complex (PAP) (Bar = 50 μm). (C) and (D) were analyzed by immunoelectron microscopy following reaction with gold-labeled (10 nm) secondary antibodies. Gold particles were detected in secretory granules (SG) (Bar = 250 nm).

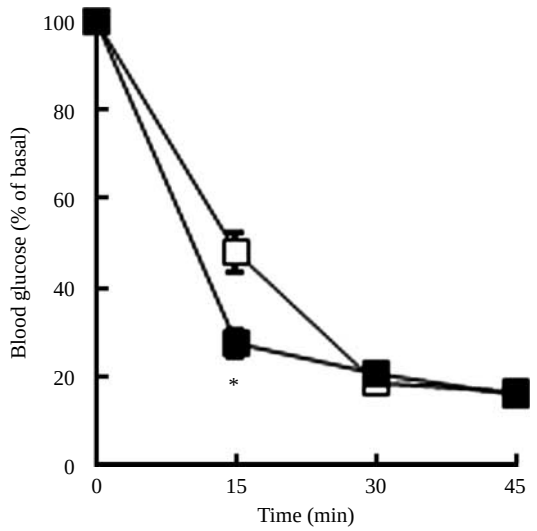

Figure 2 Insulin tolerance test. Blood glucose levels were measured at the indicated time points following i.p. insulin administration (1 mU/g body weight) to normal (□) and *grt* (■) mice. The results are expressed as a percentage of the value at time 0 (before insulin loading). Results are presented as means ± S.E.M. (n = 5–6). *Significantly different from normal mice (P < 0·05).

Effect of glucose and KCl treatment on insulin release from isolated islets

To evaluate the effects of glucose (16·1 mM) or KCl (28·9 mM) treatment on insulin secretion from isolated islets, size-matched islets from 6- to 8-month-old normal and *grt*

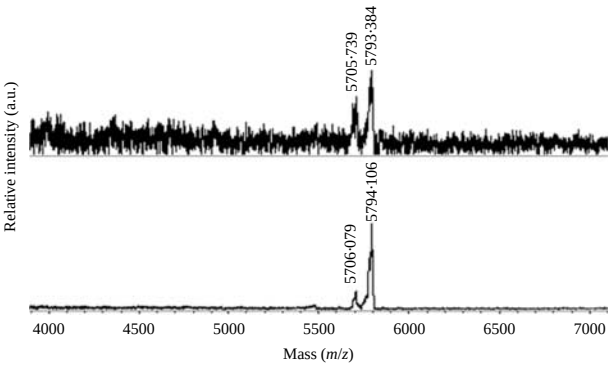

Figure 4 Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopic (MALDI-TOF/MS) analysis. MALDI-TOF mass spectra of the islets from normal (upper stand) and *grt* (lower stand) mice were acquired using a Bruker AutoflexIII MALDI-TOF mass spectrometer.

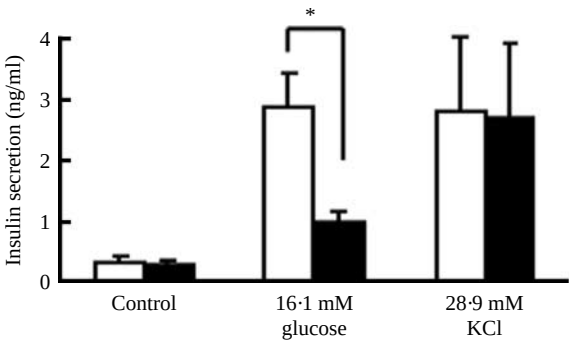

Figure 5 Glucose-mediated insulin secretion in isolated islets. Islets from normal (□) and *grt* (■) mice were isolated, and batches of ten islets were preincubated in DPBS for 60 min. Insulin secretion was assessed in the absence of glucose and KCl (control), in the presence of 16·1 mM glucose, or in the presence of 28·9 mM KCl for 60 min. The medium was collected and gently centrifuged. Insulin concentrations were determined using an ELISA. Results are presented as means±S.E.M. (n=5–6). *Significantly different from normal mice (P<0·05).

mice were statically incubated under *in vitro* conditions. Insulin secretion from islets in control medium did not differ between islets of normal and *grt* mice (0·32±0·12 vs 0·27 ±0·08 ng/ml, Fig. 5). The insulin-secretory response to glucose (16·1 mM) treatment of *grt* mice islets was markedly reduced compared with normal mouse islets (0·99±0·15 vs 2·88±0·57 ng/ml, P<0·05, Fig. 4); however, there was no difference in insulin secretion from islets in DPBS containing KCl (28·9 mM) between normal and *grt* mouse islets (2·82 ±1·22 vs 2·71±1·24 ng/ml, Fig. 5). These observations indicate that isolated islets in *grt* mice have normal K^+_{ATP} channels and postchannel activity, and that there appears to be an abnormal multistep process of glucose uptake or sensing in islets from *grt* mice.

Glut2 and Gk mRNA levels

To determine the defect in insulin secretion in *grt* mice, the mRNA levels of the glucose sensors, *Glut2* and *Gk*, in pancreatic islets were analyzed using real-time PCR. There was no difference in *Glut2* (Fig. 6A) or *Gk* (Fig. 6B) mRNA levels between islets of normal and *grt* mice. This observation indicates that reduction of insulin secretion from islets of *grt* mice involves a process other than a decrease in the expression of *Glut2* and *Gk* mRNA.

Effect of T_3 treatment to grt mice on IGTTs

Since *grt* mice are hypothyroid animals, the effect of T_3 treatment on the impaired insulin secretion of these mice was studied. The results of IGTTs of normal, *grt*, and T_3-treated *grt* mice are shown in Fig. 7. As described previously, the blood glucose levels under fasting conditions in *grt* mice were higher than in normal mice (Fig. 7A). The blood glucose levels continued to increase in *grt* mice after glucose administration, and were significantly higher than the corresponding values in normal mice at 90 and 120 min. Following T_3 treatment of *grt* mice, the blood glucose levels after fasting and blood glucose levels after glucose administration were restored to nearly the same levels as those in normal mice (Fig. 7A). The plasma insulin levels were significantly increased at 30 min after the administration of glucose as compared with the levels at time 0 in normal mice (Fig. 7B). However, this increase of the insulin levels induced by glucose administration was not observed in *grt* mice (Fig. 7C). On the other hand, the plasma insulin levels were slightly increased at 30 min after a glucose load as compared with the levels at time 0 in T_3-treated *grt* mice (P=0·056, Fig. 7D). However, this increase was not comparable to that observed in normal mice (Fig. 7B and D). Altogether, these

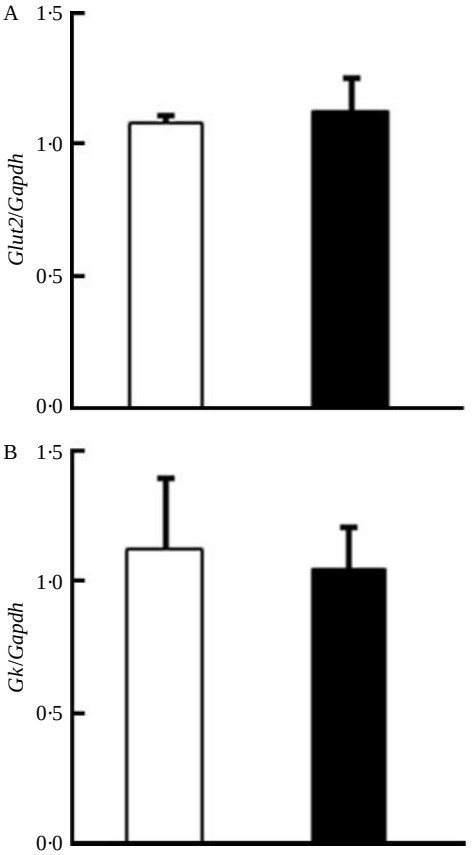

Figure 6 Real-time PCR analysis of the glucose transporter 2 (*Glut2*) and glucokinase (*Gk*) in pancreatic islets. Expression of the *Glut2* (A) and *Gk* (B) mRNA levels in pancreatic islets of normal (□) and *grt* (■) mice. Total RNA was extracted from 750 to 850 islets and reverse transcribed. The level of mRNA expression was analyzed by real-time PCR. The mRNA level was normalized by dividing the level of mRNA of the housekeeping gene, *Gapdh*, in the same sample. Results are presented as means±S.E.M. (n=3).

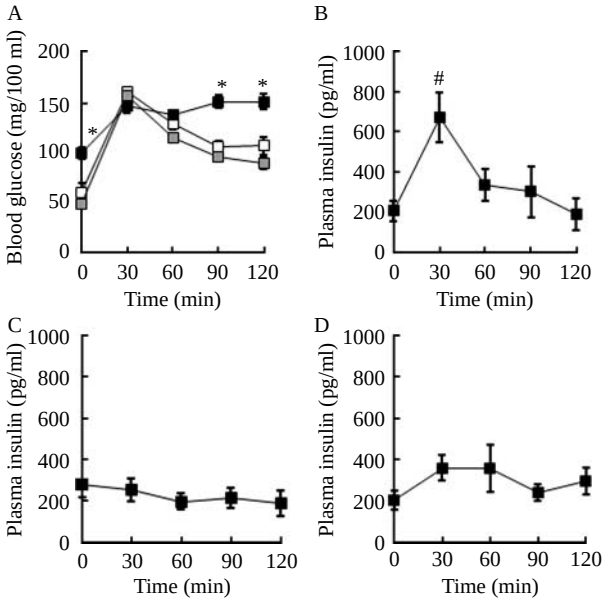

Figure 7 Effect of T_3 treatment on the results of IGTT in *grt* mice. T_3 (0·2 μg/g body weight) was injected i.p. into 3-week-old *grt* mice once every other day for 9 weeks. Mice injected with the vehicle alone were used as the control. Blood glucose levels following i.p. glucose administration (1 mg/g body weight) in normal (□), *grt* (■) and T_3-treated *grt* (▣) mice were measured by an automated blood glucose meter (A). Plasma insulin levels in normal (B), *grt* (C), and T_3-treated *grt* (D) mice were assayed by an ELISA. Results are presented as means±S.E.M. (n=7–9). *,#Significantly different from normal mice and at time 0 respectively (P<0·05).

observations indicate that there is no significant relationship between the thyroid dysfunction and the insulin hyposecretion in *grt* mice.

Effect of T_3 treatment on insulin secretion from the islets of grt mice

To confirm the effect of the thyroid hormones on the impaired insulin secretion in *grt* mice, we investigated the glucose-stimulated insulin secretion from the isolated islets of T_3-treated *grt* mice. There was no difference in the insulin secretion in response to glucose exposure between the islets of T_3-treated *grt* mice and those of control *grt* mice (Fig. 8). Thus, T_3 treatment was ineffective in correcting the impaired insulin secretion from the islets of *grt* mice.

Expression of Tpst1 and Tpst2 mRNA in pancreatic islets

In order to investigate the relationship between islet function and TPST, the mRNA expression of *Tpst1* and *Tpst2* was analyzed in isolated islets from 6- to 8-month-old normal and *grt* mice using RT-PCR and gene-specific primers (Table 2). The mRNA of these enzymes was expressed in pancreatic islets of both normal and *grt* mice (Fig. 9). This observation suggests the existence of some target proteins for TPSTs in pancreatic islets.

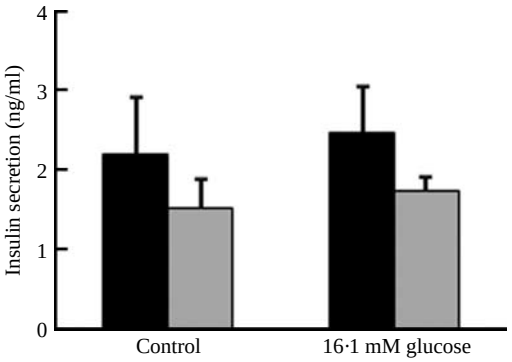

Figure 8 Effect of T_3 treatment on glucose-stimulated insulin secretion in isolated islets of *grt* mice. T_3 (0·2 μg/g body weight) was injected i.p. into 3-week-old *grt* mice once every other day for 9 weeks. Mice injected with the vehicle alone were used as the control. Isolated islets from control *grt* (■) and T_3-treated *grt* (▣) mice were preincubated in DPBS for 60 min. Insulin secretion was assessed in the absence of glucose (control) or in the presence of 16·1 mM glucose for 60 min. The medium was collected and gently centrifuged. Insulin concentrations were measured using an ELISA. Results are presented as means±S.E.M. (n=3).

Discussion

It has been reported that overt hypothyroidism is associated with profound changes in insulin sensitivity and insulin secretion, and that these changes are reversible, at least in part, after recovery of thyroid function (Rochon *et al.* 2003, Stanická *et al.* 2005). The results of the present study indicated that hypothyroidal *grt* mice also have a significant reduction in glucose-induced insulin secretion.

In this study, we showed that *grt* mice secrete less insulin, both *in vivo* and *in vitro*, in response to glucose administration compared with normal mice, indicating a functional failure of pancreatic islets. In addition, it was inferred that the insulin secretion was impaired throughout the life in *grt* mice, since significant elevation of the blood glucose levels under fasting conditions compared with those in normal mice was observed in *grt* mice ranging in age from 2 to 12 months. The function of pancreatic β-cells varies with differences in insulin

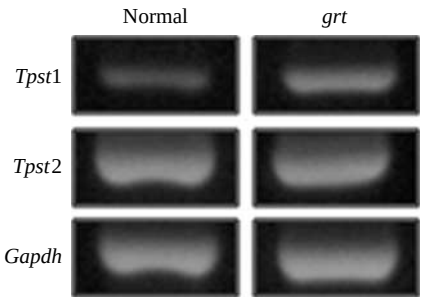

Figure 9 Expression of tyrosylprotein sulfotransferase (*Tpst*) 1 and *Tpst2* mRNA in islets of normal and *grt* mice. The mRNA expression of *Tpst1, Tpst2,* and the internal control *Gapdh* was analyzed by RT-PCR. PCR products were separated on 1·5% ethidium bromide (EtBr)–agarose gels.

sensitivity (Kahn *et al.* 1993). Insulin resistance produces a compensatory increase in insulin secretion and, ultimately, it is the failure of the β-cell that causes fasting hyperglycemia and development of type 2 diabetes (Kahn 1994). Low T_4 levels, even when within the physiological range, were significantly associated with increased insulin resistance (Roos *et al.* 2007). In addition, the level of insulin receptors was reported to be decreased in hypothyroidism (Mackowiak *et al.* 1999). On the other hand, it has also been reported that hypothyroidism has no impact on insulin sensitivity (Owecki *et al.* 2006). We therefore examined the influence of hypothyroidism on insulin sensitivity in *grt* mice, and demonstrated that blood glucose levels in response to insulin treatment were almost the same between normal and *grt* mice. Our observation suggested that thyroid dysfunction does not affect insulin sensitivity, and that impaired insulin secretion is not attributable to insulin resistance in *grt* mice.

Histological examination showed that insulin was localized in secretory granules in pancreatic β-cells of both normal and *grt* mice. In addition, the size of isolated islets and of β-cells in islets and the ratio of β-cells in the islets were almost the same between normal and *grt* mice (data not shown). These findings suggest that reduced insulin secretion in *grt* mice is not attributable to the number of β-cells in the islets or to a change in insulin synthesis or storage in pancreatic β-cells. This finding was further supported by analysis of the molecular weight of insulin in the islets of normal and *grt* mice using MALDI-TOF/MS. We, therefore, speculated that insulin of the same molecular weight as that in normal mice is synthesized and stored in the islets of *grt* mice.

Glucose is transported by membrane-bound GLUT2 into the β-cell, where it is phosphorylated by GK to yield glucose-6-phosphate, thereby initiating glycolysis. The resultant increase in the ATP:ADP ratio causes the closure of the ATP-sensitive K^+ channels (K^+_{ATP} channel) and subsequent depolarization of the plasma membrane. Oscillatory changes in the membrane potential activate the opening and subsequent closure of the voltage-gated Ca^{2+} channels to allow an influx of extracellular Ca^{2+} (Santos *et al.* 1991), and an increase in cytoplasmic Ca^{2+} triggers insulin exocytosis (Gilon *et al.* 1993, Bergsten *et al.* 1994). The insulin-secretory responses to glucose or KCl were determined *in vitro* by static incubation of pancreatic islets of normal and *grt* mice. In the islets of *grt* mice, insulin secretion in response to 16·1 mM glucose was significantly reduced. In contrast, there was no difference in insulin secretion in the presence of 28·9 mM KCl. These results indicated that the K^+_{ATP} channel and the postchannel pathway function in a normal manner in *grt* mice.

We hypothesized that the reduction in insulin release from pancreatic islets of *grt* mice might be associated with the expression of *Glut2* and *Gk* mRNA, since it has been reported that the expression of these glucose sensors is regulated by thyroid hormone. For example, T_3 can modulate the mRNA and protein levels of these molecules in pancreatic islets (García-Flores *et al.* 2001). In addition, thyroid hormones regulate hepatic glucose transport by altering the expression of *Glut2* mRNA and protein levels in hepatocytes (Weinstein *et al.* 1994, Kemp *et al.* 1997, Mokuno *et al.* 1999). Thyroid hormone is also involved in the regulation of *Gk* gene transcription in liver (Höppner & Seitz 1989). We showed that there is no difference in the level of expression of *Glut2* or *Gk* mRNA between islets of normal and *grt* mice. Therefore, we predicted that there must be a functional failure of glucose sensors in *grt* mice, including glucose sensors involved in glucose transport, GK activity, glycolysis, and glucose oxidation. Since GLUT2 has a high Michaelis–Menten constant for glucose, the action of GLUT2 is needed for a high physiological concentration of glucose. Therefore, GLUT2 would not be expected to be rate-limiting to consequent glucose utilization, indicating that it is not possible to evaluate insulin secretion only by assay of the expression of GLUT2 (Kooptiwut *et al.* 2002). In support of this idea, it has been shown in rat pancreatic β-cells that the rates of glucose transport were at least 50-fold higher than the corresponding rates of glucose utilization, and that glucose phosphorylation, not glucose transport, correlated with glucose sensitivity (Heimberg *et al.* 1993). In addition, glucose could stimulate insulin secretion from mouse islets in which there was a complete absence of GLUT2 (Guillam *et al.* 2000). On the other hand, GK activity can be elevated in the presence of increased GLUT2 protein levels (Ferber *et al.* 1994). Finally, the observed reduction in insulin secretion from pancreatic islets of *grt* mice may be associated with the protein levels and function of GLUT2 and GK rather than with their mRNA expression, as well as with other factors. Further studies will determine whether there is a link between insulin hyposecretion and glucose utilization in *grt* mice.

T_3 replacement restores growth retardation (Yoshida *et al.* 1994) and serum TSH levels (Tomita *et al.* 1995) in *grt* mice. Thyroid hormone receptor α (TRα) and TRβ mRNA and protein have been detected in pancreatic islets (Zinke *et al.* 2003). We demonstrated that T_3 replacement can completely recover the impaired glucose tolerance in *grt* mice, and that the plasma levels of insulin in response to a glucose load were only slightly increased in T_3-treated *grt* mice, indicating that hypothyroidism is associated with a deterioration of glucose metabolism. The recovery of impaired blood glucose levels in T_3-treated *grt* mice may be associated with increased GLUT4 localized in the myocytes and adipocytes that is involved in glucose transport, since GLUT4 protein expression is regulated by T_3 (Shimizu & Shimazu 2002). It has been reported that although subjects with hypothyroidism are characterized by attenuated plasma insulin concentrations and increased glucagon concentrations in the basal state, T_4 replacement can, among other effects, result in partial amelioration of insulin secretion and improved insulin sensitivity (Handisurya *et al.* 2008). In the present study, insulin sensitivity in *grt* mice was almost the same as that of normal mice, suggesting that the effect of T_3 replacement may be mediated by mechanisms other than modulation of insulin resistance in *grt* mice.

Our data further suggest that complete restoration of insulin secretion cannot be achieved by T_3 treatment of *grt* mice. In addition, we could not observe any improvement in insulin secretion from the isolated islets of T_3-treated *grt* mice. We, therefore, presumed that a mechanism other than hypothyroidism is involved in the regulation of insulin secretion. We hypothesized that there may be a relationship between insulin hyposecretion in *grt* mice and the gene responsible for the *grt* phenotype, *Tpst2*. TPST catalyzes the transfer of a sulfuryl group from the universal sulfation substrate, 3′-phosphoadenosine 5′-phosphosulfate, to a tyrosyl residue within acidic motifs of proteins that transit the Golgi apparatus (Moore 2003). In mammals, two isozymes, TPST1 and TPST2, catalyze the tyrosine sulfation of proteins. Although mammalian TPST1 and TPST2 have 65–67% amino acid sequence identity (Moore 2003), they appear to have different acceptor preferences (Seibert *et al.* 2002). Evidence indicates that posttranslational modification by tyrosine sulfation regulates many important protein–protein interactions and modulates binding affinity and specificity (Niehrs *et al.* 1990, Pittman *et al.* 1994, Sasaki *et al.* 2007). TPST2 plays a crucial role in tyrosine sulfation of the TSH receptor (Sasaki *et al.* 2007). Sulfation of the tyrosine 385 residue of the TSH receptor by TPST2 is indispensable for activation of TSH signaling. This means that *grt* mice develop hypothyroidism and growth retardation because they are unable to fully respond to TSH (Sasaki *et al.* 2007). It has been reported that TPST2 is ubiquitously expressed (Mishiro *et al.* 2006). However, there has been no report concerning the expression of TPST2 in mouse pancreatic islets. To evaluate the relationship between TPST2 and insulin secretion in *grt* mice, we analyzed the expression of *Tpst* mRNA in islets of *grt* mice using RT-PCR, to confirm that the mRNA of *Tpst1* and *Tpst2* is expressed in islets of *grt* mice. These observations indicate that target proteins of TPST2 may be expressed in pancreatic islets. Chromogranin A and secretogranin II are known to be expressed in islets, although the physiological role of insulin secretion by these molecules in pancreatic islets is unclear (Schmid *et al.* 2007, Koshimizu *et al.* 2010). Tyrosine residues of these proteins are sulfonated by TPST (Moore 2003), and they play a significant role in granule formation in endocrine cells (Koshimizu *et al.* 2010). On the other hand, it has been reported that cholecystokinin and gastrin regulate insulin secretion (Unger *et al.* 1967, Ubilluz 1994), and that these proteins also require tyrosine sulfation for optimal function (Rehfeld 1998). Then, it is of great interest to investigate whether TPST2 is directly and/or indirectly involved in the regulation of insulin secretion from the pancreatic islets. A study of the relationship between these molecules and insulin secretion from pancreatic islet is currently in progress.

In summary, the present study demonstrates that *grt* mice show reduced glucose-stimulated insulin secretion, and that the administration of T_3 to these mice improves the insulin hyposecretion, albeit only to a slight extent. Further studies are required to investigate the underlying mechanism, including the role of TPST2, in the reduction of insulin secretion in *grt* mice.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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