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Overexpression of PACAP in Islets Inhibits Hyperinsulinemia and Islet Hyperplasia in Agouti Yellow Mice

SHUHEI TOMIMOTO, HITOSHI HASHIMOTO, NORIHITO SHINTANI, KYOHEI YAMAMOTO, YUKI KAWABATA, KEN-ICHI HAMAGAMI, KAZUYA YAMAGATA, JUN-ICHIRO MIYAGAWA, and AKEMICHI BABA

Laboratory of Molecular Neuropharmacology (S.T., H.H., N.S., K.Yamam., Y.K., K.H., A.B.), Graduate School of Pharmaceutical Sciences, Osaka University; Department of Internal Medicine and Molecular Science (K.Yamag., J.M.), Graduate School of Medicine, Osaka University; Laboratory of Molecular Pharmacology (A.B.), Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan

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Running title: PACAP Overexpression in *A^y^* mouse islets

Address correspondence and reprint requests to:

Akemichi Baba

Laboratory of Molecular Neuropharmacology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

Tel: (81) (6) 6879-8180; Fax: (81) (6) 6879-8184; E-mail: baba@phs.osaka-u.ac.jp

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Abbreviations: PACAP, pituitary adenylate cyclase-activating polypeptide; Tg/+ mice, transgenic mice overexpressing PACAP in pancreatic β-cells; *A^y^*/+ mice, lethal yellow agouti (KK*A^y^*) mice; VIP, vasoactive intestinal polypeptide; GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide; PAC_1 receptor, type 1 PACAP receptor; PBS, phosphate-buffered saline.

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) is an intraislet neuropeptide and shares insulinotropic and insulin-sensitizing properties with GLP-1, however, the pathophysiological significance of PACAP in diabetes remains largely unknown. To assess this, we crossed our recently developed transgenic mice overexpressing PACAP in pancreatic β-cells (Tg/+), with lethal yellow agouti (KK*A^y*) mice (*A^y*/+), a genetic model for obesity-diabetes, and examined the metabolic and morphologic phenotypes of F_1 animals. Tg/+ mice with the *A^y* allele (Tg/+:*A^y*/+) developed maturity-onset obesity and diabetes associated with hyperglycemia, hyperlipidemia, and hyperphagia, similar to those of *A^y*/+ mice, but hyperinsulinemia was significantly ameliorated in Tg/+:*A^y*/+ mice. Although *A^y*/+ mice exhibited a marked increase in islet mass resulting from hyperplasia and hypertrophy, this increase was significantly attenuated in Tg/+:*A^y*/+ mice. Size frequency distribution analysis revealed that the very large islets comprising one-fourth of islets of *A^y*/+ mice were selectively reduced in Tg/+:*A^y*/+ mice. Since functional defects have been demonstrated in the large islets of obese animal models, taken together, these findings suggest that PACAP regulates hyperinsulinemia and the abnormal increase in islet mass that occurs during the diabetic process.

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PACAP which exists in two molecular forms, either with 27 (PACAP27) or 38 (PACAP38) amino acid residues, belongs to the vasoactive intestinal polypeptide (VIP)/secretin/glucagon superfamily (Arimura, 1998; Vaudry et al., 2000). Some members of this group, such as the gastrointestinal hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), stimulate β-cell growth, differentiation, and cell survival, in addition to their well-documented nutrient-stimulated secretion of insulin (so-called "incretin effect") (Kieffer and Habener, 1999; Pospisilik et al., 2003). GLP-1 and GIP are secreted postprandially from the L-cells of the lower small intestine and from the K-cells of the upper small intestine, respectively, and released into the circulatory system. In comparison, PACAP is a neuropeptide in pancreatic islets, where it may act as a parasympathetic and sensory neurotransmitter, and stimulate secretion of insulin in a glucose-dependent manner (Kieffer and Habener, 1999; Sherwood et al., 2000; Filipsson et al., 2001). In addition, PACAP has been shown to be expressed in islet β-cells (Portela-Gomes et al., 2003; Yada et al., 1997). PACAP stimulates insulin secretion from insulin-secreting β-cell lines (Klinteberg et al., 1996; Straub and Sharp, 1996), isolated pancreas (Yokota et al., 1993; Yada et al., 1994; Bertrand et al., 1996), and intact animals (Fridolf et al., 1992). There is recent evidence to suggest that PACAP exerts not only insulinotropic effects but also insulin-sensitizing properties like GLP-1. Yada et al. (2000) showed that intraperitoneal administration of PACAP reduces blood glucose in GK rats and mice on a high-fat diet. PACAP also enhances insulin-induced glucose uptake in 3T3-L1 adipocytes (Nakata et al., 1999). These findings implicate a potential therapeutic role for PACAP in diabetes.

ZAC/PLAGL1, the products which regulate both apoptosis and cell cycle arrest, as well as a transcriptional regulator of the type 1 PACAP receptor (PAC_1) (Ciani et al., 1999), are a strong candidate gene for transient neonatal diabetes (TNDM) (Kamiya et al., 2000). In addition, a genome-wide linkage study revealed a connection between chromosome 18p11, the site of the human PACAP gene (Hosoya et al., 1992), and a susceptibility locus for type 2 diabetes (Parker et al., 2001). These findings imply that perturbation of PACAP signaling is involved in the pathogenesis of diabetes.

The plethora of peripheral and central effects of PACAP upon whole-body glucose metabolism includes the stimulation of insulin, glucagon (Fridolf et al., 1992; Filipsson et al., 2001) and epinephrine (Hamelink et al., 2002) secretion, hormonal regulation of lipid and carbohydrate metabolism (Gray et al., 2001), suppression of appetite (Mizuno et al., 1998), and influence on higher brain functions including psychomotor activity (Hashimoto et al., 2001; Hashimoto et al., 2002). This wide range of effects makes it difficult to determine the precise role of PACAP in both the regulation of glucose homeostasis and development of diabetes. In addition, identification of the mechanisms underlying the effects of PACAP in pancreatic islets has been hampered by a lack of suitable low-molecular weight PACAP antagonists.

As an approach to understanding the physiological and pathophysiological significance of PACAP in the pancreas, we have recently generated transgenic mice overexpressing PACAP in islet β cells under the control of human insulin promoter (Tg/+ mice) (Yamamoto et al., 2003). Tg/+ mice are normoglycemic and normoinsulinemic under basal conditions. Although glucose homeostasis was maintained, Tg/+ mice showed a greater acute insulin response to glucose compared with non-transgenic wild-type (+/+) mice. Also, streptozotocin-induced increases in plasma glucose were attenuated in Tg/+ mice. Notably, streptozotocin-treated Tg/+ mice exhibited an increase in 5-bromo-2-deoxyuridine (BrdU)-positive β cells, but without any change in the number of TUNEL-positive apoptotic cells. Morphometric analysis revealed that total islet mass increased in 12-month-old transgenic mice. In sharp contrast, PACAP-deficient mice (Hashimoto et al., 2001) exhibited significantly impaired glucose-induced insulin secretion (Shintani et al., 2003).

These observations suggest that, in addition to its islet function required for a normal glucose-stimulated insulin secretion, PACAP has a protective role against type 1 diabetes from islet degeneration associated with streptozotocin administration, and long-term effects on islet β-cell mass and function. These findings motivated us to determine the role of PACAP in the development of type 2 diabetes, particularly in the regulation of islet mass in vivo. In the present study, we performed genetic crosses between Tg/+ and lethal yellow agouti (KKAy) mice (Ay/+), a mouse model of type 2 diabetes characterized by obesity, hyperlipidemia,

insulin resistance and hyperglycemia (Leibel et al., 1997), and examined the metabolic and morphologic phenotypes of F1 animals.

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Materials and Methods

Animals. The generation of mice overexpressing PACAP specifically in the pancreatic islets (Tg/+) has been reported previously (Yamamoto et al., 2003). The male KKA^y mice (A^y/+), in which the A^y gene had been transferred into the original Japanese KK strain (Japan CLEA, Tokyo, Japan), were crossed with female Tg/+ mice with a C57BL/6J background to produce F_1 offspring. For genotyping, tail DNA was analyzed by the polymerase chain reaction (PCR) using the following PACAP gene exon specific primers: exon 3, 5'-AGA AGA CGA GGC TTA CGA CCA G-3' (sense) and exon 4, 5' -ACG ACC GAC TGC AGG TAC TTC- 3' (antisense). Four genotypes of F_1 mice (Tg/+:A^y/+, Tg/+, A^y/+, and +/+) with a C57BL/6J and KK hybrid background were obtained. Male F_1 mice with the four genotypes were used in the present study. Mice were housed in a temperature-, humidity-, and light-controlled room with a 12-hour light/12-hour dark cycle *(lights on from 8:00 AM to 8:00 PM)* and allowed free access to water and food (CMF, 369 kcal/100 g, Oriental Yeast Co. Ltd., Tokyo, Japan). All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Osaka University.

Analysis of Pancreatic PACAP Content. The pancreatic PACAP content was determined as described previously (Tamakawa et al., 1998) with a few modifications. Each of pancreata was weighed, boiled in 0.5 M acetic acid for 10 minutes, homogenized using Polytron, and then boiled for another 10 minutes. After centrifugation (2,000 *g*, 10 minutes), the supernatant was applied to a Sep-Pak C-18 cartridge (Waters), which was washed with water and then eluted with 60% acetonitrile-distilled water containing 0.1% trifluoroacetic acid. The eluate was lyophilized, and the PACAP content was measured by a radioimmunoassay using a commercial kit (Peninsula Laboratories).

Measurement of Food Intake. The F_1 mice were maintained in individual metabolic cages and a food tray was placed in their cage. The cumulative food intake was measured by weighing

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the residual food in the tray after two weeks.

Measurement of Blood Glucose, Insulin, and Triglyceride Concentrations. Blood was sampled from the tail vein of fed mice from 9:00 to 11:00 AM. The plasma glucose, insulin and triglyceride concentrations were measured using the Glucose CII-Test (Wako Pure Chemicals Industries Ltd., Osaka, Japan), a Sensitive Rat Insulin RIA kit (LINCO) and a Triglyceride INT kit (Sigma-Aldrich, Tokyo, Japan), respectively.

Glucose and Insulin Tolerance Test. For the insulin tolerance test, male F_1 mice were injected intraperitoneally with 0.5 or 2 U/kg porcine insulin (Sigma-Aldrich) after a two-hour fast. For the glucose tolerance test, mice were orally injected with 2 mg/g glucose after a 14-hour fast. Blood was sampled from the tail vein just prior to (time 0) and at 10, 30, 60, 90 and 120 minutes after the injection. The plasma glucose and insulin concentrations were determined as described above.

Immunohistochemistry and Quantitative Pancreatic Histomorphometry. The pancreas was carefully removed from each F_1 mouse. After weighing, each pancreas was immediately fixed in a 4% paraformaldehyde phosphate-buffered saline (PBS) solution and left overnight. It was then successively immersed in a 30% sucrose PBS solution for 48 hours, frozen under nitrogen fumes and stored at -80°C until sectioning. Serial sections (14 μm thick) were obtained from each pancreas and immunostained with a guinea pig anti-insulin antibody (DAKO, CA, USA), a rabbit anti-glucagon antibody (DAKO), and a rabbit anti-PACAP antibody (Yanaihara Institute Inc., Shizuoka, Japan). Quantitative islet histomorphometry was performed as described previously with a few modifications (Masuzaki et al., 1999). The whole pancreas was removed from sacrificed F_1 mice, and sections from each pancreas (representing the pancreatic head, body and tail) were obtained. Each section was stained with hematoxylin-eosin to obtain an enhanced view of the islets compared to the other areas of the pancreas. Total islet and pancreatic area were measured using a Nikon TE300 microscope (Nikon Inc., Tokyo, Japan)

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and Mac Scope image analysis software (Mitani Co., Fukui, Japan).

Insulin Content in the Whole Pancreas. Each pancreas removed from a 28-week-old F_1 mouse was homogenized in 4 ml of ice-cold acid-ethanol solution (75% ethanol, 0.15 N HCl), and insulin was extracted for 48 hours at 4°C. After centrifugation (2,000 *g*, 30 minutes, 4°C), the supernatant was neutralized and diluted with PBS. The insulin concentrations in the 5000-fold diluted samples were assayed using the above-mentioned RIA kit.

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Results

Overexpression of PACAP in Tg/+ and Tg/+:A^y/+ Mice. Genetic crosses between female Tg/+ mice and male *A^y*/+ mice produced F_1 offspring with four genotypes (Tg/+:*A^y*/+, Tg/+, *A^y*/+, and +/+). Immunohistochemical staining for insulin and glucagon showed a normal distribution and topology of insulin- and glucagon-containing cells in F_1 mouse islets (Fig. 1A-D). In addition, there was no apparent difference in α-cell to β-cell ratio between Tg/+:*A^y*/+ and *A^y*/+ mice, as well as between Tg/+ and +/+ mice. PACAP immunoreactivity was weakly detected in the islets of +/+ and *A^y*/+ mice, whereas it was strongly detected throughout the islets of Tg/+ and Tg/+:*A^y*/+ mice (Fig. 1E-H). A radioimmunoassay revealed that pancreatic PACAP content in Tg/+ mice was 141 ± 18 pg/mg tissue, which was ~ three-fold higher than that in +/+ mice (48 ± 12 pg/mg) (Fig. 1I). Pancreatic PACAP content in *A^y*/+ mice (38.3 ± 1.2 pg/mg) was similar to that in +/+ mice, whereas PACAP concentrations in Tg/+:*A^y*/+ mice (489 ± 86 pg/mg) was ~ 13-fold higher than that in *A^y*/+ mice ($P < 0.001$) and ~3.5-fold higher than that in Tg/+ mice ($P < 0.01$) (Fig. 1I).

Body Weight Changes in F_1 Mice. The time course of changes in the body weight of 5 to 17 week old F_1 mice was observed (Fig. 2A). By ~ 5 – 7 weeks of age, the four genotypes of mice had a similar body weight. Thereafter, the body weight of Tg/+ mice remained similar to that of +/+ mice (at 17 weeks of age, 41.4 ± 2.6 g and 40.9 ± 3.1 g, respectively). Tg/+:*A^y*/+ mice gradually developed obesity similar to that of *A^y*/+ mice, and both genotypes remained obese compared with the lean mice (+/+ and Tg/+) (at 17 weeks of age, Tg/+:*A^y*/+ mice, 48.3 ± 0.8 g; *A^y*/+ mice, 52.6 ± 1.0 g) (*A^y*/+ *vs.* +/+, $P < 0.01$; Tg/+:*A^y*/+ *vs.* Tg/+, $P < 0.01$).

Plasma Insulin, Glucose, and Triglyceride Concentrations in F_1 Mice. The plasma insulin, glucose, and triglyceride concentrations were determined in F_1 mice in the fed state (Fig. 2B-D). From eight weeks of age the plasma glucose and triglyceride levels in Tg/+:*A^y*/+ mice gradually increased in a similar manner to *A^y*/+ mice, but remained virtually unchanged in

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+/+ and Tg/+ mice (Fig. 2C, D). In *A^y*/+ mice from eight weeks old, the plasma insulin levels increased markedly and showed a dependence on age. Plasma insulin levels were also elevated in Tg/+:*A^y*/+ mice, but they were significantly decreased in Tg/+:*A^y*/+ mice by ~ 40-60% in comparison with *A^y*/+ mice ($P < 0.001$, ANOVA; at 17 weeks of age, Tg/+:*A^y*/+ mice, 92 ± 16 ng/ml; *A^y*/+ mice, 233 ± 14 ng/ml) (Fig. 2B). The levels of plasma insulin in +/+ and Tg/+ mice remained normal (at 17 weeks of age, +/+ mice, 3.37 ± 0.36; Tg/+ mice, 3.38 ± 0.74 ng/ml).

Food Intake in F_1 Mice. For two weeks, the cumulative (ad libitum) food intake of F_1 mice was determined. Fig. 3 shows total the food intake in F_1 animals between 15 and 17 weeks of age. There was no significant difference in the food intake between +/+ and Tg/+ mice (4.64 ± 0.18 and 4.48 ± 0.12 g/day, respectively). The food intake in *A^y*/+ mice (6.36 ± 0.34 g/day) was 37% higher than that in +/+ mice ($P < 0.01$). The food intake in Tg/+:*A^y*/+ mice (5.81 ± 0.30 g/day) was not significantly different from that in *A^y*/+ mice, but it was a significant 30% higher than in Tg/+ mice ($P < 0.05$). Similar results were obtained from animals between 5 and 7 weeks of age and between 21 and 23 weeks of age (data not shown).

Insulin Tolerance Test. The insulin tolerance test was performed to evaluate the insulin sensitivity of F_1 mice at between 21 and 30 weeks of age (Fig. 4). No significant hypoglycemic effects were observed in *A^y*/+ and Tg/+:*A^y*/+ mice when a normal dose of 0.5 U/kg insulin was administered (data not shown). A high dose of insulin (2 U/kg) was therefore chosen for the insulin tolerance test. Both +/+ and Tg/+ mice showed similar responses to an intraperitoneal injection of insulin (2 U/kg; $P = 0.48$, ANOVA). The hypoglycemic response in Tg/+:*A^y*/+ mice was not significantly different from that in *A^y*/+ mice ($P = 0.62$, ANOVA), but throughout the observation period, plasma glucose levels in the obese (*A^y*/+ and Tg/+:*A^y*/+) mice were significantly higher compared with the lean (+/+ and Tg/+) mice, (*A^y*/+ *vs.* +/+, $P < 0.01$; Tg/+:*A^y*/+ *vs.* Tg/+, $P < 0.01$, ANOVA). Similar results were obtained in mice at 8 weeks of age (data not shown).

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Glucose Tolerance Test. An oral glucose tolerance test (2 mg/g body weight) revealed that after 30 minutes, plasma glucose concentrations were significantly lower in Tg/+ mice compared with the other genotypes ($P < 0.05$) (Fig. 5A). Plasma glucose levels in the obese mice (*Ay*/+ and Tg/+:*Ay*/+) were significantly higher compared with the lean (+/+ and Tg/+) mice (*Ay*/+ *vs.* +/+, $P < 0.05$; Tg/+:*Ay*/+ *vs.* Tg/+, $P < 0.01$, ANOVA), but there was no difference between Tg/+:*Ay*/+ and *Ay*/+ mice ($P = 0.63$, ANOVA).

In contrast, 10 minutes after glucose injection plasma insulin levels were significantly higher in *Ay*/+ mice compared with Tg/+:*Ay*/+ mice and the other genotypes ($P < 0.001$), but thereafter declined to similar levels found in Tg/+:*Ay*/+ mice (Fig. 5B). Plasma insulin levels in the obese mice (*Ay*/+ and Tg/+:*Ay*/+) were again significantly higher compared with the lean mice (+/+ and Tg/+) (*Ay*/+ *vs.* +/+, $P < 0.001$; Tg/+:*Ay*/+ *vs.* Tg/+, $P < 0.001$, ANOVA). There was no significant difference between +/+ and Tg/+ mice ($P = 0.75$, ANOVA).

Histomorphometric Analysis of Pancreatic Islets in F_1 Mice. Histological examination of the pancreas of F_1 mice revealed that the islets of *Ay*/+ mice showed marked hyperplasia in comparison with +/+ mice (Fig. 6). In contrast, the islets of Tg/+:*Ay*/+ mice were characterized by a smaller islet area in comparison with *Ay*/+ mice. A systematic quantitative histomorphometric analysis of the pancreas of F_1 mice was then performed (Fig. 7). The mean islet area in *Ay*/+ mice ($80{,}000 \pm 8{,}000\ \mu m^2$) was significantly increased compared with +/+ mice ($21{,}900 \pm 2{,}300\ \mu m^2$) ($P < 0.001$), but this increase was significantly attenuated in Tg/+:*Ay*/+ mice ($49{,}500 \pm 5{,}200\ \mu m^2$) ($P < 0.01$). The mean islet area in Tg/+:*Ay*/+ was significantly higher than that in Tg/+ mice ($31{,}400 \pm 4{,}100\ \mu m^2$) ($P < 0.05$) (Fig. 7A). Although there was no significant difference in the number of islets per unit pancreas area between +/+, Tg/+, and Tg/+:*Ay*/+ mice (0.337 ± 0.034, 0.367 ± 0.038, and $0.454 \pm 0.070\ mm^{-2}$, respectively), it was markedly increased in *Ay*/+ mice ($0.685 \pm 0.053\ mm^{-2}$) compared with the three other genotypes ($P < 0.01$) (Fig. 7B). The islet mass (which is the ratio of islet area to pancreatic area multiplied by the pancreatic wet weight) of *Ay*/+ mice (40.9 ± 6.2 mg/pancreas) was significantly greater compared with +/+ and Tg/+ mice (+/+, 3.86 ± 0.56 mg/pancreas, $P <$

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0.001; Tg/+, 6.23 ± 0.79 mg/pancreas, $P < 0.001$). The islet mass in Tg/+:*A*^{y}/+ mice (19.4 ± 2.9 mg/pancreas) was ~ 50% lower than that in *A*^{y}/+ mice ($P < 0.01$) (Fig. 7C). This result is in good agreement with the plasma insulin levels (Fig. 2B) and pancreatic insulin content: the insulin content of the whole pancreas of Tg/+:*A*^{y}/+ mice was 61% lower than in *A*^{y}/+ mice (5.4 ± 1.7 and 13.7 ± 3.7 μg/g pancreas, respectively) (n = 4). Finally, a reduction in islets size was caused by a decrease in the number of largest islets; the very large (>100,000 μm^2) islets composing 26.2% of islets of *A*^{y}/+ mice were selectively and significantly reduced in Tg/+:*A*^{y}/+ mice (14.2%, $P < 0.05$) (Fig. 7D).

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Discussion

The aim of the present study was to determine the effects of overexpressed PACAP in the pancreas on the development of type 2 diabetes, by examining the metabolic and morphologic phenotypes of F_1 animals generated from genetic crosses of Tg/+ with *Ay*/+ mice. Here, we show that 1) overexpression of PACAP does not influence the obesity phenotype (including hyperglycemia, hyperlipidemia, and hyperphagia) in *Ay*/+ mice, but the hyperinsulinemia observed in *Ay*/+ mice in the ad libitum-fed state was markedly ameliorated in Tg/+:*Ay*/+ mice (about 60% inhibition at 17 weeks of age); 2) plasma glucose levels in the fed state as well as during glucose and insulin tolerance testings were not different between *Ay*/+ and Tg/+:*Ay*/+ mice; however, 3) an enhanced first-phase insulin response (0 – 10 minutes) to a glucose load in *Ay*/+ mice was significantly attenuated in Tg/+:*Ay*/+ mice; 4) although *Ay*/+ mice exhibit a marked increase in islet mass resulting from islet hyperplasia and hypertrophy, this increase in islet mass was markedly reduced in Tg/+:*Ay*/+ mice. It is noteworthy that 4) the very large islets (>100,000 μm^2) composing one-fourth of islets of *Ay*/+ mice were selectively and significantly reduced in Tg/+:*Ay*/+ mice.

In the present study, immunohistochemical analysis indicated that endogenous PACAP expression in the islet β-cells of +/+ and *Ay*/+ mice is weak. As expected, Tg/+ and Tg/+:*Ay*/+ mice showed a marked increase in pancreatic PACAP content, and high levels of PACAP expression throughout the islets. A 3.5-fold increase in pancreatic PACAP content in Tg/+:*Ay*/+ compared with Tg/+ mice is in accordance with three-fold elevation in the total islet mass in Tg/+:*Ay*/+ *vs.* Tg/+ mice (Figs. 1I and 7C).

However irrespective of the genotypes, plasma PACAP levels were undetectable (< 40 pg/ml) by RIA. Such a low level of plasma free PACAP is possibly due to the presence of the PACAP38-specific binding factor, ceruloplasmin, whose physiological significance in PACAP-binding activity is currently unknown (Tams et al., 1999).

Mice with the *Ay* allele developed maturity-onset obesity and diabetes (hyperglycemia), but Tg/+:*Ay*/+ mice showed a blunted increase in plasma insulin levels. This finding is in contrast

to previous results of ours obtained from Tg/+ mice (with a C57BL/6 genetic background), in which the insulin response to glucose was enhanced without affecting the plasma glucose response (Yamamoto et al., 2003). The most plausible explanation for this discrepancy between the levels of insulin and glucose is an altered sensitivity to insulin due to changes in insulin resistance and/or glycogenolysis. However, our previous study with Tg/+ mice did not provide evidence to support these explanations. In the present study, insulin sensitivity, assessed by the insulin tolerance test after a two-hour fast, and fasting insulin levels after a 14-hour fast (time 0 in Fig. 5B) were unchanged between Tg/+:*A^y*/+ and *A^y*/+ mice. However, the first-phase insulin response (10 minutes after glucose load) during the glucose tolerance test was higher in *A^y*/+ mice than in Tg/+:*A^y*/+ mice. This occurred despite a similar plasma glucose response exhibited by these genotypes and may suggest an increased insulin resistance in *A^y*/+ *vs.* Tg/+:*A^y*/+ mice.

It has been demonstrated that all three PACAP receptor types are found in adipose tissue (Wei and Mojsov, 1996), and that PACAP enhances insulin-induced glucose uptake in 3T3-L1 adipocytes (Nakata et al., 1999). Given that PACAP overexpressed in Tg/+:*A^y*/+ mice is released from pancreatic islets into the circulation, while it may be bound to some plasma binding proteins (see above), it is likely that PACAP exerts an insulin-sensitizing effect through increased glucose uptake in adipose tissue. A more precise investigation of insulin sensitivity (e.g. insulin clamp technique) is clearly required.

The enlargement of pancreatic islets in various obese rodent models has been observed previously (Like and Chick, 1970; Chan et al., 1998). To assess the impact of PACAP overexpression on islet morphology, we performed a histomorphometric analysis of the pancreas. As anticipated, *A^y*/+ mice showed marked hyperplasia and hypertrophy, but PACAP overexpression significantly reduced the increases in mean islet area, the number of islets per pancreas area, and islet mass caused by the *A^y* allele. The most striking finding was that very large islets observed in *A^y*/+ mice were selectively reduced in Tg/+:*Ay*/+ mice.

Previous morphological studies have shown that obese rodents have an increased proportion of very large islets (Chan et al., 1998). In Zucker (*fa*/*fa*) rats, a widely used rodent model of type

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2 diabetes characterized by obesity, hyperlipidemia, hyperinsulinemia, and islet hypertrophy, the large islets are inefficient releasers of insulin compared to small islets on the basis of mass (Hayek and Woodside, 1979). Furthermore, large *fa*/*fa* islets have been shown to suffer multifocal to coalescing β cell necrosis after overnight culture (Chan et al., 1998). With these previous findings, the present results imply a regulatory effect of PACAP on the abnormal increase in islet mass that occurs during the diabetic process.

It has been demonstrated that PACAP acts as an intra-islet autocrine/paracrine regulator of glucose-induced insulin secretion (Yada et al., 1997). Such a loop is likely implicated in the action of overexpressed PACAP, and may also be implicated in the regulation of β-cell growth and/or differentiation in normal animals.

PACAP, and related peptide GLP-1, stimulate insulin secretion in β cells in a glucose-dependent manner, possibly via a similar signal transduction pathway (Leech et al., 1996). GLP-1 stimulates the proliferation of INS-1 cells (Buteau et al., 1999) and increases the mass of β-cells in adult rodents in vivo, via both proliferation and neogenesis of βcells (Kieffer and Habener, 1999). However, there have been few investigations into the physiological roles of PACAP in pancreatic islets in vivo. In many cell types, PACAP is involved in a variety of cellular processes, including proliferation, differentiation and cytoprotection (Arimura, 1998; Vaudry et al., 2000), where the actions of PACAP are sometimes biphasic with stimulation occurring at lower doses (for instance, at subnanomolar concentrations), and inhibition at higher doses, or vice versa. PACAP stimulates several different signaling cascades, leading to the activation of adenylate cyclase, phospholipase C, extracellular regulated kinase (ERK), and also p38 mitogen-activated protein (MAP) kinases (Arimura, 1998; Hashimoto et al., 2000; Vaudry et al., 2000; Sakai et al., 2001; Sakai et al., 2002). Future research should focus on the mechanisms underlying the PACAP-mediated regulation ofβ-cell fate and function.

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FIGURE LEGENDS

Fig. 1. Characterization of overexpressed PACAP in F_1 mice. Double immunostaining for insulin (green) and glucagon (red) (A-D), and single immunostaining for PACAP (E-H) were performed in pancreatic sections from each genotype of F_1 mice. Scale bar in A, 100 μm. I, PACAP content in the pancreas of F_1 mice. Statistically significant differences were assessed by ANOVA, followed by a post-hoc Scheffe's test.

Fig. 2. Time course of changes in body weight, plasma insulin, glucose and triglyceride levels in F_1 mice. Body weight (A), plasma levels of insulin (B), glucose (C), and triglyceride (D) were determined in +/+ (open squares; n = 7), Tg/+ (closed squares; n = 4), *Ay*/+ (open circles; n = 6), and Tg/+:*Ay*/+ (closed circles; n = 13 - 14) mice in the fed state. Data are expressed as the mean ±SE. $P < 0.01$, $P < 0.001$, compared with +/+ mice; $P < 0.01$, $P < 0.001$, compared with Tg/+ mice; §§§ $P < 0.001$, compared with *Ay*/+ mice; two-way ANOVA.

Fig. 3. Food intake in F_1 mice. Total food intake was measured in +/+ (n = 11), Tg/+ (n = 10), *Ay*/+ (n = 15), and Tg/+:*Ay*/+ (n = 17) mice during a two-week period (between 15 and 17 weeks of age). Data are expressed as the mean ± SE. Statistically significant differences were assessed by ANOVA, followed by a post-hoc Scheffe's test. N.S., not significant.

Fig. 4. Insulin tolerance test. After two hours of fasting, insulin (2 U/kg body weight) was intraperitoneally injected into 21-30-week-old +/+ (open squares; n = 20), Tg/+ (closed squares; n = 18), *Ay*/+ (open circles; n = 23), and Tg/+:*Ay*/+ (closed circles; n = 31) mice, and plasma glucose levels were measured. Data are expressed as the mean ± SE. $P < 0.01$, compared with +/+ mice; $P < 0.01$, compared with Tg/+ mice, two-way ANOVA.

Fig. 5. Glucose tolerance test. After 14 hours of fasting, glucose (2 mg/g body weight) was orally injected into 21-24-week-old +/+ (open squares; n = 10), Tg/+ (closed squares; n = 14),

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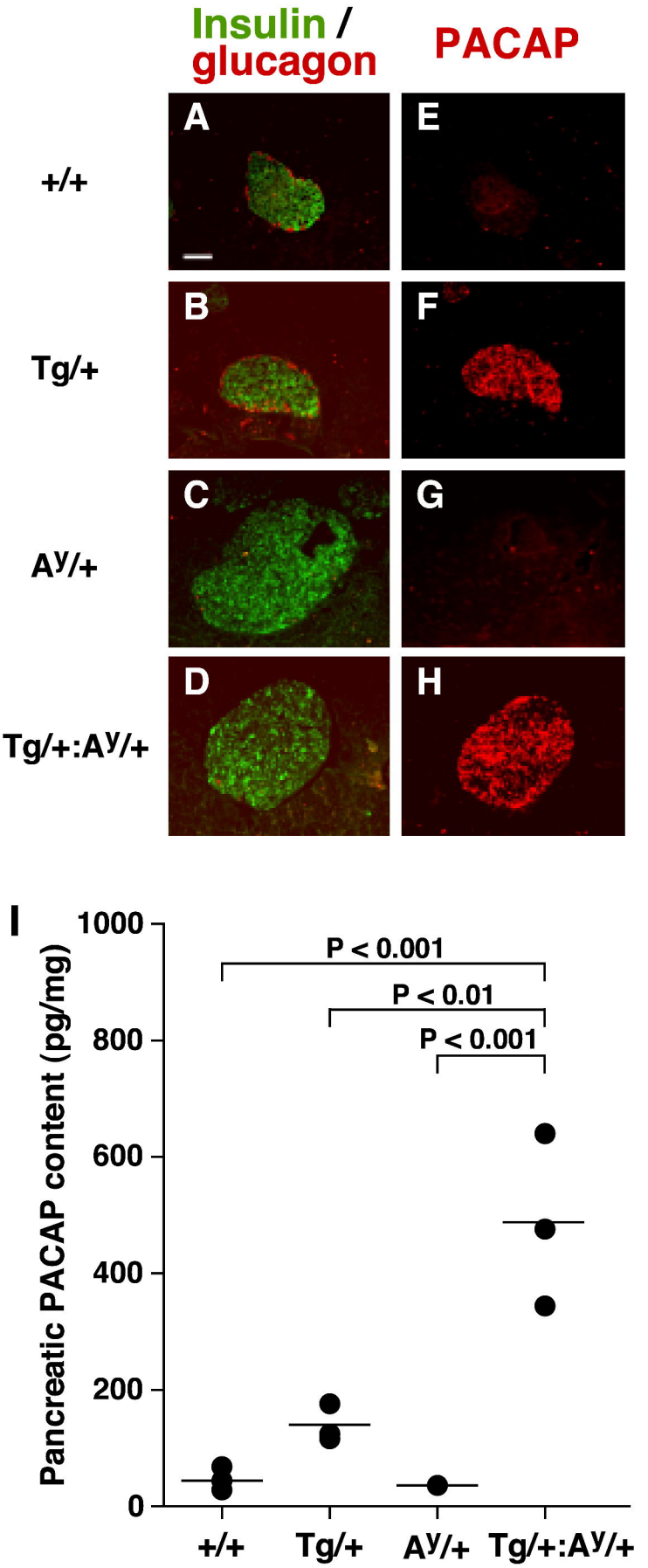
A^{y}/+ (open circles; n = 20), and Tg/+:*A*^{y}/+ (closed circles; n = 21) mice, and plasma glucose (A) and insulin (B) responses were measured. Data are expressed as the mean ±SE. * $P < 0.05$, ** $P < 0.001$, compared with +/+ mice; ‡ $P < 0.01$, ‡‡ $P < 0.001$, compared with Tg/+ mice, repeated measures ANOVA; † $P < 0.05$, compared with +/+ mice; § $P < 0.05$, §§§ $P < 0.001$, compared with Tg/+:A^{y}/+ mice; post-hoc Fisher PLSD test.

Fig. 6. Hematoxylin and eosin staining in pancreatic sections from each genotype of F_1 mice. Pancreatic sections from 27-28-week-old F_1 mice were stained with hematoxylin and eosin. Note that Tg/+:*A*^{y}/+ mice have a lower number and smaller islets than *A*^{y}/+ mice. Scale bar, 500 μm.

Fig. 7. Quantitative islet histomorphometry in F_1 mice. The mean of an islet area (A), the number of islets per square millimeter of total pancreatic area (B), total islet mass (C), and frequency distribution of islet areas (D) in F_1 mice. Data are expressed as the mean ±SE. Statistically significant differences were assessed by ANOVA, followed by a post-hoc Fisher PLSD test. N.S., not significant.



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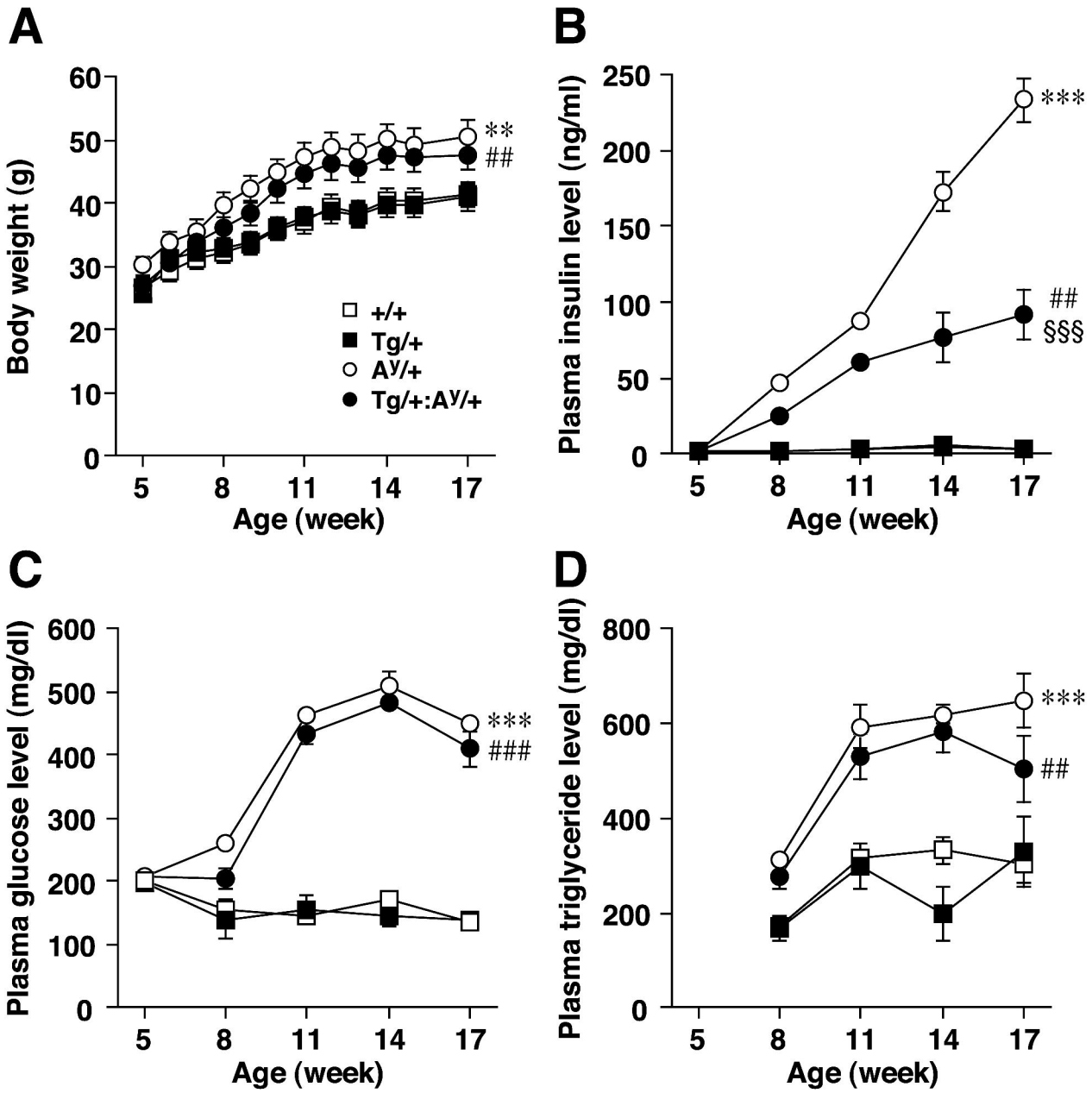

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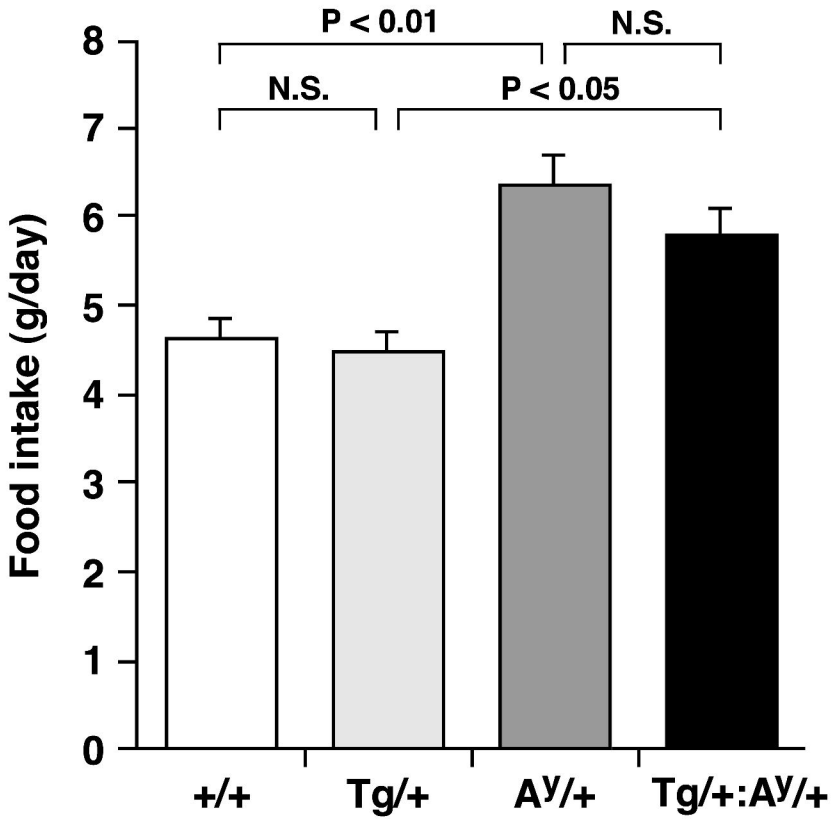

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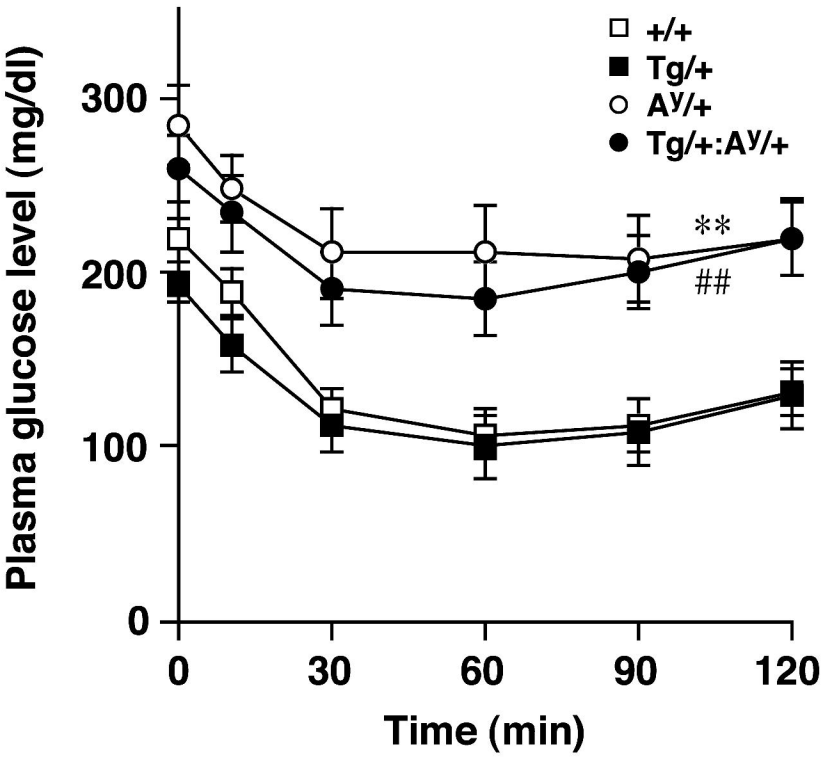

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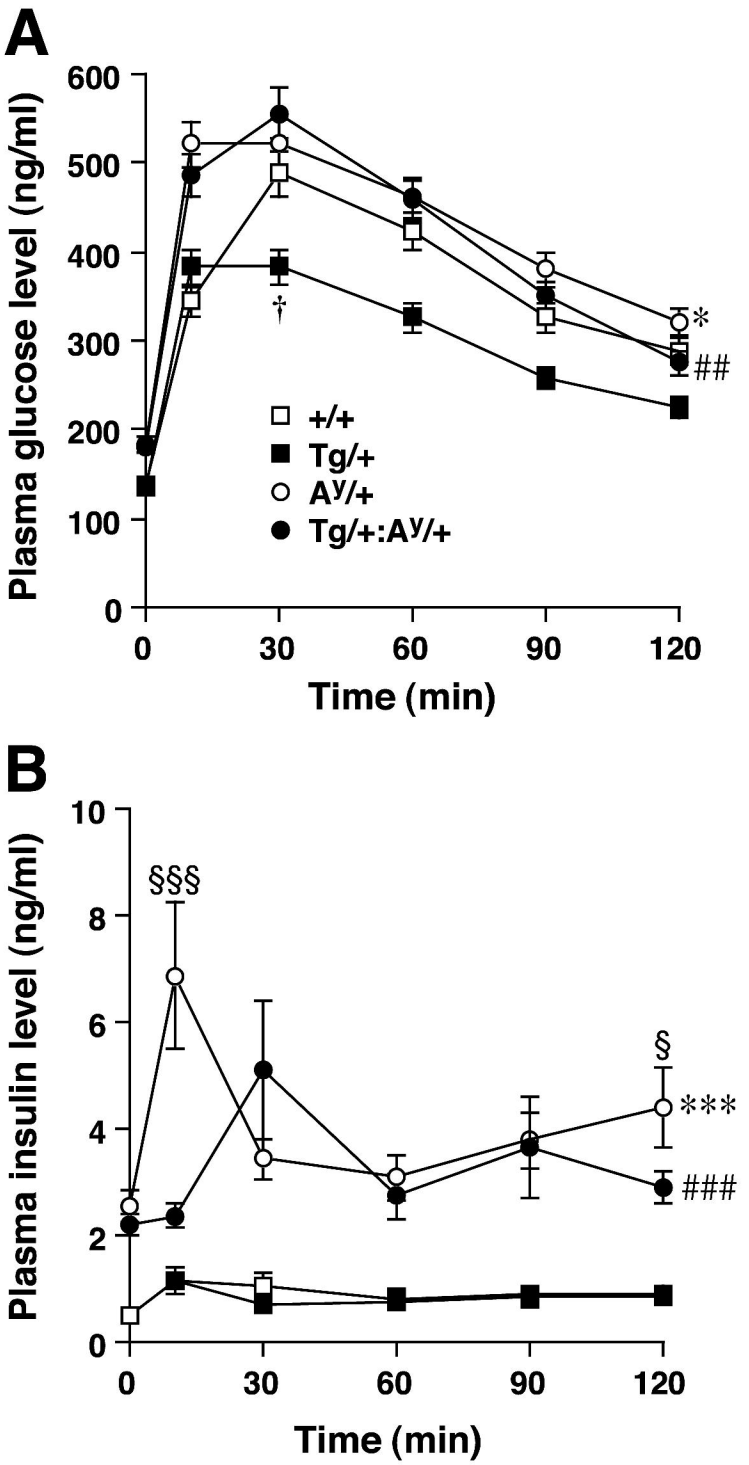

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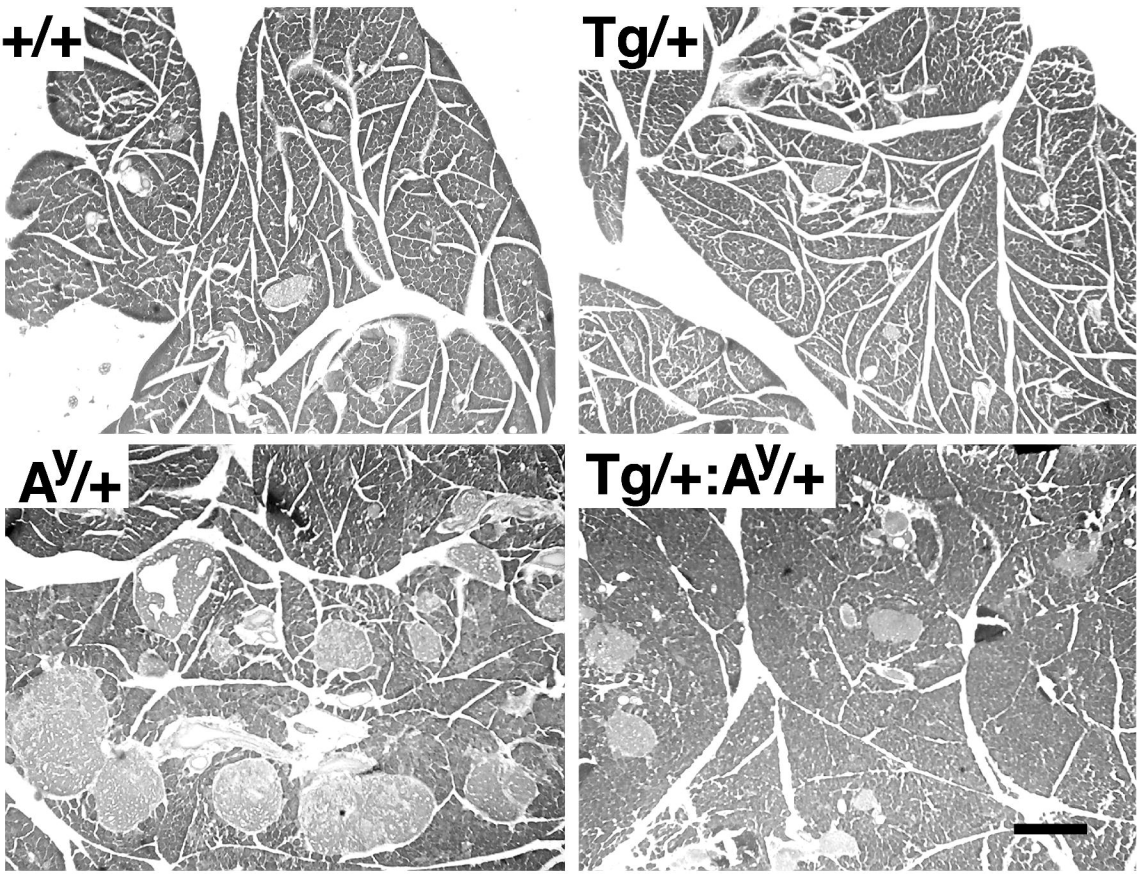

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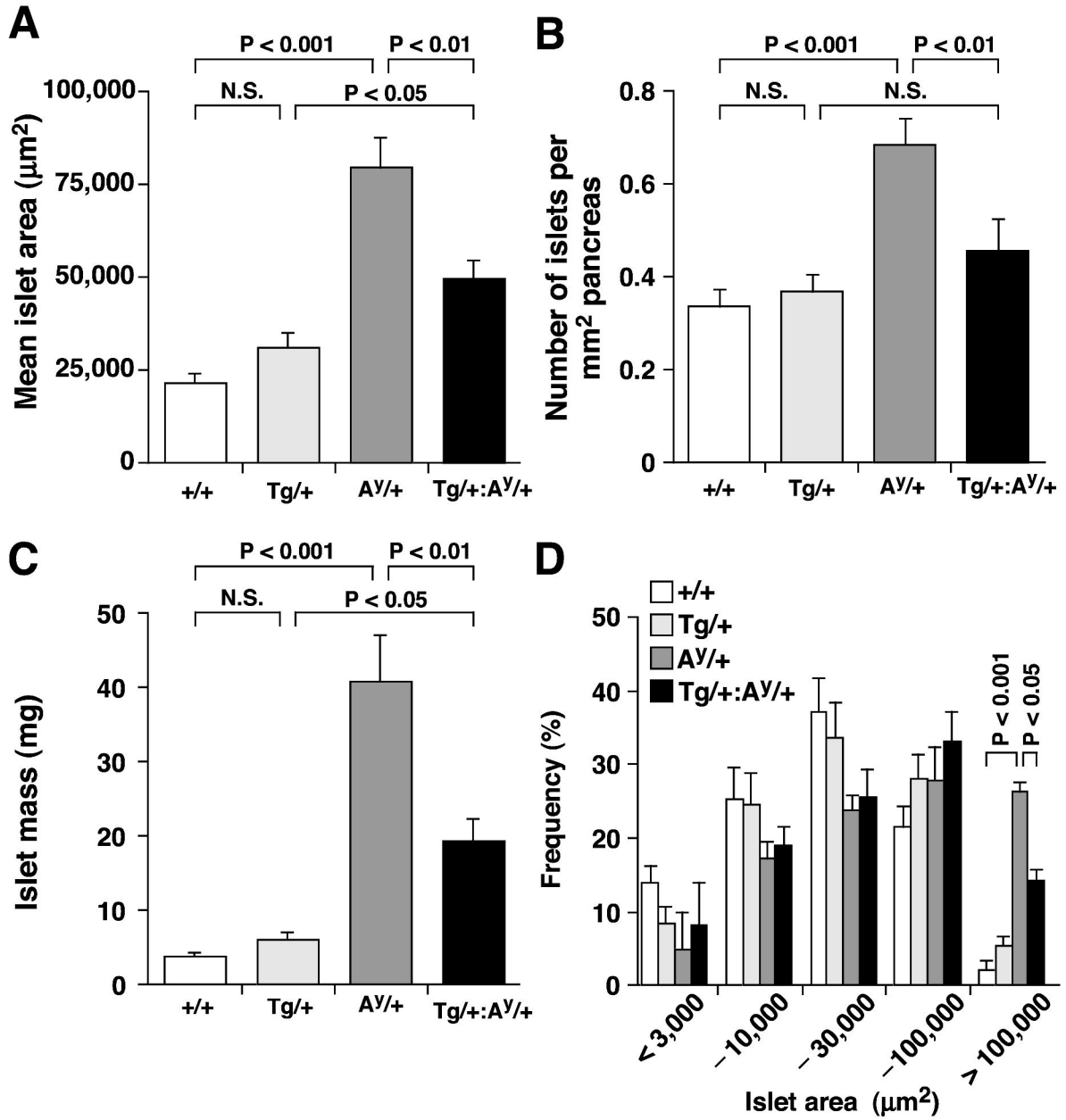

Fig. 7. Tomimoto, S., et al.