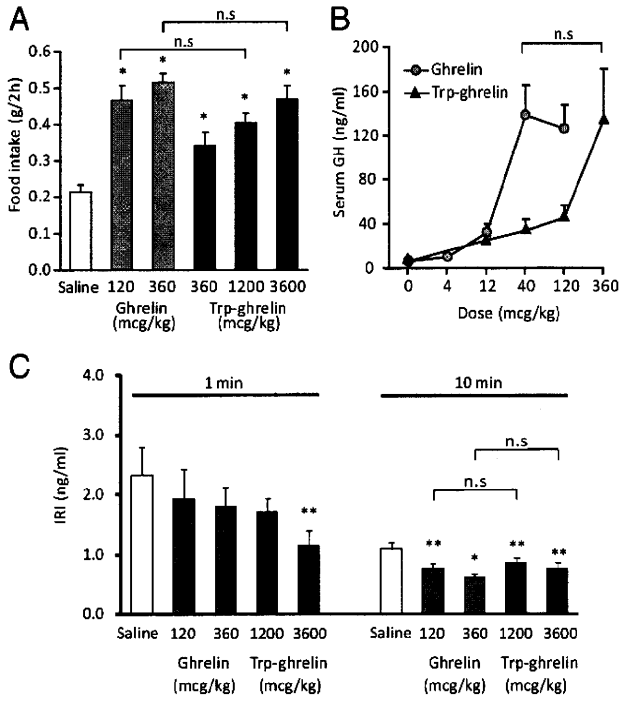

FIG. 1. Effects of the exogenous administration of Trp^3-ghrelin on food intake, GH secretion, and inhibition of glucose stimulated insulin release. A, Two-hour food intake after saline (*open bar*), ghrelin at 120 or 360 mcg/kg (*shaded bars*), or Trp^3-ghrelin at 360, 1200, or 3600 mcg/kg (*closed bars*) ip injection (n = 8–10). B, Serum GH levels were measured 10 min after 4, 12, 40, or 120 mcg/kg ghrelin (*shaded circles*) or 12, 40, 120, or 360 mcg/kg Trp^3-ghrelin (*closed triangles*) iv injection (n = 8–10). C, Serum insulin levels were measured 1 and 10 min after iv injection of 1.0 g/kg glucose, together with saline (*open bars*), ghrlein (120 and 360 mcg) (*shaded bars*), or Trp^3-ghrelin (1200 and 3600 mcg/kg) (*closed bars*) (n = 8). *, $P < 0.01$; **, $P < 0.05$; n.s, not significant compared with saline group; IRI, immunoreactive insulin. Data are presented as the means ± SEM.

inhibition of glucose stimulated insulin release) that of ghrelin.

Experiment 2, generation of Tg mice overexpressing Trp^3-ghrelin

Two Tg mouse lines, Tg6-2 and Tg6-5, were obtained. Hepatic transgene expression in Tg6-2 and Tg6-5 mice was 3.02 ± 1.15 and 0.07 ± 0.01 in arbitrary units, respectively, after normalization to preproghrelin mRNA expression levels seen in the stomachs of non-Tg littermates (non-Tg mice) (1.00 ± 0.18). No expression of preproghrelin mRNA was seen in the livers of non-Tg mice (Fig. 2C).

Two RIA methods [RIA recognizing the N-terminal region of ghrelin (N-RIA) and RIA recognizing the C-terminal region of ghrelin (C-RIA)] were performed to measure plasma ghrelin, des-acyl ghrelin, and Trp^3-ghrelin concentrations. In a previous study, N-RIA has been demonstrated to recognize only the acylated N-terminal region of ghrelin, whereas C-RIA recognizes the C-terminal region of ghrelin, making it possible to detect both acylated ghrelin and des-acyl ghrelin (12). We determined whether Trp^3-ghrelin could be detected by one or both of these RIA systems. When synthetic Trp^3-ghrelin was added to plasma samples from wild-type mice, Trp^3-ghrelin could only be detected by C-RIA, not N-RIA (data not shown). At 8 wk of age, plasma ghrelin concentrations measured by N-RIA did not differ among genotypes. Total plasma ghrelin concentrations, including ghrelin, des-acyl ghrelin, and Trp^3-ghrelin, measured by C-RIA were significantly elevated in Tg mice (Fig. 2, D and E). To determine precise plasma Trp^3-ghrelin concentration, we also performed HPLC on Tg6-2 samples (Fig. 2F). Plasma ghrelin (40.5 ± 10.2 *vs.* 36.6 ± 4.4 fmol/ml) and des-acyl ghrelin (167.5 ± 51.8 *vs.* 235.7 ± 44.8 fmol/ml) concentrations did not differ among genotypes.

Plasma Trp^3-ghrelin concentrations in Tg6-2 was 3437.8 ± 571.6 (2546.4–5101.7) fmol/ml, which was approximately 85-fold (3437.8/40.5 = 84.9-fold) higher than plasma ghrelin (acylated ghrelin) concentrations seen in non-Tg mice. Because Trp^3-ghrelin is approximately 1/10–1/20 less potent than ghrelin *in vivo* (experiment 1), plasma Trp^3-ghrelin concentrations in Tg6-2 were calculated to have an activity approximately 6-fold greater than that of ghrelin (acylated ghrelin) seen in non-Tg mice (84.9-fold × 1/10–1/20 = 4.2–8.5-fold). Total ghrelin concentrations measured by C-RIA in the Tg mice were roughly constant throughout the day.

We then analyzed the phenotype of the Tg6-2 line. Tg mice overexpressing Trp^3-ghrelin (Tg6-2 line) were abbreviated as Trp^3-ghrelin-Tg mice.

The analysis of the phenotypes of Trp^3-ghrelin-Tg mice

During postnatal development, there were no significant differences in somatic growth between Trp^3-ghrelin-Tg and non-Tg mice (Supplemental Fig. 1, A and B). Consistent with these results, no changes in serum GH and IGF-I concentrations were observed in Trp^3-ghrelin-Tg mice (Supplemental Fig. 1, C and D). The average food intake of Trp^3-ghrelin-Tg mice did not differ from that of non-Tg mice (Supplemental Fig. 1E). Trp^3-ghrelin-Tg mice consumed the largest food portions during the dark phase (75.4 ± 2.7%), similar to the behavior seen in non-Tg mice (75.9 ± 1.6%). There were no differences between 10-wk-old Trp^3-ghrelin-Tg and non-Tg mice in pituitary and hypothalamic mRNA levels of factors involved in GH secretion and food intake (Supplemental Fig. 2, A and B). In addition, glucose metabolism in Trp^3-ghrelin-Tg mice did not differ from that seen in non-Tg mice in early life (Supplemental Fig. 1, F and G).

We conducted a precise evaluation of glucose metabolism using more aged mice. Thus we continued rearing

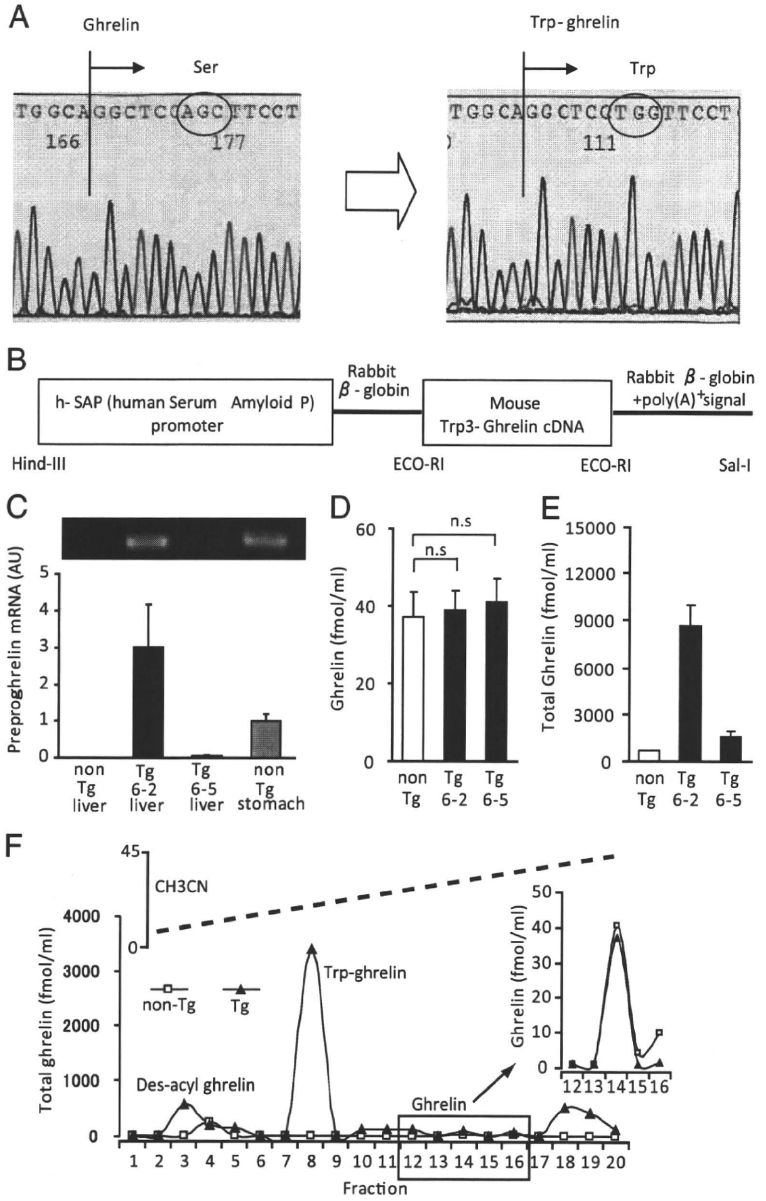

FIG. 2. Generation of Trp^3-ghrelin overexpressing Tg mice. A, A mutant construct in which the AGC codon encoding Ser, the third amino acid of ghrelin that is modified by n-octanoic acid, was replaced by a TGG codon encoding Trp. B, The construct encoding Trp^3-ghrelin used to generate Tg mice was a fusion gene of the hSAP promoter combined with the mutated cDNA of mouse ghrelin. C, The expression levels of preproghrelin mRNA or mutated preproghrelin mRNA. D, Plasma concentrations of ghrelin (acylated form) were measured by N-RIA (n = 8–10). E, Plasma concentrations of total ghrelin, which included ghrelin, des-acyl ghrelin, and Trp^3-ghrelin, were measured by C-RIA (n = 8–10). F, Representative results of HPLC analysis (non-Tg, *open square*; Tg6-2, *closed triangle*). n.s, Not significant. Data are presented as the means ± SEM (C–E).

these mice to 1 yr of age. Some intriguing results on glucose metabolism were obtained from 1-yr-old Trp^3-ghrelin-Tg mice. Although there were no differences between Trp^3-ghrelin-Tg and non-Tg mice in anthropometric parameters, including body weight, total body fat percentage, and lean body mass, Trp^3-ghrelin-Tg mice exhibited impaired glucose tolerance and reduced insulin sensitivity (Fig. 3, A–F); blood glucose levels after glucose injection were significantly higher than those in non-Tg mice. The acute phase of insulin secretion typically seen in response to glucose tended to be suppressed in Trp^3-ghrelin-Tg mice (P = 0.11) (Fig. 3, C and D). In addition, the hypoglycemic response after the injection with insulin was blunted in Trp^3-ghrelin-Tg mice (Fig. 3E). There were no differences, however, in pancreatic insulin mRNA levels between 1-yr-old Trp^3-ghrelin-Tg and non-Tg mice (Fig. 3F). Because glucose tolerance and insulin sensitivity are influenced by GH, we examined whether GH secretion was augmented in 1-yr-old Trp^3-ghrelin-Tg mice. Serum GH and IGF-I levels were unchanged in Trp^3-ghrelin-Tg mice in comparison with those seen in non-Tg mice at 1 yr of age (Fig. 4, A and B). There was no difference between 1-yr-old Trp^3-ghrelin-Tg and non-Tg mice in ghrelin or GOAT mRNA within the stomach or in plasma acylated ghrelin concentrations, which reflects the intrinsic secretion of ghrelin (Fig. 4, C and D). Because ghrelin can also affect the lipid metabolism, we measured serum nonesterified fatty acid, total cholesterol, and triglyceride levels. However, there was no significant difference in them (non-Tg *vs.* Tg: nonesterified fatty acid, 0.74 ± 0.03 *vs.* 0.82 ± 0.04 mEq/liter, P = 0.12; total cholesterol, 122.5 ± 9.8 *vs.* 143.4 ± 7.4 mg/dl, P = 0.10; triglyceride, 162.6 ± 12.8 *vs.* 159.5 ± 10.8 mg/dl, P = 0.85).

Discussion

It is challenging to generate ghrelin gain-of-activity models, because ghrelin requires posttranscriptional modification, an octanoylation of Ser^3. GOAT is responsible for this octanoylation of ghrelin, which confers its biological activity (10, 13). In this study, we succeeded in generating Tg mice overexpressing Trp^3-ghrelin, a ghrelin analog that does not require posttranscriptional modification with GOAT for activity. Because expression of the mutated-ghrelin transgene was driven by the hSAP promoter, Trp^3-ghrelin was continuously secreted from the liver after birth. Plasma concentrations of Trp^3-ghrelin of Tg mice were calculated to have an equivalent activity

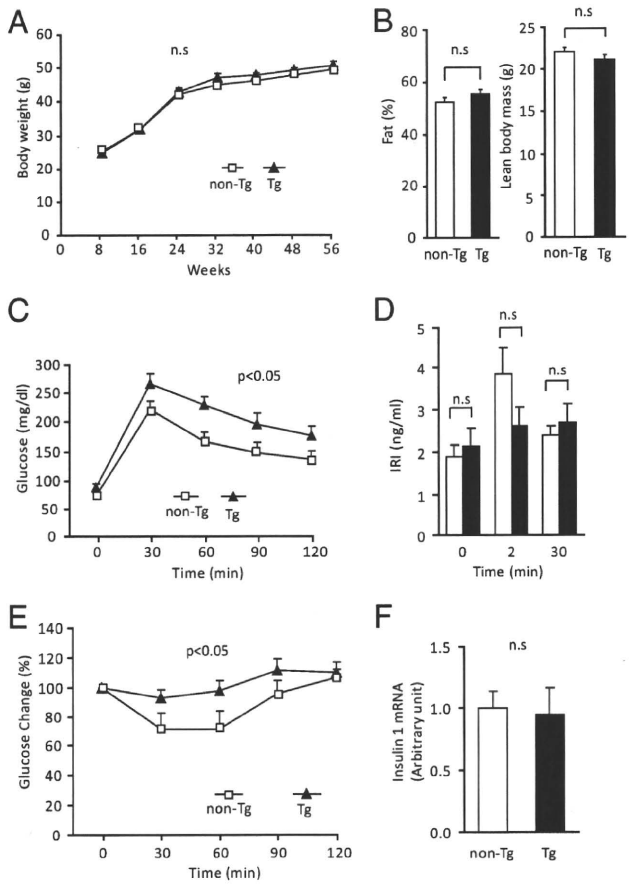

FIG. 3. Analysis of Trp^3-ghrelin Tg mice. A, Changes of body weight in Trp^3-ghrelin-Tg mice (*closed triangles*) and non-Tg littermates (*open squares*) (n = 20–25). B, Body fat percentage and lean body mass, as determined by computer tomography, in 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 14–16). C, Glucose tolerance test (0.75 g/kg) was performed in 52-wk-old Trp^3-ghrelin-Tg mice (*closed triangles*) and non-Tg littermates (*open squares*) (n = 14–16; *, $P < 0.05$ in comparison with non-Tg littermates). D, Serum insulin levels at baseline, 2 min, and 30 min after ip glucose injection of 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 14–16, $P = 0.11$ in comparison with non-Tg littermates). E, Insulin tolerance test after treatment with 1.5 U/kg regular insulin in 52-wk-old Trp^3-ghrelin-Tg mice (*closed triangles*) and non-Tg littermates (*open squares*) (n = 14–16; *, $P < 0.05$ in comparison with non-Tg littermates). F, Insulin 1 mRNA levels in the pancreases of 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 14). IRI, Immunoreactive insulin; n.s, not significant. Data are presented as the means ± SEM.

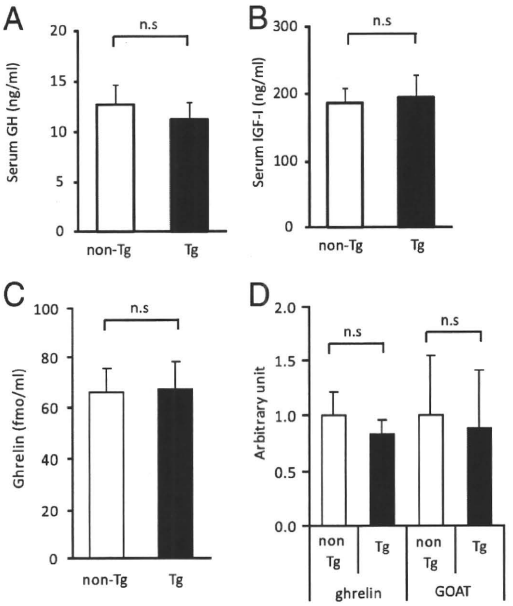

FIG. 4. We examined the levels of GH/IGF-I axis factors, plasma ghrelin levels, and ghrelin and GOAT mRNA levels in the stomachs of 52-wk-old Trp^3-ghrelin-Tg mice. A and B, We measured serum GH (A) and IGF-I (B) levels in 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 10). C, Plasma ghrelin levels in 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 10). D, The mRNA levels of ghrelin and GOAT in the stomachs of 52-wk-old Trp^3-ghrelin-Tg mice (*closed bars*) and non-Tg littermates (*open bars*) (n = 14). n.s, Not significant. Data are presented as the means ± SEM.

as 4.2- to 8.5-fold higher levels of acylated ghrelin in non-Tg mice. We think that this unique mouse model is a useful tool to evaluate the long-term pathophysiological and/or pharmacological effects of ghrelin or ghrelin analogs and provides insight into the physiological roles of ghrelin/GHS-R systems.

Bewick *et al.* (8) developed ghrelin-overexpressing mice using the endogenous ghrelin promoter. Although this mouse model was suitable to investigate the physiological role of ghrelin, it is not suitable to explore the pathophysiological or pharmacological effects of ghrelin, because the plasma ghrelin levels achieved in these mice were only 1.5-fold greater than that seen in non-Tg mice at the highest. Reed *et al.* (5) also developed ghrelin-overexpressing mice using the neuron-specific enolase promoter, reaching circulating ghrelin levels approximately 5-fold higher than those seen in non-Tg mice. Because these mice primarily produced ghrelin in the brain, it remains unclear whether the phenotype of these mice resulted from elevations in peripheral ghrelin and/or central ghrelin. Kirchner *et al.* (13) generated Tg mice simultaneously expressing human ghrelin and GOAT in the liver under the control of the human apolipoprotein E promoter. When fed a standard diet, these mice lack the circulating fatty-acid-modified forms of ghrelin, demonstrating high circulating concentrations of des-acyl ghrelin only. These mice exhibited elevated concentrations of fatty-acid-modified forms of ghrelin only when given a diet rich in medium-chain triglycerides. It may be difficult to characterize the phenotype of the mice precisely, especially the metabolic phenotype, under such a diet.

Trp^3-ghrelin-Tg mice exhibited normal growth patterns and feeding behaviors. These results are consistent with previous results; ghrelin loss-of-function mice, ghrelin-deficient mice, or ghrelin-receptor-null mice all have normal growth rates, food intake, and body compositions (14–17). One-year-old Trp^3-ghrelin-Tg mice demon-

strated impaired glucose tolerance and reduced insulin sensitivity, although there were no differences in body weight or composition between Trp^3-ghrelin-Tg and non-Tg mice. When ghrelin-receptor-null mice were maintained on long-term standard chow, they had lower blood glucose levels with low-to-normal insulin levels in comparison with wild-type mice, although they exhibited similar body weights and composition (14). Ghrelin-receptor-null mice appeared to have enhanced insulin sensitivity in comparison with wild-type mice. In addition, Gauna *et al.* (18) demonstrated that administration of ghrelin to wild-type mice reduced insulin sensitivity. It was also reported that ghrelin inhibited glucose-stimulated insulin release (19–21).

In conclusion, we succeeded in generating Tg mice overexpressing a ghrelin analog. The mice presented in this study will serve as a useful tool for evaluating the long-term effects of ghrelin or ghrelin analogs. In addition, the method provided in this study may be useful in the generation of gain-of-function models for hormones that require posttranscriptional modification.

Acknowledgment

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Disclosure Summary: The authors have nothing to disclose.

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A Postweaning Reduction in Circulating Ghrelin Temporarily Alters Growth Hormone (GH) Responsiveness to GH-Releasing Hormone in Male Mice But Does Not Affect Somatic Growth

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Ghrelin was initially identified as an endogenous ligand for the GH secretagogue receptor. When administrated exogenously, ghrelin stimulates GH release and food intake. Previous reports in ghrelin-null mice, which do not exhibit impaired growth nor appetite, question the physiologic role of ghrelin in the regulation of the GH/IGF-I axis. In this study, we generated a transgenic mouse that expresses human diphtheria toxin (DT) receptor (DTR) cDNA in ghrelin-secretion cells [ghrelin-promoter DTR-transgenic (GPDTR-Tg) mice]. Administration of DT to this mouse ablates ghrelin-secretion cells in a controlled manner. After injection of DT into GPDTR-Tg mice, ghrelin-secreting cells were ablated, and plasma levels of ghrelin were markedly decreased [nontransgenic littermates, 70.6 ± 10.2 fmol/ml *vs.* GPDTR-Tg, 5.3 ± 2.3 fmol/ml]. To elucidate the physiological roles of circulating ghrelin on GH secretion and somatic growth, 3-wk-old GPDTR-Tg mice were treated with DT twice a week for 5 wk. The GH responses to GHRH in male GPDTR-Tg mice were significantly lower than those in wild-type mice at 5 wk of age. However, those were normalized at 8 wk of age. In contrast, in female mice, there was no difference in GH response to GHRH between GPDTR-Tg mice and controls at 5 or 8 wk of age. The gender-dependent differences in response to GHRH were observed in ghrelin-ablated mice. However, GPDTR-Tg mice did not display any decreases in IGF-I levels or any growth retardation. Our results strongly suggest that circulating ghrelin does not play a crucial role in somatic growth. **(*Endocrinology* 151: 1743–1750, 2010)**

GH secretion is predominantly regulated by two hypothalamic peptides, one factor is GHRH and a second is somatostatin (SST). In 1999, Kojima *et al.* (1) discovered ghrelin as an endogenous ligand for the GH secretagogue receptor (GHS-R or ghrelin receptor) from rats' stomach. Ghrelin, an acylated peptide of 28 amino acids, is synthesized primarily in endocrine cells of the stomach, named X/A-like or ghrelin cells (2). Peripheral administration of ghrelin strongly stimulates GH secretion (1, 3). Because coadministration of GHRH and ghrelin produces synergistic effects on pituitary GH release (4), circulating ghrelin may play a role in augmentation of GHRH-stimulated GH pulses. Therefore, circulating ghrelin was thought to be the third peptide which

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Abbreviations: BMD, Bone mineral density; CT, computed tomography; DT, diphtheria toxin; DTR, DT receptor; GHS-R, GH secretagogue receptor; GPDTR-Tg, ghrelin-promoter DTR-transgenic; HB-EGF, heparin-binding epidermal growth factor-like growth factor; SST, somatostatin; WT, nontransgenic littermates.

regulates GH secretion. Indeed, patients with a functional mutation in GHS-R, ghrelin receptor, display familial short stature (5). Okimura *et al.* (6), however, demonstrated that circulating ghrelin levels do not correlated with those of GH; also, administration of a GHS antagosist to freely moving rats did not reduce plasma GH levels. Ghrelin knockout mice also exhibit normal growth patterns (7). On the other hand, ghrelin receptor knockout mice exhibit modest, but significant, body weight reductions and decreased serum IGF-I levels (8). Together, these findings question the physiologic significance of ghrelin in the regulation of GH secretion. As always with such model mice, there may be confounding factors, such as developmental adaptation and other compensatory mechanisms. To avoid these factors, it may be necessary to ablate ghrelin after birth or before puberty. Moreover, during the prepubertal and pubertal period, GH-dependent proportional body growth is observed in many mammalian species. The fetal growth is GH-independent, and growth during the early postnatal is only partial dependent upon GH. Therefore, to evaluate whether an absence of circulating ghrelin can influence a somatic growth through GH/IGF-I axis modification, we think that it is appropriate to choose a postweaning model.

In this study, we adopted a diphtheria toxin (DT) receptor (DTR)-mediated conditional and targeted cell ablation strategy to ablate ghrelin secretion cells, X/A-like cell, in a specific and controlled manner (9). We generated a transgenic mouse expressing human DTR cDNA, which encodes human heparin-binding epidermal growth factor-like growth factor (HB-EGF), under the control of the transcriptional regulatory regions of ghrelin. In this mouse, ghrelin-secreting cells express the human DTR and can be ablated after the administration of a small amount of DT. By using this transgenic mouse, we ablated ghrelin-secretion cells after weaning, which allowed us to evaluate the physiologic significance of ghrelin in GH secretion and somatic growth.

Materials and Methods

All animal experiments were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research. Procedures were performed in accordance with the principles and guidelines established by that committee.

Plasmid construction and generation of transgenic mice [ghrelin-promoter DTR-transgenic (GPDTR-Tg) mice]

The pGPDTR plasmid was constructed by replacement of the mouse albumin enhancer/promoter region of pMS7 (9) with a 4.1-kb *MulI-Hin*dIII fragment containing the 5′-flanking region of the human ghrelin gene (−4110/−33) derived from the p-4110/-33GHRE plasmid (human ghrelin promoter in pGL3) (Fig. 1A) (10). The 6.4-kb *Not*I-*Xho*I fragment of pGPDTR was microinjected into the pronucleus of fertilized eggs obtained from C57/B6 mice (SLC, Shizuoka, Japan). The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (Japan CLEA, Osaka, Japan) by using standard techniques (11). Founder transgenic mice, identified by PCR analysis, were bred with C57BL/6 mice. Mice were housed in air-conditioned animal quarters, with light between 0800 and 2000 h. Except where noted, animals were fed standard rat chow (CE-2, 352 kcal/100 g; Japan CLEA) and water *ad libitum*.

Semiquantitative PCR

Total RNA was extracted using a Sepasol-RNA kit (Nacalai Tesque, Kyoto, Japan). RT used a high capacity cDNA RT kit (Applied Biosystems, Foster City, CA).

Semiquantitative PCR determined the distribution of the DTR in GPDTR-Tg mice, using the following primers: sense 5′-CCTCCTCTCGGTGCGGG-3′ and antisense 5′-AGTCAC-CAGTGCCGAGAGAACTG-3′. Thirty-five cycles of thermal was performed with 94 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec. Human heart mRNA (purchased from Clontech, Palo Alto, CA) was used as a positive control.

DT injection

DT was purchased from Sigma-Aldrich Japan (Tokyo, Japan). According to the previous report using DTR-mediated cell ablation systems (9), DT was injected im.

Histological procedures

Formalin-fixed, paraffin-embedded tissue sections were immunostained using avidin-biotin peroxidase complex methods (Vectastain "ABC" Elite kit; Vector Laboratories, Burlingame, CA) as described (11). Sections were incubated overnight at 4 C with antighrelin-(1–11) antiserum that specifically recognizes acylated ghrelin (final dilution, 1:5000). Tissue sections were also stained with hematoxylin and eosine.

Measurement of plasma ghrelin levels

Measurement of plasma ghrelin levels was performed as reported previously (12). Blood samples drawn from the retro-orbital vein at 1000 h were immediately transferred to chilled siliconized glass tubes containing Na2EDTA (1 mg/ml) and

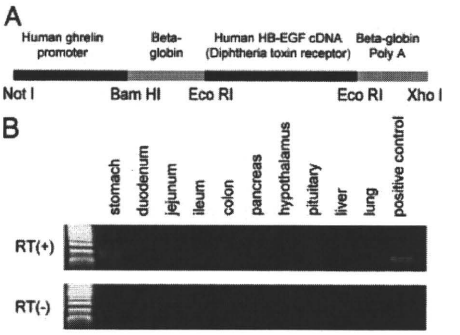

FIG. 1. Generation of GPDTR-Tg mice. A, The GPDTR-Tg construct contained a fusion gene comprised of the 5′flanking lesion of human ghrelin (4085 bp) and the DTR cDNA (human HB-EGF). B, Expression of DTR mRNA in various tissues of GPDTR-Tg mice at 8 wk of age. The human heart mRNA was used as a positive control.

TABLE 1. PCR primers and TaqMan probes

Ghrelin	Sense	5′-GCATGCTCTGGATGGACATG-3′
	Antisense	5′-TGGTGGCTTCTTGGATTCCT-3′
	Probe	5′-AGCCCAGAGCACCAGAAAGCCCA-3′
GH	Sense	5′-AAGAGTTCGAGCGTGCCTACA-3′
	Antisense	5′-GAAGCAATTCCATGTCGGTTC-3′
	Probe	5′-CCATTCAGAATGCCCAGGCTGCTTTC-3′
GHRH	Sense	5′-AGGATGCAGCGACACGTAGA-3′
	Antisense	5′-TCTCCCCTTGCTTGTTCATGA-3′
	Probe	5′-CCACCAACTACAGGAAACTCCTGAGCCA-3′
SST	Sense	5′-AGCTGAGCAGGACGAGATGAG-3′
	Antisense	5′-ACAGGATGTGAATGTCTTCCAGTT-3′
	Probe	5′-CGAACCCAGCAATGGCACCCC-3′
IGF-1	Sense	5′-ACCCGGACCTACCAAAATGAC-3′
	Antisense	5′-GGTGTGAAGACGACATGATGTGT-3′
	Probe	5′-CACCTGCAATAAAG-3′
GHS-R	Sense	5′-CACCAACCTCTATCCAGCAT-3′
	Antisense	5′-CTGACAAACTGGAAGCGTTTGCA-3′
	Probe	5′-TCCGATCTGCTCATCTTCCTGTGCATG-3′

aprotinin (1000 KIU/ml; Ohkura Pharmaceutical, Kyoto, Japan). After centrifugation at 4 C to separate out the plasma, hydrochloric acid was added to samples at a final concentration of 0.1 N. Plasma was immediately frozen and stored at −80 C until assayed. Plasma ghrelin concentrations were determined using a ghrelin ELISA kit (Mitsubishi Kagaku Iatron, Tokyo, Japan).

Real-time PCR analysis

Extraction of total RNA from various tissues and RT was performed as described above. Real-time quantitative PCR used an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using the primers and TaqMan probes described in Table 1. The mRNA expression levels of each gene were normalized to that of 18S rRNA. All samples were examined in triplicate in 96-well plates using an ABI Prism 7500 sequence detector according to the manufacturer's protocol.

GH provocative test

GH provocative test was carried out as previously described (12). These experiments were conducted in unanesthelized mice. Human GHRH was purchased from Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan). Serum samples were collected at 15 and 30 min after sc injection of 180 mcg/kg of GHRH.

Ghrelin-rescue experiments

Osmotic infusion pumps (Alzet Micro-Osmotic pump, Model 1002; Durect Corp., Cupertino, CA) were implanted sc in 3-wk-old male GPDTR-Tg mice. Ghrelin (60 mcg/kg·d; Peptide Institute, Osaka, Japan) or saline was continuously infused through the osmotic infusion pumps. Then mice were started to treat with DT (50 ng/kg twice a week) a day after pump implantation. The average plasma ghrelin levels during continuous infusion of ghrelin were 31.6 ± 5.3 fmol/ml in the DT-treated GPDTR-Tg mice, whereas those without ghrelin infusion were 1.7 ± 0.2 fmol/ml. GH provocative test were carried out in these mice at the age of 5 wk.

Measurement of serum GH and IGF-I levels

Blood samples were collected from the tail veins of mice. Serum was isolated by centrifugation and stored at −20 C until assayed. Serum GH levels and IGF-I levels were measured using the appropriate EIA kits from SPI-BIO (Bonde, France) and Diagnostic Systems Laboratories, Inc. (Webster, TX), respectively, according to the manufacturers' instructions.

Measurement of body lengths

Mouse body length was measured by manual immobilization and extension of mice to determine nose-to-anus length. All measurements were performed by the same individual in a blind fashion.

Measurement of fat mass and bone mineral density (BMD)

The fat mass (% fat) and BMD of mice were measured by computed tomography (CT) (Laboratory CT; Lacita, Aloka, Japan) under pentobarbital anesthesia.

Statistical analysis

Results are expressed as the means ± SEM. Multiple comparisons between groups were made by Turkey-Krammer test, with α set at $P < 0.05$. The results on body weight and serum GH levels after GHRH injection were analyzed by a two-way ANOVA followed by Tukey's *post hoc* test, with α set at $P < 0.05$. Statistical analyses were carried out with STATVIE 4.0 software (Abacus Concepts, Inc., Berkley, CA).

Results

Generation of transgenic mice in which ghrelin can be ablated in a controlled manner

Transgenic mice

To elucidate physiologic role of ghrelin in GH secretion and somatic growth, we developed transgenic mice in which ghrelin can be ablated in controlled manner. We adopted a DTR-mediated conditional and targeted cell ablation strategy. We created transgenic mice that expressed the gene for the human DTR, human HB-EGF,

under the control of the ghrelin promoter. By injecting transgenes into 184 eggs, we obtained three lines of transgenic mouse (Tg 1-2, Tg 5-1, and Tg 5-8). We continued with the Tg 5-1 transgenic line, because Tg 1-2 animals did not exhibit decreases in plasma ghrelin levels after injection of high-dose DT and Tg 5-8 required high doses of DT (50 mcg/kg) to ablate ghrelin-producing cells (data not shown). In Tg 5-1 transgenic animals, semiquantitative PCR analysis revealed high expression of DTR mRNA in the stomach and weak expression in the duodenum and jejunum. No expression, however, could be detected in the ileum, colon, pancreas, hypothalamus, pituitary, liver, or lung (Fig. 1B). In Tg 5-1 mice, the ghrelin-producing cells of the stomach were ablated by injection with low-dose DT (10 or 50 ng/kg) (Fig. 2, A, B, and D). We therefore designated the Tg 5-1 transgenic line and nontransgenic littermates as GPDTR-Tg mice and wild-type (WT) mice, respectively.

Ablation of ghrelin-producing cell

To determine the dose and timeframe of DT injection, preliminarily studies were performed: GPDTR-Tg mice were injected with saline or DT twice a week at a dose of 10, 30, 50, 100, and 500 ng/kg (on d 0 and 2). Plasma ghrelin levels on d 4 were decreased to approximately 60, 30, 5, 5, and 5% of control mice (Tg mice treated with saline) after 10, 30, 50, 100, and 500 ng/kg of DT injection, respectively. Thus, we judged that 50 ng/kg of DT is the smallest effective dose to reduce plasma ghrelin. The final results using 10 and 50 ng/kg of DT were described below. Next, GPDTR-Tg mice were injected with 50 ng/kg of DT with four schedules: once a week (on d 0), twice a week (on d 0 and 2), three times a week (on d 0, 2, and 4), or daily (from d 0 to 6), and plasma ghrelin levels were measured on d 7. The once-a-week injection of DT was insufficient, but the twice-a-week injection of DT had enough effect on reduction in plasma ghrelin concentration.

To ablate ghrelin-producing cells, 8-wk-old male WT and GPDTR-Tg mice were injected im with 10 or 50 ng/kg DT daily on d 0 and 2 and analyzed on d 4. WT mice treated with saline or DT and GPDTR-Tg mice treated with saline were used as control mice.

To evaluate the effects of DT injection on ghrelin-producing cell, we analyzed stomach by immunohistochemical analysis with antighrelin antisera (Fig. 2A) and real-time PCR (Fig. 2, B and C). DT injection reduced in a dose-dependent manner both the number of ghrelin-positive cells and the expression of ghrelin mRNA in the stomach of GPDTR-Tg mice (Fig. 2, A and B). DT injection did not produce in any abnormalities in WT mice, because

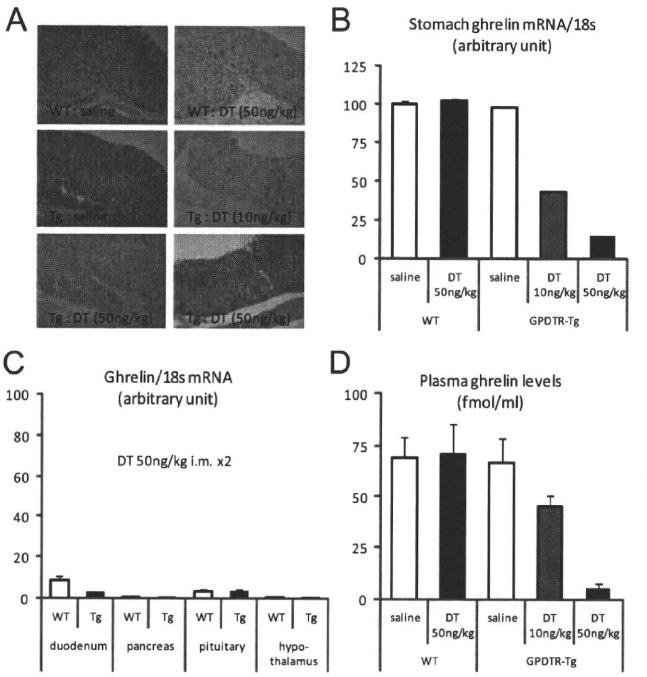

FIG. 2. Ablation of ghrelin-secretion cells. Eight-week-old male GPDTR-Tg mice (Tg) and nontransgenic littermates (WT) were injected with saline or 10 or 50 ng/kg of DT (im) on d 0 and 2, then analyzed on d 4. A, Histological analysis of stomach sections. Immunohistochemical analysis of ghrelin peptide expression and hematoxylin and eosin staining. Original magnification, ×100. B, Ghrelin mRNA levels in the stomach. C, Ghrelin mRNA levels in the duodenum, pancreas, pituitary, and hypothalamus of GPDTR-Tg and WT mice injected with 50 ng/kg of DT. D, Plasma ghrelin levels in GPDTR-Tg and WT mice. For B–D, data represent the means ± SEM (n = 8).

these mice do not possess the DTR, making them insensitive to DT. In transgenic animals, DT injection also reduced ghrelin mRNA expression in the duodenum, but not the pancreas, pituitary, or hypothalamus (Fig. 2C). Plasma ghrelin levels in GPDTR-Tg mice treated with 10 and 50 ng/kg of DT were decreased to approximately 60 and 5–7% of control mice, respectively (Fig. 2D). These results suggested that this transgenic mouse model is a useful tool for evaluating the physiologic role of circulating ghrelin.

Histological analysis with hematoxylin and eosin staining revealed that no inflammatory cell infiltration was seen in the stomach (Fig. 2A), small intestine, colon, pancreas, pituitary, and hypothalamus of the GPDTR-Tg mice with 50 ng/kg of DT injection. Other historical abnormalities were also not observed in these tissues (data not shown).

The effects of a reduction in circulating ghrelin after weaning on the GH/IGF-I axis and somatic growth

To study the effects of postweaning reductions in circulating ghrelin on the GH/IGF-I axis and somatic growth, 3-wk-old WT and GPDTR-Tg mice were treated with DT

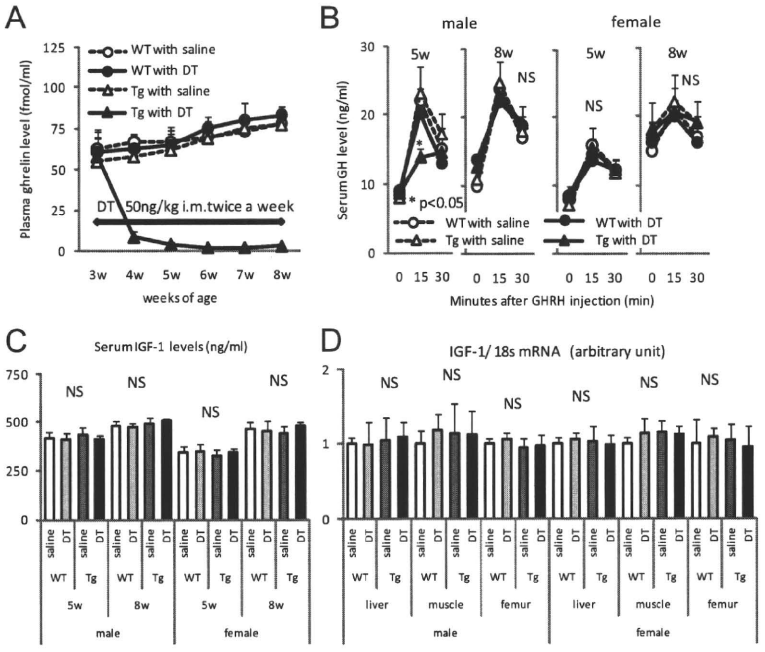

FIG. 3. The effects of a postweaning reduction in circulating ghrelin on the GH/IGF-I axis. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). A, Plasma ghrelin levels before and after DT injection. B, GH response to GHRH administration (180 μg/kg sc) in GPDTR-Tg and WT mice at 5 and 8 wk of age. C, Serum IGF-I levels of GPDTR-Tg and WT mice at 5 and 8 wk of age. D, IGF-I mRNA levels in liver, skeletal muscle, and femur in GPDTR-Tg and WT mice at 5 wk of age. Data represent the means ± SEM (n = 12).

(50 ng/kg) or saline twice a week for 5 wk (from 3 to 8 wk old). After DT injection, plasma ghrelin levels of GPDTR-Tg mice decreased rapidly. In GPDTR-Tg mice, ghrelin levels were undetectable by 5 wk of age, remaining so thereafter (Fig. 3A). The data obtained from GPDTR-Tg mice were compared with those from three groups of control mice (WT with saline, WT with DT, and GPDTR-Tg with saline).

To elucidate whether a postweaning reduction in circulating ghrelin can influence GH secretion, we measured basal serum GH levels and performed GH provocative test with GHRH. There were no differences in basal serum GH levels between GPDTR-Tg mice treated with DT and control mice in either males or females at 5 or 8 wk of age. GH provocative test with GHRH showed some intriguing results (Fig. 3B). The GH responses to GHRH in male GPDTR-Tg mice treated DT were significantly lower than those in three controls at 5 wk of age. However, those responses were normalized at 8 wk of age. On the other hand, there were no differences in GH response to GHRH among four groups (WT with saline or DT, and Tg with saline or DT) in females at 5 or 8 wk of age.

To elucidate whether temporarily attenuation of GH responses to GHRH can affect IGF-I regulation, we investigated serum IGF-I levels and IGF-I mRNA expressions in the liver, skeletal muscle, and distal femur. There were no differences in serum IGF-I levels among any animal groups in either males or females at 5 or 8 wk of age (Fig. 3C). There were also no differences in IGF-I mRNA expressions in the liver, skeletal muscle, or distal femur among any animal groups at 5 wk of age (Fig. 3D). We then investigated the effects of decreases in circulating ghrelin on the expression of mRNA encoding GHRH and SST within the hypothalamus and encoding GH and GHS-R in the pituitary. There were no differences in mRNA expression levels of these mediators among any animal groups in male and female at 5 wk of age (Fig. 4).

As expected from the results of the IGF-I studies, no evidence of growth retardation could be found in either male or female GPDTR-Tg mice treated with DT during the observation period. There were no difference in body weight or length in comparison with three groups of control mice at any point (Fig. 5, A and B, for male; and Fig. 5, D and E, for female animals). CT analysis of body composition demonstrated that there were no differences in percent fat or BMD among any animal groups at 5 and 8 wk of age (Fig. 5C for male, and Fig. 5F for female animals).

There were no differences in weekly food intake from 3 to 8 wk of age [WT *vs.* GPDTR-Tg (treated with DT); male, 18.4 ± 0.5 *vs.* 18.9 ± 0.7; female, 18.4 ± 1.0 *vs.* 18.5 ± 0.6 (g/wk)]. These results suggested that although GH responses to GHRH were temporarily reduced under conditions of decrease in circulating ghrelin, somatic growth was not impaired.

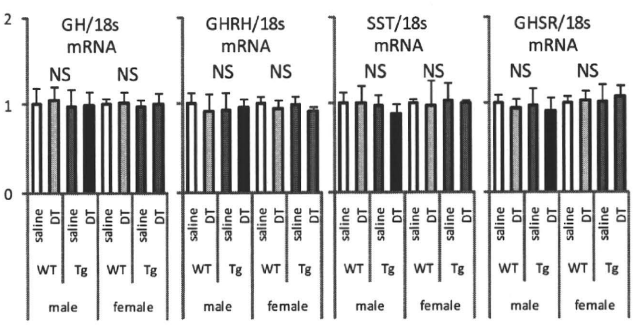

FIG. 4. The effects of a postweaning reduction in circulating ghrelin on the expression of mRNA encoding GHRH, SST,GH and GHS-R. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). Pituitary mRNA levels of GH and GHS-R and hypothalamic mRNA levels of GHRH and SST in GPDTR-Tg and WT mice at 5 wk of age. Data represent the means ± SEM (n = 12).

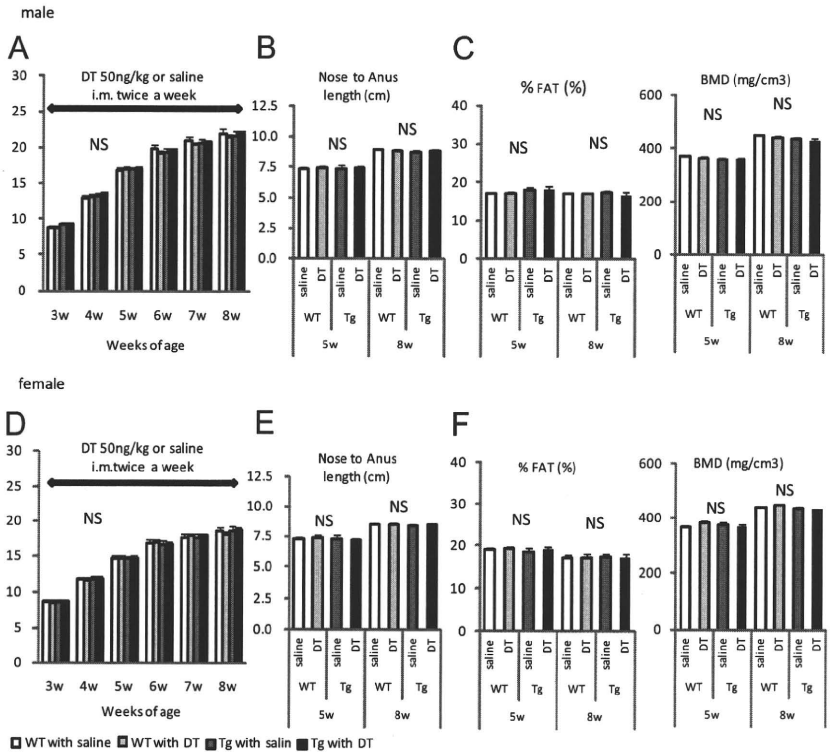

FIG. 5. The effects of a postweaning reduction in circulating ghrelin levels on somatic growth. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). A and D, Changes in body weight in male (A) and female mice (D). B and E, Nose to anus length in male (B) and in female mice (E) at 5 and 8 wk of age. C and F, Body composition (% Fat) and BMD as analyzed by CT in male (C) and in female mice (F) at 5 and 8 wk of age. Data represent the means ± SEM (n = 12).

GH response to GHRH in the ghrelin-rescued GPDTR-Tg mice

To elucidate whether GH responsiveness to GHRH can be ameliorated by ghrelin replacement in the ghrelin-ablated mice, GH provocative test were carried out in the DT-treated GPDTR-Tg mice whose circulating ghrelin were rescued by continuously administration of ghrelin with osmotic pump. The average plasma ghrelin levels during continuous infusion of ghrelin were 31.6 ± 5.3 fmol/ml in the DT-treated GPDTR-Tg mice, whereas those without ghrelin infusion were 1.7 ± 0.2 fmol/ml. GH provocative test were carried out at the age of 5 wk.

GH responsiveness to GHRH was ameliorated by ghrelin replacement. Serum GH levels at 0, 15, and 30 min after GHRH administration in the ghrelin-rescued mice were 7.8 ± 1.6, 26.2 ± 4.2, and 12.3 ± 0.8 ng/ml, respectively, whereas those in mice without ghrelin replacement were 6.8 ± 1.5, 10.9 ± 2.6, and 11.3 ± 1.6 ng/ml, respectively. These results suggested that attenuated response to GHRH seen in ghrelin-ablated mice without ghrelin replacement was due to acute ghrelin deficiency.

Discussion

In this study, we generated transgenic mice expressing the DTR driven by the transcriptional regulatory machinery of ghrelin. Injection of DT into this mouse can ablate ghrelin-secreting cells. Approximately 70–80% of circulating ghrelin originates from the stomach (13). Ghrelin-producing cells are also found throughout the small intestine, with the duodenum producing approximately one-tenth that of the stomach (14). Semiquantitative PCR revealed that DTR was only expressed in stomach and not in pituitary, hypothalamus, and pancreas and the intensity of the band of DTR in stomach was very low. Three possibilities might be considered to explain this result. The first is the low efficiency of gene transfection. Three lines of GPDTR-Tg mice that we generated in this study were inserted with low copy numbers of transgene (DTR cDNA). Thus, the expression levels of DTR mRNA could be very low even in stomach. The second is the efficiency of gene expression. In this study, we designed a fusion gene comprising the 4085-bp fragment contained a partial sequence of the 5′-flanking region of the human ghrelin gene and human DTR. The efficiency of gene expression driven by this fragment might be lower than those driven by the original ghrelin promoter region. The last, except gastrointestinal tract, transcription of ghrelin gene might be driven by a different size of fragment of the 5′-franking region. Immunohistochemical and PCR analyses demonstrated that ghrelin-secreting cells in the stomach and duodenum were ablated after DT injection into GPDTR-Tg mice, resulting in marked reduction of plasma ghrelin levels. In contrast, ghrelin-producing cells of the pituitary and hypothalamus were unaffected. Thus, this transgenic mouse is a useful model to explore the role of circulating ghrelin, because plasma ghrelin levels can be abrogated in a controlled manner without altering pituitary and hypothalamic ghrelin mRNA expression levels.

The physiologic roles of ghrelin in the regulation of GH secretion remain unclear, because previous reports using rodents deficient or reduced in ghrelin signals have given conflicting results (7, 8, 15, 16). Sun *et al.* (7) reported that ghrelin-deficient mice did not exhibit any growth retardation or decreases in serum IGF-I levels. Wortley *et al.*

(15) also were unable to observe any significant differences between ghrelin-deficient mice and WT mice in body weight or basal serum GH levels, when fed a standard diet. Moreover, Zigman *et al.* (16) demonstrated there was no significant difference in serum IGF-I levels between ghrelin receptor knockout and WT mice. Sun *et al.* (8), however, showed that ghrelin receptor knockout mice exhibited only a small reduction in body weight and serum IGF-I levels. In addition, Pantel *et al.* (5) showed that two unrelated families with short statue have a missense mutation of GHS-R. This mutation impairs the constitutive activity of the GHS-R. They also reported a young patient with growth delay who has a recessive partial isolated GH deficiency due to GHS-R mutations (17). These results indicate importance of ghrelin/GHS-R signals in GH secretion and somatic growth.

The purpose of this study is to evaluate whether an absence of circulating ghrelin can influence GH secretion and somatic growth via GH/IGF-I axis in mammals. First, we investigated basal serum GH levels and the GH response to GHRH. Although basal serum GH levels in the ghrelin-abrogated mice did not differ from those seen in WT mice, the GH responses to GHRH in male GPDTR-Tg mice were significantly lower than those in WT mice at 5 wk of age. As coadministration of GHRH and ghrelin produces synergistic effects on pituitary GH release (4), circulating ghrelin may play a role in augmentation of GHRH-stimulated GH pulses. Indeed, GH responsiveness to GHRH was ameliorated by ghrelin replacement in the ghrelin-ablated mice. However, the attenuated response to GHRH in the ghrelin-ablated mice had persisted only for a short term. The GH responses to GHRH in male GPDTR-Tg mice were recovered and were not different from those in WT mice at 8 wk of age. It is possible that an adaptation to reduced circulating ghrelin occurred within a short term. Indeed, Popovic *et al.* (18) reported that 10 patients who underwent total-gastrectomy at least 2 yr ago, a state of acquired chronic hypoghrelinemia, exhibited normal GH response to GHRH compared with normal subjects. Meanwhile, in female mice, there were no differences in either basal serum GH levels or GH response to GHRH between WT and GPDTR-Tg mice at 5 or 8 wk of age. The secretory pattern of GH in rodents is sexually differentiated. In male rats, GH is secreted in episodic pattern with low levels between pulses, whereas in females, the pulses are lower and plasma GH levels between pluses are higher than males (19). The secretory pattern of GH differs between male and female by 30 d of age (20). Gonadal steroids are thought to produce the sexual differences in GH secretion. We assumed that the sexual differences in GH response to GHRH in ghrelin-ablated mice may depend on gonadal steroids.

As GH secretion is pulsatile in nature, a single measurement of GH concentration in blood would not adequately reflect endogenous GH secretion. To estimate the amplitude and frequency of GH pulses, short-interval blood sampling under a conscious state is required. Such studies are difficult to perform in mice. Instead, we investigated serum IGF-I levels, skeletal muscle IGF-I mRNA expression, and anthropometric parameters that reflect pulsatile GH release under similar nutritional conditions (21). Serum IGF-I levels and IGF-I mRNA expression in skeletal muscle did not decrease in the ghrelin-abrogated mice in comparison with WT mice. These results suggest that circulating ghrelin does not play a dominant role in the GH/IGF-I axis. Due to significant differences between species in the regulation of GH secretion (21), we have to give careful considerations to apply the results of animal experiments concerning GH secretion directly to humans; insulin-induced hypoglycemia is a potent stimulus of GH secretion in humans, whereas rats respond to the stress of hypoglycemia by decreasing GH secretion (22, 23). L-arginine is a potent GH secretagogue in humans, but does not (or less overtly) stimulate GH secretion in rats (21, 24).

Somatic growth is affected not only by GH and IGF-I but also by thyroid hormones, sex steroids, and glucocorticoids. It also depends on genetic background and nutrition. Adequate nutrition is one of the most important factors affecting somatic growth. In present study, there were no differences in food intake between the ghrelin-abrogated mice and WT mice. Body weight, length, and body composition also were not influenced by plasma ghrelin levels. These results suggest that circulating ghrelin does not play a dominant role in somatic growth.

We cannot exclude the possibility that hypothalamic ghrelin may regulate GH secretion, as hypothalamic ghrelin-secreting cells were preserved in this animal model. Shuto *et al.* (25) demonstrated that transgenic rats expressing antisense GHS-R mRNA within the arcuate nucleus of the hypothalamus displayed growth retardation, suggesting that ghrelin/GHS-R systems in the hypothalamus function in the regulation of GH. Further studies will be needed to elucidate the role of hypothalamic ghrelin in GH secretion.

In summary, we have succeeded in generating transgenic mice in which circulating ghrelin can be abrogated in a controlled manner after birth. Our results suggest that circulating ghrelin does not play a crucial role in somatic growth.

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Is Pulse Pressure a Predictor of New-Onset Diabetes in High-Risk Hypertensive Patients?

A subanalysis of the Candesartan Antihypertensive Survival Evaluation in Japan (CASE-J) trial

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OBJECTIVE — Hypertensive patients have an increased risk of developing diabetes. Accumulating evidence suggests a close relation between metabolic disturbance and increased arterial stiffness. Here, we examined the association between pulse pressure and the risk of new-onset diabetes in high-risk Japanese hypertensive patients.

RESEARCH DESIGN AND METHODS — The Candesartan Antihypertensive Survival Evaluation in Japan (CASE-J) trial examined the effects of candesartan and amlodipine on the incidence of cardiovascular events in 4,728 high-risk Japanese hypertensive patients. In the present study, we analyzed the relationship between pulse pressure at baseline and new-onset diabetes in 2,685 patients without diabetes at baseline (male 1,471; mean age 63.7 years; mean BMI 24.8 kg/m^2) as a subanalysis of the CASE-J trial.

RESULTS — During 3.3 ± 0.8 years of follow-up, 97 patients (3.6%) developed diabetes. In multiple Cox regression analysis, pulse pressure was an independent predictor for new-onset diabetes (hazard ratio [HR] per 1 SD increase 1.44 [95% CI 1.15–1.79]) as were male sex, BMI, and additional use of diuretics, whereas age and heart rate were not. Plots of HRs for new-onset diabetes considering both systolic and diastolic blood pressure (DBP) revealed that a higher pulse pressure with a lower DBP, indicating that the increased pulse pressure was largely due to increased arterial stiffness, was strongly associated with the risk of new-onset diabetes.

CONCLUSIONS — Pulse pressure is an independent predictor of new-onset diabetes in high-risk Japanese hypertensive patients. Increased arterial stiffness may be involved in the development of diabetes.

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Deaths from cardiovascular disease (CVD), which, as the leading cause of death, accounts for one-third of all deaths globally, are forecast to increase from 17.1 million in 2004 to 23.4 million in 2030 (1). Hypertension is an established risk factor for cardiovascular mortality and morbidity through its effect on several target organs, including the brain, heart, and kidneys (2). Diabetes is also strongly associated with an increased risk of cardiovascular events (3). Because hypertensive patients have an increased risk of developing diabetes (new-onset diabetes), the two conditions frequently cluster together and synergistically increase the propensity to CVD (4). Further, a recent study has shown that new-onset diabetes negatively affects the incidence of cardiovascular morbidity and mortality to the same degree as known diabetes (5). Prevention of new-onset diabetes is therefore an important issue in the management of hypertension, and several studies with the aim of determining predictors of new-onset diabetes have been reported (6–8).

One independent predictor of cardiovascular morbidity and mortality in hypertensive patients is pulse pressure (9). Although pulse pressure derives from the interaction of cardiac ejection (stroke volume) and the properties of arterial circulation (arterial stiffness and wave reflection), elevated pulse pressure is thought to be largely associated with increased arterial stiffness due to aging, arteriosclerosis, or both (9,10), and several recent studies have reported an association among increased arterial stiffness and impaired glucose metabolism, metabolic syndrome, and insulin resistance (11–13). These findings suggest a possible association between increased pulse pressure and new-onset diabetes, but this association has not been examined in hypertensive patients.

The CASE-J trial was designed to compare the long-term effects of the angiotensin II receptor blocker (ARB) candesartan cilexetil and the calcium channel blocker (CCB) amlodipine besylate on the incidence of cardiovascular events in 4,728 high-risk Japanese hypertensive patients (14). Results showed that both treatment-based regimens lowered systolic (SBP) and diastolic blood pressure (DBP) levels to <140/80 mmHg, and no statistically significant difference was seen in the incidence of primary cardiovascular events. However, candesartan-based regimens significantly suppressed the incidence of new-onset diabetes compared with amlodipine-based regimens (15).

Here, we report a subanalysis of the CASE-J trial with the aim of determining whether pulse pressure is associated with the risk of new-onset diabetes independent of the effects of antihypertensive

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treatment and other possible risk factors for diabetes.

RESEARCH DESIGN AND METHODS

— The CASE-J trial was a prospective, multicenter, randomized, open-label, active-controlled, two-arm parallel-group comparison with response-dependent dose titration and blinded assessment of end points conducted in high-risk Japanese hypertensive patients. The trial protocol was approved by the Ethics Committee of Kyoto University Graduate School of Medicine in accordance with the principles of the Declaration of Helsinki. Details of the study and the main results have been reported previously (14,15). In brief, 4,728 high-risk Japanese hypertensive patients aged 20–84 years were randomly assigned to either candesartan- or amlodipine-based regimens. Blood pressure was measured at a clinic with the patient in the sitting position. The average of two consecutive measurements of blood pressure on separate visits was used. High-risk was defined as the presence of any one or more of the following: *1*) severe hypertension (SBP/DBP ≥180/110 mmHg); *2*) type 2 diabetes (fasting blood glucose ≥126 mg/dl, casual blood glucose ≥200 mg/dl, A1C ≥6.5%, 2-h blood glucose on a 75-g oral glucose tolerance test ≥200 mg/dl, or current treatment with a hypoglycemic agent at baseline); *3*) a history of stroke or transient ischemic attack > 6 months before screening; *4*) left ventricular hypertrophy (LVH), angina pectoris, or a history of myocardial infarction >6 months before screening; *5*) proteinuria or renal dysfunction (serum creatinine ≥1.3 mg/dl); or *6*) arteriosclerotic peripheral artery obstruction. Exclusion criteria have been reported elsewhere (14,15).

Enrolled patients were randomly assigned to receive candesartan by oral administration at 4–12 mg/day or amlodipine by oral administration at 2.5–10 mg/day. Patients already under treatment with diuretics, α-blockers, and β-blockers at enrollment were allowed to continue taking these drugs, but the new addition of other ARBs and CCBs or any ACE inhibitors was prohibited.

Outcome measurement

Of the 4,703 high-risk hypertensive patients analyzed in the CASE-J trial, 2,018 who had diabetes at baseline were excluded, leaving 2,685 patients for inclusion in the present study. New-onset diabetes was prespecified as the end point on 17 September 2005, which was after the beginning but before the completion of the CASE-J trial (15). To detect the occurrence of new-onset diabetes, individual case report forms and adverse-event databases were monitored. A case of new-onset diabetes was defined as a patient reported as having developed diabetes on the adverse event form or a patient who had newly started antidiabetic agent therapy in the case report form. Written informed consent was obtained from each participating patient before allocation.

Statistical analysis

Data are expressed as means ± SD or proportions. Continuous variables were compared using Student's *t* test. Frequency analysis was performed with the χ^2 test. Pulse pressure was calculated as the difference between SBP and DBP. Multiple Cox regression analysis was used to examine the association between each blood pressure index (SBP, DBP, and pulse pressure) at baseline and the risk of new-onset diabetes with adjustment for baseline characteristics (prior antihypertensive treatment, allocated drug, age, sex, BMI, heart rate, history of cerebrovascular events, LVH, history of ischemic heart disease, renal dysfunction, peripheral vascular disease, hyperlipidemia, and smoking) as standard covariates and additional drugs (diuretics, α-blockers, and β-blockers) as time-varying covariates. Fractional pulse pressure (PP_f), which is calculated as pulse pressure divided by mean arterial pressure, has recently been proposed as a new parameter of the pulsatile component of blood pressure (16). PP_f is thought to more directly reflect arterial stiffness than pulse pressure, because dividing by mean arterial pressure theoretically cancels out the influence of cardiac output and peripheral vascular resistance. We also evaluated the predictive value of this variable for new-onset diabetes by multiple Cox regression analysis. Because each blood pressure index is affected by aging (10), we also conducted subgroup analyses stratified by age (cutoff point: age 65 years), using the median age at baseline of all included patients. The test for interaction in the multiple Cox model was evaluated with the interaction term. In addition, to clarify the significance of pulse pressure for new-onset diabetes, the associations of both SBP and DBP with the incidence of new-onset diabetes were examined by multiple Cox regression analysis with SBP grouped into two categories (SBP <160 mmHg and 160 mmHg ≤SBP) and DBP plotted as a continuous variable. This model was plotted with the middle 80% of the distribution of DBP for each SBP group, and the HR of a DBP of 90 mmHg in the SBP <160 mmHg category was assigned a reference value of 1.0. All statistical tests were two-sided with an α level of 0.05 and were performed using SAS (version 9.1; SAS Institute, Cary, NC).

RESULTS

Baseline characteristics

During 3.3 ± 0.8 years of follow-up, 97 patients (3.6%) developed new-onset diabetes. Baseline characteristics of patients with and without new-onset diabetes are shown in Table 1. Patients developing diabetes were more likely to be male and obese, less likely to have been randomly assigned to a candesartan-based regimen, and more likely to have had lower DBP, higher pulse pressure, and LVH at baseline. At the time of randomization, 1,702 (65.8%) patients without and 65 (67.0%) patients with new-onset diabetes were under treatment with antihypertensive drugs (CCB 40.1 vs. 34.0%, $P = 0.229$; ACE inhibitor 13.3 vs. 16.5%, $P = 0.363$; ARB 17.9 vs. 22.7%, $P = 0.229$; diuretic 3.1 vs. 5.2%, $P = 0.255$; β-blocker 12.9 vs. 16.5%, $P = 0.297$; and α-blocker 5.6 vs. 4.1%, $P = 0.542$, respectively).

Predictors of new-onset diabetes

Multiple Cox regression analysis revealed that pulse pressure (per 1 SD increase) was an independent predictor of new-onset diabetes (HR 1.44 [95% CI 1.15–1.79], $P = 0.001$) (Table 2). In addition, risk was also significantly associated with male sex, BMI, LVH, and concomitant use of diuretics. As reported previously, candesartan-based regimens significantly reduced the risk of new-onset diabetes compared with amlodipine-based regimens (15).

Because pulse pressure was calculated as the difference between SBP and DBP, we conducted separate analyses for SBP and DBP and found that DBP (per 1 SD decrease) was also an independent predictor for new-onset diabetes, whereas SBP (per 1 SD increase) was not (HR for SBP 1.13 [95% CI 0.90–1.41], $P = 0.284$; and HR for DBP 1.45 [1.16–1.81], $P < 0.001$). Subgroup analysis stratified by age (cutoff point: age 65 years) revealed that pulse pressure remained significantly associated with the risk of new-onset diabetes in both age-groups (aged <65 years: HR 1.72 [95% CI 1.18–2.49],

Table 1—*Baseline characteristics*

	Total	NOD (−)	NOD (+)
n	2,685	2,588	97
Candesartan*	1,343 (50.0)	1,305 (50.4)	38 (39.2)
Prior antihypertensive treatment	1,767 (65.8)	1,702 (65.8)	65 (67.0)
Age (years)	63.7 ± 11.1	63.7 ± 11.2	64.9 ± 10.0
Male sex*	1,471 (54.8)	1,406 (54.3)	65 (67.0)
BMI (kg/m^2)*	24.8 ± 3.6	24.1 ± 3.5	25.2 ± 3.4
SBP (mmHg)	165.0 ± 14.8	165.0 ± 14.8	165.7 ± 16.1
DBP (mmHg)*	94.3 ± 11.3	94.4 ± 11.3	90.5 ± 11.7
Pulse pressure (mmHg)*	70.8 ± 15.8	70.6 ± 15.7	75.2 ± 18.4
Heart rate (beats/min)	71.4 ± 10.9	71.4 ± 10.9	71.2 ± 9.5
Hyperlipidemia	1,178 (43.9)	1,136 (43.9)	42 (43.3)
Smoking			
Never	1,825 (68.0)	1,766 (68.2)	59 (60.8)
Ever	273 (10.2)	261 (10.1)	12 (12.4)
Current	587 (21.9)	561 (21.7)	26 (26.8)
Cerebrovascular disease†	344 (12.8)	330 (12.8)	14 (14.4)
LVH*	1,139 (42.4)	1,088 (42.0)	51 (52.6)
Ischemic heart disease	393 (14.6)	381 (14.7)	12 (12.3)
Proteinuria	548 (20.4)	530 (20.5)	18 (18.6)
Renal dysfunction	205 (7.6)	196 (7.6)	9 (9.3)
Peripheral vascular disease	37 (1.4)	35 (1.4)	2 (2.1)

Data are *n* (%) or means ± SD. *$P < 0.05$, NOD (−) vs. NOD (+). †Stroke and transient ischemic attack. NOD, new-onset diabetes.

$P = 0.004$; aged ≥65 years: 1.34 [1.01–1.77], $P = 0.042$; and $P_{interaction} = 0.152$). However, DBP was significantly associated with risk only in the group aged <65 years, whereas whole SBP was not associated in either age-group (for SBP, aged <65 years: 1.20 [0.86–1.67], $P = 0.284$; aged ≥65 years: 1.16 [0.84–1.59], $P = 0.374$; and $P_{interaction} = 0.780$; for DBP, aged <65 years: 1.58 [1.10–2.28], $P = 0.014$; aged ≥65 years: 1.32 [0.99–1.76], $P = 0.057$; and $P_{interaction} = 0.290$).

Because different combinations of SBP and DBP give the same pulse pressure value (e.g., blood pressures of 130/60 and 180/110 mmHg both give a pulse pressure of 70 mmHg), we evaluated the association of combinations of SBP and DBP with the risk of new-onset diabetes. As shown in Fig. 1, a strong association with risk was seen for higher pulse pressures arising mainly due to a lower DBP. From this result, we hypothesized that patients at high risk of new-onset diabetes had increased arterial stiffness. Accordingly, we next examined the association between PP_f and the risk of new-onset diabetes and found that PP_f (per 1 SD increase) was an independent predictor of new-onset diabetes (HR 1.49 [95% CI 1.21–1.84], $P < 0.001$). In subgroup analysis stratified by age, PP_f (per 1 SD increase) was significantly associated with the risk of new-onset diabetes in both age-groups (aged <65: 1.88 [1.29–2.73], $P < 0.001$; aged ≥65: 1.34 [1.03–1.74], $P = 0.027$; and $P_{interaction} = 0.057$). Because fewer patients developed diabetes with candesartan- than amlodipine-based regimens, we examined the difference in this effect stratified by quartile of PP_f. As shown in Fig. 2, a trend to an increased incidence of new-onset diabetes with increasing PP_f was seen in patients with amlodipine-based regimens, but not in those with candesartan-based regimens ($P = 0.0234$ for interaction in the quadratic term). Candesartan-based regimens significantly suppressed the incidence of new-onset diabetes in the highest quartile of PP_f. This result was not changed after adjustment for baseline characteristics (data not shown).

Table 2—*Predictors of new-onset diabetes by multiple Cox regression analysis*

Variables, unit of increase	HR (95% CI)	*P* value
Pulse pressure, per 1 SD increase	1.44 (1.15−1.79)	0.001
Prior antihypertensive treatment, yes	0.97 (0.61−1.54)	0.901
Allocated drug, candesartan	0.64 (0.42−0.97)	0.037
Sex, male	1.77 (1.07−2.92)	0.026
Age, per 10 years	1.09 (0.87−1.36)	0.460
BMI, per 1 kg/m^2 increase	1.11 (1.06−1.17)	<0.001
Heart rate, per 1 SD increase	1.01 (0.82−1.23)	0.960
Hyperlipidemia, yes	1.04 (0.68−1.57)	0.867
Smoking		
Ever	1.03 (0.52−2.04)	0.942
Current	1.22 (0.72−2.06)	0.458
Cerebrovascular disease, yes	1.48 (0.80−2.75)	0.214
LVH, yes	1.75 (1.13−2.72)	0.013
Ischemic heart disease, yes	0.91 (0.47−1.76)	0.777
Renal damage, yes*	1.10 (0.68−1.79)	0.694
Peripheral vascular disease, yes	1.49 (0.36−6.16)	0.581
Additional use of diuretics, yes	2.10 (1.25−3.52)	0.005
Additional use of β-blockers, yes	0.70 (0.40−1.24)	0.226
Additional use of α-blockers, yes	0.63 (0.32−1.24)	0.185

Data are HR (95% CI) and are adjusted for each variable. *Renal damage, proteinuria, and renal dysfunction.

CONCLUSIONS — In this study, we demonstrated that pulse pressure was a predictor of new-onset diabetes in high-risk hypertensive patients, independent of the effects of antihypertensive treatment and other possible risk factors for new-onset diabetes. Further, a higher pulse pressure arising mainly due to a lower DBP, indicating that the increased pulse pressure resulted largely from increased arterial stiffness, was associated with a higher risk of new-onset diabetes. This finding suggests that increased arterial stiffness, reflected in an increased pulse pressure, may be related to the process of new-onset diabetes in high-risk hypertensive patients, albeit that the mechanism of this association remains to be elucidated.

Two potential interpretations may explain these results. First, increased pulse pressure may be a surrogate marker for the risk of new-onset diabetes. Support-

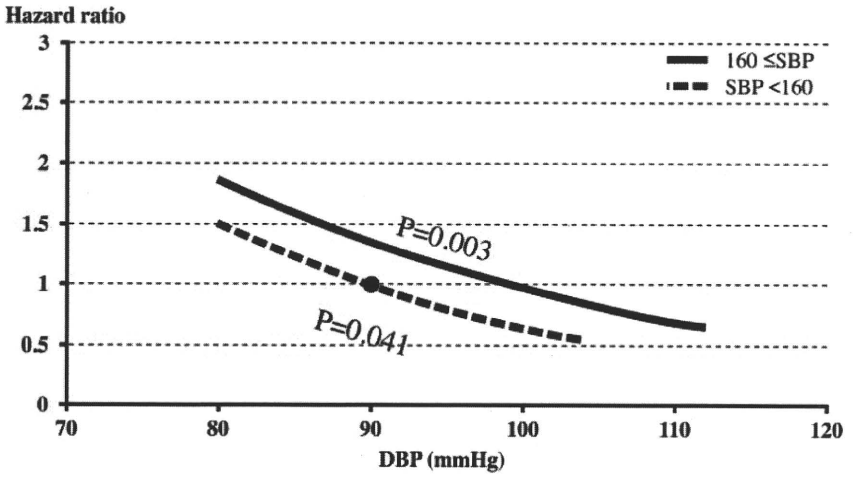

Figure 1—*Risk of new-onset diabetes by SBP and DBP at enrollment. HR of DBP of 90 mmHg in the SBP <160 mmHg category was assigned a reference value of 1.0.*

ing this suggestion, a higher pulse pressure, reflecting increased arterial stiffness, was observed in hypertensive patients with metabolic syndrome than in those without (17). Further, accumulating evidence supports the concept of increased arterial stiffness in patients with a metabolic disturbance, which is considered a potential mechanism linking metabolic disturbance to increased CVD risk (11–13). Arterial properties are affected both functionally and structurally by many factors, including aging, blood pressure, sympathetic nervous system function, endothelial function, inflammation, bioactive peptides, and other cardiovascular risk factors. Impaired glucose metabolism, including metabolic syndrome and insulin resistance, usually precedes the development of overt type 2 diabetes (18). Prolonged exposure to hyperglycemic conditions can lead to increased arterial stiffness via collagen cross-linking due to nonenzymatic glycation, endothelial dysfunction, inflammation, and local activation of the renin-angiotensin-aldosterone system in pre-diabetic as well as diabetic individuals (18). Indeed, PP_f, represented as a parameter of the pulsatile component of blood pressure, was superior to pulse pressure in terms of the risk stratification of new-onset diabetes.

Second, increased pulse pressure may directly affect glucose metabolism. Recent findings have clarified that microvascular dysfunction may be a cause rather than a consequence of hypertension (19). Microvascular dysfunction may also contribute to impaired insulin-mediated changes in muscle perfusion and glucose metabolism, providing a novel pathophysiological framework for understanding the association among hypertension, obesity, and impaired insulin-mediated glucose disposal (19,20). Microvascular dysfunction is thus a potential mechanism explaining the clustering of hypertension and type 2 diabetes. Interestingly, relations between microvascular function and both aortic stiffness and pressure pulsatility have been reported (21). Abnormalities in peripheral vascular resistance may have deleterious consequences for aortic stiffness, and microvascular dysfunction may in turn be further aggravated by increased transmission of the forward wave into the microcirculation. Accordingly, increased pulse pressure, reflecting increased arterial stiffness, may be both a cause and a consequence of microvascular dysfunction, leading to a "vicious cycle" in impaired glucose metabolism as well as arteriosclerosis (9,19,20).

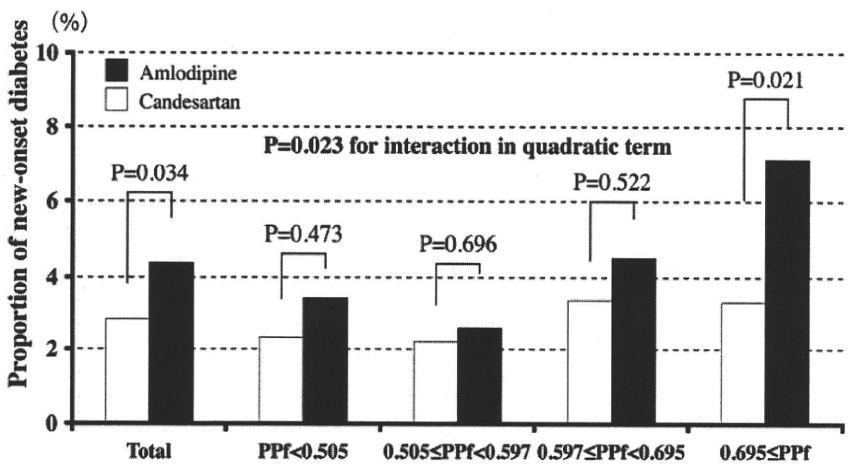

Figure 2—*Effect of candesartan and amlodipine on the incidence of new-onset diabetes stratified by quartile of PP_f. PP_f (linear and quadratic terms), the allocated drugs, and their interaction terms were entered in multiple Cox regression model.* P *value was calculated based on the Wald test.*

The present study also revealed that electrocardiographic or echocardiographic LVH at baseline was an independent predictor of new-onset diabetes. In their recent subanalysis of the Losartan Intervention for Endpoint Reduction in Hypertension (LIFE) study, Oki et al. (22) reported that in-treatment resolution or continued absence of electrocardiographic LVH was associated with a lower incidence of diabetes. Because pulse pressure was positively related to LVH (23), our study might validate their findings from a different perspective. Interestingly, in another subanalysis of the LIFE study, Olsen et al. (24) found that treatment with the ARB losartan was associated with less peripheral vascular hypertrophy/rarefaction and higher insulin sensitivity than that with atenolol, supporting the hypothesis that microvascular dysfunction in hypertension may induce insulin resistance. In the present study, the suppressive effect of the ARB candesartan against new-onset diabetes tended to strengthen as PP_f increased. These results suggest that ARBs decrease the risk of new-onset diabetes partly via the improvement of microcirculation.

Although the prevalence of diabetes increases with age (25), it remains unclear whether age is a risk factor for new-onset diabetes (6–8). In the present study, age at baseline was not an independent predictor of new-onset diabetes. We assumed that high-risk elderly hypertensive patients who did not have diabetes at baseline were survivors who had avoided the development of diabetes and that their

underlying risk of new-onset diabetes and ability to metabolize glucose may thus have differed from those of younger subjects. We also observed a strong association between pulse pressure and new-onset diabetes in patients aged <65 years, possibly owing to the same mechanism.

Several limitations of this study warrant mention. First, it was conducted as a post hoc analysis. Second, although we found an interesting association between pulse pressure and the risk of new-onset diabetes, the CASE-J trial was not designed to prospectively evaluate this association, and we were consequently unable to elucidate causality, because we did not directly measure parameters of arterial stiffness or collect the data to clarify the underlying mechanism. Third, we were unable to include baseline data regarding glucose metabolism into the multiple Cox regression analysis or information about a family history of diabetes, physical activity, or diet, which are well-known and important risk factors for new-onset diabetes. Fourth, new-onset diabetes was prespecified as the end point just before the completion of the CASE-J trial. Accordingly, there was a possibility of nonreporting bias, because the definition of new-onset diabetes was not in the original protocol and determination of whether new-onset diabetes had occurred depended on the participating investigators' reports. Thus, we may have underestimated the overall incidence of new-onset diabetes. Nevertheless, the present study is the first to examine the association of pulse pressure with new-onset diabetes in hypertensive patients and may provide useful information in understanding the underlying mechanism between hypertension and new-onset diabetes. Finally, because the study population consisted of Japanese patients with high-risk hypertension, the generalizability of our findings to other ethnic groups or general populations may be limited.

In summary, we found that pulse pressure is an independent predictor of new-onset diabetes in high-risk Japanese hypertensive patients. The development of type 2 diabetes may involve increased arterial stiffness, suggesting the importance of the "microvascular dysfunction" theory in the underlying pathophysiological mechanism between hypertension and new-onset diabetes. To our knowledge, this study is the first to report the relation between pulse pressure and new-onset diabetes in hypertensive patients. Further studies are required to elucidate the significance of pulse pressure in new-onset diabetes in hypertensive patients.

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No potential conflicts of interest relevant to this article were reported.

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ORIGINAL ARTICLE
Hypertension and Circulatory Control

Impact of Left Ventricular Hypertrophy on the Time-Course of Renal Function in Hypertensive Patients

– A Subanalysis of the CASE-J Trial –

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Background: In this subanalysis of the CASE-J, which was conducted to compare the effects of candesartan and amlodipine in Japanese high-risk hypertensive patients, the association of left ventricular hypertrophy (LVH) with renal function is clarified.

Methods and Results: Patients were divided into 2 groups: 1,082 patients with LVH and 2,119 patients without LVH. The primary endpoint was the change in the estimated glomerular filtration rate (eGFR). The eGFRs were increased from 63.6 to 65.1 $ml \cdot min^{-1} \cdot 1.73 m^{-2}$ in patients with LVH and from 63.6 to 68.5 $ml \cdot min^{-1} \cdot 1.73 m^{-2}$ in those without LVH. The improvement in the eGFR was greater in patients without LVH than in those with LVH (P=0.004). In patients with chronic kidney disease (CKD) patients, the eGFR increased from 52.7 to 60.5 $ml \cdot min^{-1} \cdot 1.73 m^{-2}$ in patients without LVH, but from 53.1 to 57.2 $ml \cdot min^{-1} \cdot 1.73 m^{-2}$ in those with LVH (P<0.001, patients without LVH vs patients with LVH). Furthermore, because the eGFR changed from 76.5 to 75.4 $ml \cdot min^{-1} \cdot 1.73 m^{-2}$ in patients without CKD but with LVH, and from 76.5 to 77.5 $ml \cdot min^{-1} \cdot 1.73 m^{-2}$ in those without either CKD or LVH, the final eGFR was higher in patients without LVH than in those with LVH (P=0.048).

Conclusions: LVH related to the time-course of renal function in Japanese hypertensive patients. (*Circ J* 2010; **74:** 2132–2138)

Key Words: Chronic kidney disease; Estimated glomerular filtration rate; Hypertension; Left ventricular hypertrophy

With progressive aging of the population and an increasing prevalence of hypertension and diabetes mellitus, chronic kidney disease (CKD) remains a worldwide public health problem. As many patients with CKD die of cardiovascular (CV) disease before reaching end-stage renal disease, measures against CKD should be undertaken from the viewpoint of improving their prognosis.[1,2]

Left ventricular hypertrophy (LVH) is a manifestation of target organ damage and an independent risk factor for CV morbidity and mortality.[3,4] Several studies have examined the association of renal dysfunction with LVH and have reported that reduced renal function and albuminuria are risk factors for it.[5–7] LVH is thus common in patients with CKD, indicating kidney–heart interaction. To date, however, few studies have examined the impact of LVH on renal function in hypertensive patients.[8,9] Our previous subanalysis of the CASE-J trial reported that cardiac complications, including LVH and ischemic heart disease, were independent predictors of CV events, but not of renal events.[10] In contrast, Boner et al reported that LVH was associated with a significantly increased risk of not only CV events but also the progression of kidney disease in patients with type 2 diabetes and nephropathy.[9] Of 4,703 patients in the CASE-J trial, only 46 (1.0%) experienced a renal event, a much smaller proportion than the 32.9% in the RENALL study, indicating that the CASE-J trial lacked sufficient statistical power to evaluate the impact of LVH on renal events.

In this context, the present study was conducted as a subanalysis of the CASE-J trial aimed at investigating the impact of LVH on the time-course of renal function in high-risk Japanese hypertensive patients.

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Table 1. Baseline Characteristics of the Study Patients

Characteristics	LVH (–)	LVH (+)
No. of participants	2,119	1,082
Candesartan(%)	1,071 (50.5)	537 (49.6)
Age (years)	64.4±10.0	63.6±10.3
Men (%)*	1,065 (50.3)	687 (63.5)
Body mass index (kg/m^2)*	24.6±3.6	24.4±3.5
SBP (mmHg)*	162.7±13.9	160.7±13.8
DBP (mmHg)*	91.1±11.3	90.9±10.6
Heart rate (beats/min)*	72.6±10.8	71.3±11.4
Severe HT (SBP ≥180 and/or DBP ≥110mmHg)*	448 (21.1)	120 (11.1)
Type 2 diabetes†,*	1,089 (51.4)	324 (29.9)
Ischemic heart disease (AP and/or OMI)	299 (14.1)	134 (12.4)
Cerebrovascular disease		
Cerebral hemorrhage*	52 (2.5)	12 (1.1)
Cerebral infarction*	155 (7.3)	48 (4.4)
TIA*	39 (1.8)	7 (0.6)
Renal dysfunction		
Proteinuria*	471 (22.2)	171 (15.8)
sCr ≥1.3mg/dl*	175 (8.3)	74 (6.8)
Vascular disease		
ASO*	32 (1.5)	7 (0.6)

Data are shown as number of patients (%) or mean±SD.
*P<0.05; cardiac risk (–) vs cardiac risk (+).
†Type 2 diabetes mellitus was defined by fasting blood glucose ≥126mg/dl, casual blood glucose ≥200mg/dl, hemoglobin A_{1c} ≥6.5%, 2h blood glucose on 75g OGTT ≥200mg/dl, or current treatment with hypoglycemic agent at baseline.
LVH, left ventricular hypertrophy; SBP, systolic blood pressure; DBP, diastolic blood pressure; HT, hypertension; AP, angina pectoris; OMI, old myocardial infarction; TIA, transient ischemic attack; sCr, serum creatinine; ASO, atherosclerosis obliterans; OGTT, oral glucose tolerance test.

Methods

Study Design

The CASE-J trial was a prospective, multicenter, randomized, open-label, active-controlled, 2-arm parallel-group comparison study, which evaluated the efficacy of angiotensin receptor blocker candesartan and Ca channel blocker amlodipine in reducing the incidence of CV events in high-risk hypertensive patients.[11,12] The rationale and complete design of the CASE-J trial and main outcome of the primary endpoint have been reported elsewhere.[11,12] Briefly, 4,728 patients with high-risk hypertension were randomly assigned to either a candesartan- or amlodipine-based treatment regimen. High-risk was defined as the presence of any one of the following: (a) severe hypertension: systolic blood pressure (SBP)/diastolic blood pressure (DBP) ≥180/110mmHg; (b) type 2 diabetes mellitus; (c) history of stroke or transient ischemic attack more than 6 months prior to screening; (d) LVH (SV1+RV5 ≥3.5mV in electrocardiography (ECG) and/or LV wall thickness ≥12mm in echocardiography), angina pectoris, or a history of myocardial infarction more than 6 months prior to screening; (e) proteinuria or serum creatinine concentration ≥1.3mg/dl; and (f) arteriosclerotic peripheral artery obstruction. The exclusion criteria have been reported elsewhere.[11] Enrolled patients were given one of the following medications after randomization, namely candesartan administered orally at a dose of 4–12mg/day or amlodipine administered orally at a dose of 2.5–10mg/day. Finally, 4,703 randomly assigned patients were included in the analysis. Mean follow-up period was 3.2 years and follow-up rate was 97.1%.[12]

In the present analysis, we focused on LVH, which was one of inclusion criteria in the trial. Enrolled patients, whose serum creatinine values were available every 6 months during the follow-up period, were divided into 1,082 patients with and 2,119 patients without LVH. Among the 1,082 patients with LVH, 633 met the ECG criteria for LVH, 297 met the echocardiographic criteria, and 152 met both the ECG and echocardiographic criteria. The primary endpoint in this subanalysis was change in estimated glomerular filtration rate (eGFR) in patients with or without LVH. Based on CKD Guidelines of the Japanese Society of Nephrology,[13,14] eGFR was calculated by the following equation:

$$eGFR = 194 \times Cr^{-1.094} \times Age^{-0.28}$$
(×0.739, if female; Cr, serum creatinine)

Further, to evaluate the impact of CKD on the time-course of renal function, the patients were also divided into 2 additional groups, namely those with (n=1,455) and without CKD (n=1,746). Patients at enrollment with positive urinary protein tests by either or both dipstick analysis or an eGFR of <60 $ml \cdot min^{-1} \cdot 1.73\,m^{-2}$ were defined as having CKD in this study.[15]

Baseline Characteristics

Table 1 shows the baseline characteristics of patients with and without LVH in the present analysis. As LVH was one of the inclusion criteria, there were statistical differences in baseline characteristics between the 2 groups. Thus, analyses were adjusted for baseline characteristics as described below.

Statistical Analysis

Data are expressed as the mean±standard deviation or proportion. We compared continuous variables using the Student's