was abolished after the AM mice were subjected to BCAS operation, suggesting that feedback inhibition is a plausible cause for the downregulation (Figure 6D).

Discussion

Three major conclusions may be drawn from the present study. First, it was demonstrated that increased levels of circulating AM restored cerebral hemodynamics, promoted arteriogenesis as well as angiogenesis, alleviated oxidative damage in the cerebral microvessels, and preserved WM integrity; this subsequently attenuated working memory deficits in a mouse model of chronic cerebral hypoperfusion. Second, AM selectively upregulated brain levels of cAMP, VEGF, and bFGF in the hypoperfused brain but not in the normoperfused brain. Finally, it was found that such proangiogenic/arteriogenic changes did not occur in sham-operated AM-Tg mice in which the expression of AM receptor component RAMP2 was significantly suppressed, possibly through feedback inhibition.

We have found a significant correlation in the averaged number of revisiting errors at 1 month after BCAS with CBF on Days 7, 14, and 28, but not with CBF immediately after BCAS or on Days 1 and 3. Therefore, the recovery of CBF is one of the substrates for the functional improvements, whereas several phenomena other than CBF recovery may play roles in the pathophysiology of this BCAS model. In fact, we demonstrated that AM induced not only CBF recovery as a result of arteriogenesis, but also angiogenesis (not associated with CBF recovery), antioxidative activity in the microvessels, and attenuation of microglial inflammatory responses. The other effects of AM, including antiapoptotic effects and regulation of endothelial permeability or the blood–brain barrier,[3] need further investigation. Positive effects of AM may be mediated by multiple pathways.

Previous reports showed that the AM/cAMP/PKA cascade blocks oxidative damage in ischemic injury[8] and promotes angiogenic effects of the endothelial cells in vitro.[9] We found that chronic ischemic insult and circulating AM are both required to raise cAMP levels in the brain; this may be associated with alleviating oxidative damage and promoting angiogenesis.

The elevation of VEGF is consistent with the previous report that AM administration upregulates the expression of VEGF in both in vitro and in vivo hindlimb ischemia models.[6] Although no previous studies have reported that AM enhances the expression of bFGF after ischemia, we demonstrated the AM-induced upregulation of bFGF after BCAS in vivo. AM was also found to upregulate bFGF as well as VEGF in the cultured endothelial cells (unpublished data). Previous reports have demonstrated that combined gene delivery of VEGF and bFGF produces additive or synergistic effects on angiogenesis or collateral development, probably due to the protective effects of bFGF against VEGF-induced fluid leakage.[10] Thus, AM-induced elevation of bFGF may be associated with the development of functional vessels.

AM acts through 2 subtypes of receptor (AM1 and AM2), which derive from the interaction of the calcitonin receptor-like receptors with RAMP2 or 3.[11] Interestingly, RAMP2 mRNA level in sham-operated AM-Tg mice was significantly decreased compared with sham-operated WT mice but nearly reached normalization after BCAS. This may explain why arteriogenesis and angiogenesis were significantly promoted in AM-Tg mice only after ischemic insult. Shindo et al have reported that RAMP2 rather than RAMP3 is a key determinant of the effects of AM on the vasculature and is essential for angiogenesis and vascular integrity.[11] These results suggest that the AM-initiated signaling pathway is suppressed by downregulation of RAMP2 in the normoperfused brain but that such suppression is abolished by chronic ischemic stress, leading to AM-induced arteriogenesis and angiogenesis. Such tissue selectivity could be an advantage for clinical application of AM in patients with subcortical vascular dementia.

Recently, the concept of an "oligovascular niche" has been proposed, in which crosstalk between endothelial cells and oligodendrocytes, mediated by an exchange of soluble signals such as fibroblast growth factor, are thought to play an important role in sustaining oligodendrocyte homeostasis and WM integrity.[12] Because cerebral endothelial cells contribute to numerous signaling cascades that help regulate brain homeostasis and function,[13] angio-/arteriogenesis and inhibition of oxidative damage in the cerebral endothelial cells induced by AM might lead to oligovascular protection—namely, successful vascular growth and vasoprotection and preservation of white matter/oligodendrocyte integrity—and prevention of cognitive decline after chronic cerebral hypoperfusion in mice.

In conclusion, this study demonstrates that circulating AM is a highly potent and effective modality for restoring perfusion, promoting arteriogenesis and angiogenesis in the chronically ischemic brain, inhibiting oxidative damage in the cerebral microvessels, preserving ischemic WM integrity, and attenuating working memory deficits in a mouse model of subcortical vascular dementia. Future clinical studies are required to evaluate and confirm the efficacy of AM in chronic cerebral vascular diseases, especially subcortical vascular dementia.

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Disclosures

None.

References

1. Pantoni L. Leukoaraiosis: from an ancient term to an actual marker of poor prognosis. *Stroke*. 2008;39:1401–1403.
2. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun*. 1993;192: 553–560.
3. Kato J, Tsuruda T, Kita T, Kitamura K, Eto T. Adrenomedullin: a protective factor for blood vessels. *Arterioscler Thromb Vasc Biol*. 2005; 25:2480–2487.
4. Shibata M, Ohtani R, Ihara M, Tomimoto H. White matter lesions and glial activation in a novel mouse model of chronic cerebral hypoperfusion. *Stroke*. 2004;35:2598–2603.
5. Shibata M, Yamasaki N, Miyakawa T, Kalaria RN, Fujita Y, Ohtani R, Ihara M, Takahashi R, Tomimoto H. Selective impairment of working memory in a mouse model of chronic cerebral hypoperfusion. *Stroke*. 2007;38:2826–2832.
6. Iimuro S, Shindo T, Moriyama N, Amaki T, Niu P, Takeda N, Iwata H, Zhang Y, Ebihara A, Nagai R. Angiogenic effects of adrenomedullin in ischemia and tumor growth. *Circ Res*. 2004;95:415–423.
7. Miyashita K, Itoh H, Arai H, Suganami T, Sawada N, Fukunaga Y, Sone M, Yamahara K, Yurugi-Kobayashi T, Park K, Oyamada N, Taura D, Tsujimoto H, Chao TH, Tamura N, Mukoyama M, Nakao K. The neuroprotective and vasculo-neuro-regenerative roles of adrenomedullin in ischemic brain and its therapeutic potential. *Endocrinology*. 2006;147: 1642–1653.
8. Miyamoto N, Tanaka R, Shimosawa T, Yatomi Y, Fujita T, Hattori N, Urabe T. Protein kinase A-dependent suppression of reactive oxygen species in transient focal ischemia in adrenomedullin-deficient mice. *J Cereb Blood Flow Metab*. 2009;29:1769–1779.
9. Miyashita K, Itoh H, Sawada N, Fukunaga Y, Sone M, Yamahara K, Yurugi T, Nakao K. Adrenomedullin promotes proliferation and migration of cultured endothelial cells. *Hypertens Res*. 2003;26(suppl): S93–S98.
10. Kondoh K, Koyama H, Miyata T, Takato T, Hamada H, Shigematsu H. Conduction performance of collateral vessels induced by vascular endothelial growth factor or basic fibroblast growth factor. *Cardiovasc Res*. 2004;61:132–142.
11. Ichikawa-Shindo Y, Sakurai T, Kamiyoshi A, Kawate H, Iinuma N, Yoshizawa T, Koyama T, Fukuchi J, Iimuro S, Moriyama N, Kawakami H, Murata T, Kangawa K, Nagai R, Shindo T. The GPCR modulator protein RAMP2 is essential for angiogenesis and vascular integrity. *J Clin Invest*. 2008;118:29–39.
12. Arai K, Lo EH. An oligovascular niche: cerebral endothelial cells promote the survival and proliferation of oligodendrocyte precursor cells. *J Neurosci*. 2009;29:4351–4355.
13. Iadecola C, Nedergaard M. Glial regulation of the cerebral microvasculature. *Nat Neurosci*. 2007;10:1369–1376.

ORIGINAL ARTICLE

Therapeutic Impact of Leptin on Diabetes, Diabetic Complications, and Longevity in Insulin-Deficient Diabetic Mice

Masaki Naito, Junji Fujikura, Ken Ebihara, Fumiko Miyanaga, Hideki Yokoi, Toru Kusakabe, Yuji Yamamoto, Cheol Son, Masashi Mukoyama, Kiminori Hosoda, and Kazuwa Nakao

OBJECTIVE—The aim of the current study was to evaluate the long-term effects of leptin on glucose metabolism, diabetes complications, and life span in an insulin-dependent diabetes model, the Akita mouse.

RESEARCH DESIGN AND METHODS—We cross-mated Akita mice with leptin-expressing transgenic (LepTg) mice to produce Akita mice with physiological hyperleptinemia (LepTg:Akita). Metabolic parameters were monitored for 10 months. Pair-fed studies and glucose and insulin tolerance tests were performed. The pancreata and kidneys were analyzed histologically. The plasma levels and pancreatic contents of insulin and glucagon, the plasma levels of lipids and a marker of oxidative stress, and urinary albumin excretion were measured. Survival rates were calculated.

RESULTS—Akita mice began to exhibit severe hyperglycemia and hyperphagia as early as weaning. LepTg:Akita mice exhibited normoglycemia after an extended fast even at 10 months of age. The 6-h fasting blood glucose levels in LepTg:Akita mice remained about half the level of Akita mice throughout the study. Food intake in LepTg:Akita mice was suppressed to a level comparable to that in WT mice, but pair feeding did not affect blood glucose levels in Akita mice. LepTg:Akita mice maintained insulin hypersensitivity and displayed better glucose tolerance than did Akita mice throughout the follow-up. LepTg:Akita mice had normal levels of plasma glucagon, a marker of oxidative stress, and urinary albumin excretion rates. All of the LepTg:Akita mice survived for >12 months, the median mortality time of Akita mice.

CONCLUSIONS—These results indicate that leptin is therapeutically useful in the long-term treatment of insulin-deficient diabetes. ***Diabetes*** **60:2265–2273, 2011**

Leptin is an adipocyte-derived hormone that is involved in the regulation of food intake and energy expenditure (1). We previously created transgenic mice that overexpress leptin under the control of the liver-specific promoter (LepTg) (2). The plasma leptin level is stable in LepTg mice and similar to that in obese rodents and humans, suggesting that the phenotypic changes found in these animals are physiologically relevant (3,4). LepTg mice provide a unique experimental system to investigate the chronic in vivo effects of leptin. LepTg mice exhibit increased glucose metabolism, which is accompanied by the activation of insulin signaling in skeletal muscle and the liver (2). These findings indicate that leptin acts as an antidiabetic hormone. We have demonstrated the efficacy of leptin in various mouse models of diabetes (5–8).

Akita mice are an animal model of diabetes caused by pancreatic β-cell failure. Endoplasmic reticulum stress induced by misfolded proinsulin is responsible for the β-cell dysfunction and destruction in Akita mice. Male Akita mice start to develop hyperglycemia as early as weaning, when 71% decrease of β-cell mass is present. Plasma insulin levels in the mice are reduced to 41% of the control mice at 7 weeks of age. The blood glucose levels in male Akita mice increase irreversibly up to 700 mg/dL at 10 weeks of age, and about half the male Akita mice die of extreme hyperglycemia within the 1st year of life (9). Thus, the Akita mouse is a suitable model for evaluating the therapeutic impact of interventions on the onset, progression, and prognosis of diabetes.

In the current study, to clarify how and to what extent chronic leptin therapy affects the long-term course of diabetes, we genetically crossed LepTg and Akita mice to create a unique mouse model of nonobese diabetes with elevated plasma leptin level. Using this mouse diabetes model, we investigated the chronic lifelong effects of leptin on diabetes, diabetic nephropathy, and longevity in Akita mice.

RESEARCH DESIGN AND METHODS

Animals. Generation of LepTg mice was reported previously (2). Briefly, a fusion gene comprising the human serum amyloid P component promoter upstream of the mouse leptin cDNA coding sequences was designed to target hormone expression to the liver (2,8). The highest expressing transgenic line was used in this study (2). The genotype for LepTg mice was determined by PCR (5′-GCTGGTTGTTGTGCTGTCTC-3′; 5′-CAGGCTGGTGAGGACCTGTT-3′). B6-Ins2Akita (*Ins2*$^{WT/C96Y}$; referred to hereafter as Akita) mice were purchased from Japan SLC (Shizuoka, Japan). Presence of the Akita mutation was verified by absence of an Fnu4HI restriction site in the PCR product of the *Ins2* gene (5′-TGCTGATGCCCTGGCCTGCT-3′; 5′-TGGTCCCACATATGCACATG-3′). Both LepTg and Akita mice were on the same C57BL/6 J background. Hemizygous male LepTg mice were cross-mated with female heterozygous Akita mice. Male F1 mice were used in this study. Mice were maintained in a temperature-, humidity-, and light-controlled room and allowed free access to standard diet (F-2 diet; Oriental BioService, Kyoto, Japan).

The care of the animals and all experimental procedures were conducted in accordance with the guidelines for animal experiments of Kyoto University and were approved by the Animal Research Committee of Kyoto University.

Metabolic parameters measurements. Levels of leptin (Mouse Leptin ELISA, Millipore, St. Charles, MO), glucose (Glutest Neo Super, Sanwa, Nagoya, Japan, or Glucose C2-test, Wako, Osaka, Japan), HbA_{1c} (DCA2000 analyzer, Bayel-Sankyo, Tokyo, Japan), insulin (Ultra-Sensitive PLUS Mouse Insulin Kit, Morinaga, Yokohama, Japan), glucagon (Glucagon EIA Kit, Yanaihara, Shizuoka, Japan), triglyceride (TG; Triglyceride E-test, Wako), nonesterified fatty acid (NEFA; NEFA C-test, Wako), β-hydroxybutyrate (Precision Xtra, Abbott, Bedford, MA), and thiobarbituric acid reactive substances (TBARS; TBARS

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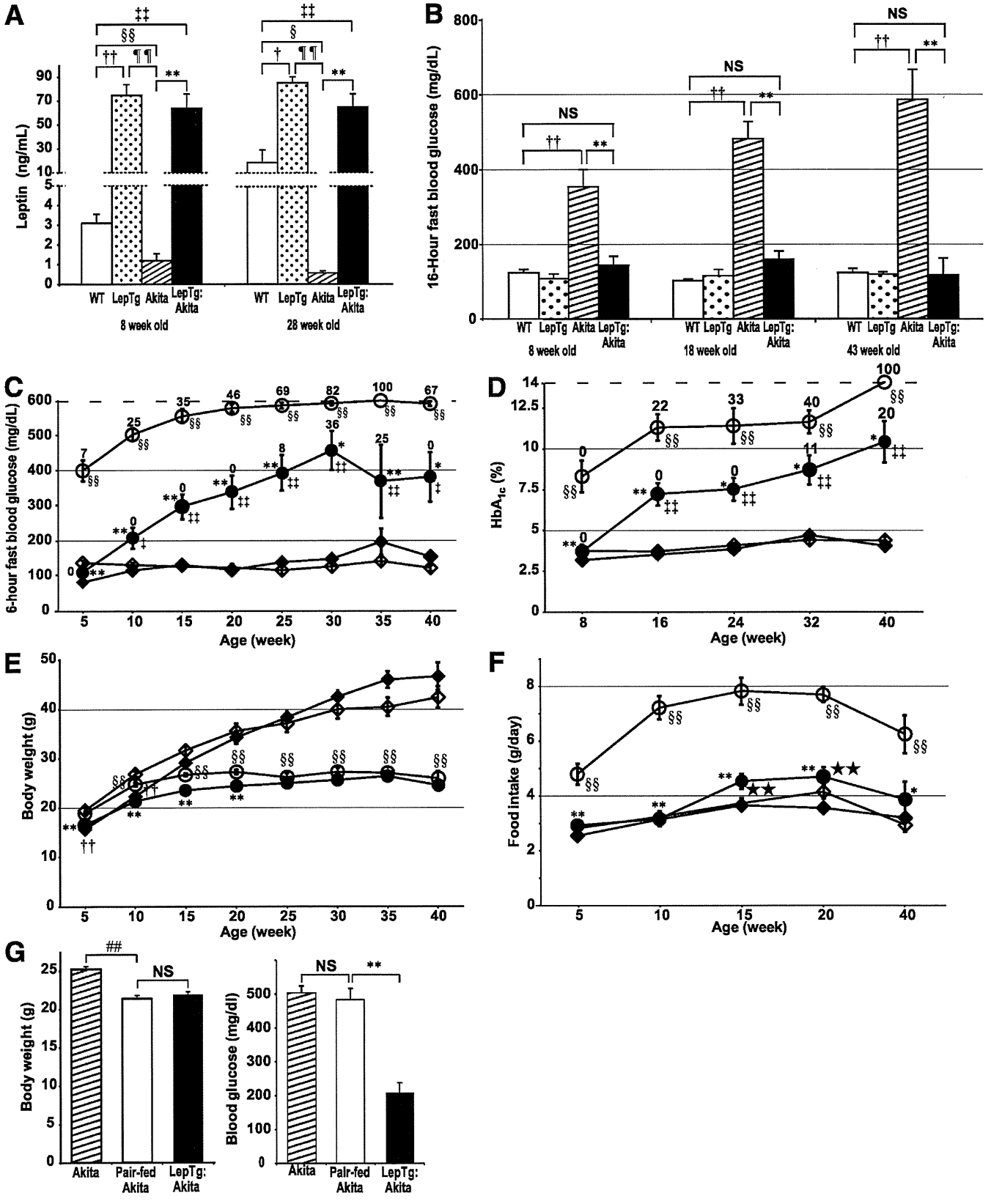

FIG. 1. Time course of changes in plasma leptin, blood glucose, HbA_{1c}, body weight, and food intake. *A*: Plasma leptin levels of WT, LepTg, Akita, and LepTg:Akita mice at 8 and 28 weeks of age ($n \geq 4$ in each group). *B*: Sixteen-hour fasting blood glucose levels of WT, LepTg, Akita, and LepTg:Akita mice at 8, 18, and 43 weeks of age ($n \geq 4$ in each group). *C*: Time course of 6-h fasting blood glucose concentrations of WT (◇), LepTg (◆), Akita (○), and LepTg:Akita (●) mice ($n \geq 11$ in each group, except $n = 5$ for data of 40 weeks of age). Since the glucometer has a detection limit up to 600 mg/dL, values above the detection limit were treated as 601 mg/dL. Dashed line indicates detection limit of 600 mg/dL. The numbers along the curves indicate the percent of samples above detection limit. *D*: Time course of glycated hemoglobin (HbA_{1c}) levels of WT (◇), LepTg (◆), Akita (○), and LepTg:Akita (●) mice ($n \geq 4$ in each group). Since the analyzer has a detection limit up to 14%, values above the detection limit were treated as 14.1%. Dashed line indicates detection limit of 14%. The numbers along the curves indicate the percent of samples above detection limit.

Assay Kit, Cayman, Ann Arbor, MI) were measured. Percent body fat was measured by Latheta LTC-100 (ALOKA, Tokyo, Japan). For glucose tolerance tests (GTTs), after 12-h fast, the mice were injected with 1.0 g/kg i.p. glucose. For insulin tolerance tests (ITTs), after a 6-hour fast, the mice were injected with 0.5 units/kg i.p. human insulin (Novo Nordisk, Bagsvaerd, Denmark).

Pair-feeding experiment. Akita mice were given the amount of food consumed by ad libitum–fed LepTg:Akita mice on the previous day. A pair-feeding study was conducted from 8 through 11 weeks of age. Body weights and blood glucose concentrations were measured at the end of the period.

Pancreatic hormone secretion and content. After 12-h fast, the mice were injected with 3.0 g/kg i.p. glucose. Blood samples were obtained from the retro-orbital venous sinus using heparin-coated glass capillaries. For hormone content, pancreata were homogenized in acid ethanol.

Histology. Pancreata were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were immunostained with the following antibodies: guinea pig anti-insulin antibody (Dako, Glostrup, Denmark), rabbit antiglucagon antibody (Dako), and Alexa488 anti–guinea pig antibody and Alexa546 anti-rabbit antibody (both from Molecular Probes, Eugene, OR). Kidney tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Periodic acid Schiff (PAS) was used to stain 1-μm sections. Mesangial area was determined by the presence of PAS-positive and nuclei-free area in the mesangium. Measurement of the mesangial area of more than 22 glomeruli randomly selected in each mouse was performed with a computer-assisted microscopy (Keyence, Osaka, Japan).

Albumin in urine. A metabolic cage was used to collect 24-h urine. Urinary albumin concentration was measured using Albuwell M (Exocell, Philadelphia, PA). Blood pressure was measured by the indirect tail-cuff method.

Survival rates. Survival data were analyzed by Kaplan-Meier analysis, and comparisons between genotypes were done by the log-rank test.

Statistical analyses. Data are expressed as means ± SE. Comparison between or among groups was by Student *t* test, Mann-Whitney *U* test, or ANOVA with Scheffé *F* test. $P < 0.05$ was considered statistically significant.

RESULTS

Generation of LepTg:Akita mice. Plasma leptin levels were measured periodically (Fig. 1*A*). At 8 weeks of age, the plasma leptin levels in Akita mice declined to 39% of the levels in WT mice (3.1 ng/mL for WT vs. 1.2 ng/mL for Akita; $P < 0.01$). Five months later, at 28 weeks of age, an age-dependent increase in plasma leptin levels in WT mice and decrease in Akita mice were observed (18.8 ng/mL for WT vs. 0.58 ng/mL for Akita; $P < 0.05$). The transgenic expression of leptin was associated with markedly and stably increased plasma leptin levels in both LepTg and LepTg:Akita mice (74.8 ng/mL for LepTg and 64.2 ng/mL for LepTg:Akita at 8 weeks of age and 85.1 ng/mL for LepTg and 65.3 ng/mL for LepTg:Akita at 28 weeks of age).

Time course of changes in blood glucose and HbA_{1c} levels. Blood glucose levels were measured after a 16-h fast at 8, 18, and 43 weeks of age (Fig. 1*B*). Akita mice showed hyperglycemia with blood glucose levels >300 mg/dL even after the 16-h fast at 8 weeks of age, and this hyperglycemia worsened progressively with time. By contrast, the glucose levels remained <160 mg/dL and were indistinguishable between LepTg:Akita and WT mice at all times studied.

Six-hour fasting blood glucose levels, which correlate closely with the daily averaged blood glucose level, and HbA_{1c} levels were followed for ~10 months (Fig. 1*C* and *D*) (10). In Akita mice, 6-h fasting blood glucose levels were >500 mg/dL after 10 weeks of age, and more than half of the mice had blood glucose levels >600 mg/dL after 15 weeks of age (Fig. 1*C*). The blood glucose levels were lower in LepTg:Akita mice than in WT mice at 5 weeks of age and increased gradually after 10 weeks of age but remained <400 mg/dL at 40 weeks of age (Fig. 1*C*). Average 6-h fasting blood glucose levels were 552.4 ± 24.1 mg/dL in Akita, 321.0 ± 39.6 mg/dL in LepTg:Akita, 136.3 ± 11.8 mg/dL in LepTg, and 128.7 ± 3.0 mg/dL in WT mice from 5 to 40 weeks of age. Thus, the average blood glucose level in LepTg:Akita mice was ~58.1% of that in Akita mice.

HbA_{1c} levels changed in a similar pattern to the 6-h fasting blood glucose levels (Fig. 1*D*). By 8 weeks of age, Akita mice had markedly elevated HbA_{1c} levels (8.4 ± 1.0%), whereas HbA_{1c} levels were the same in LepTg:Akita mice (3.7 ± 0.1%) as in WT mice (3.8 ± 0.4%) at that age. Of Akita mice >16 weeks of age, >22% had HbA_{1c} levels above the detection limit (14%). The HbA_{1c} levels of LepTg:

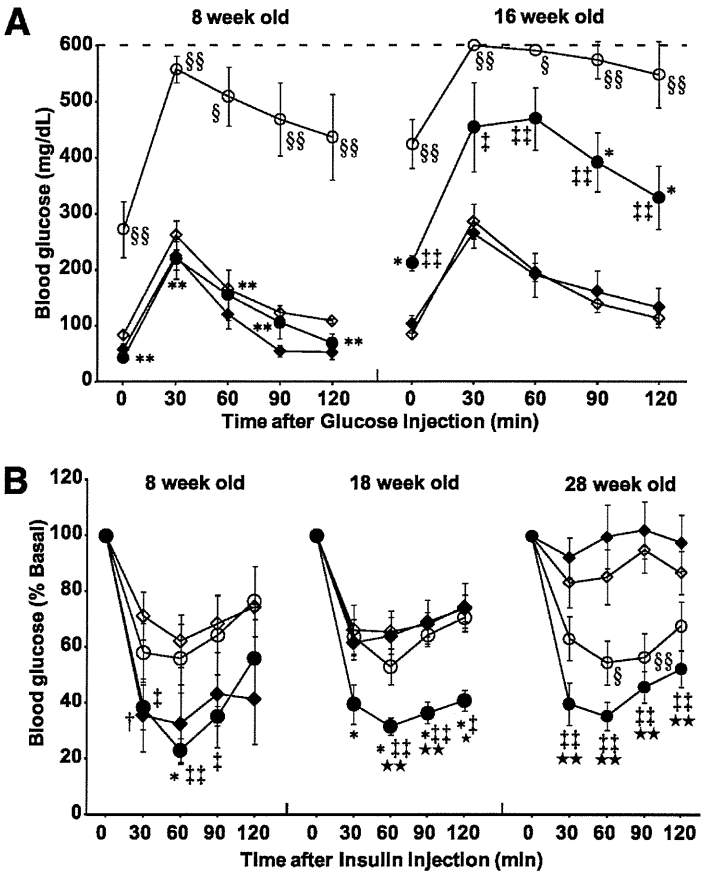

FIG. 2. GTTs and ITTs. *A*: GTTs of WT (◇), LepTg (◆), Akita (open circles), and LepTg:Akita (closed circles) mice at 8 and 16 weeks of age. Blood glucose levels are shown at indicated times after glucose injections (1 g/kg body wt i.p.; $n \geq 4$ in each group). *B*: ITTs of WT (◇), LepTg (◆), Akita (○), and LepTg:Akita (●) mice at 8, 18, and 28 weeks of age. Percent changes in blood glucose levels are shown at indicated times after injection of insulin (0.5 units/kg body wt i.p.; $n \geq 4$ in each group). Dashed line indicates detection limit of 600 mg/dL. Data are expressed as means ± SE. §$P < 0.05$, §§$P < 0.01$ for WT vs. Akita, ‡$P < 0.05$, ‡‡$P < 0.01$ for WT vs. LepTg:Akita, ★$P < 0.05$, ★★$P < 0.01$ for LepTg vs. LepTg:Akita, *$P < 0.05$, and **$P < 0.01$ for Akita vs. LepTg:Akita.

E: Time course of body weight changes of WT (◇), LepTg (◆), Akita (○), and LepTg:Akita (●) mice ($n \geq 11$ in each group, except $n = 5$ for data of 40 weeks of age). *F*: Time course of 24-h food intake of WT (◇), LepTg (◆), Akita (○), and LepTg:Akita (●) mice ($n \geq 11$ in each group, except $n = 4$ for data of 40 weeks of age). *G*: Body weight of Akita, pair-fed Akita, and LepTg:Akita mice at the end of 3 weeks of pair feeding ($n = 4$ in each group). *H*: Six-hour fasting blood glucose concentrations of Akita, pair-fed Akita, and LepTg:Akita mice at the end of 3 weeks of pair feeding ($n = 4$ in each group). Data are expressed as means ± SE. In *A–F*, †$P < 0.05$, ††$P < 0.01$ for WT vs. LepTg, §$P < 0.05$, §§$P < 0.01$ for WT vs. Akita, ‡$P < 0.05$, ‡‡$P < 0.01$ for WT vs. LepTg:Akita, ¶¶$P < 0.01$ for LepTg vs. Akita, ★★$P < 0.01$ for LepTg vs. LepTg:Akita, *$P < 0.05$, and **$P < 0.01$ for Akita vs. LepTg:Akita. In *G* and *H*, ##$P < 0.01$ for Akita vs. pair-fed Akita and **$P < 0.01$ for LepTg:Akita vs. pair-fed Akita.

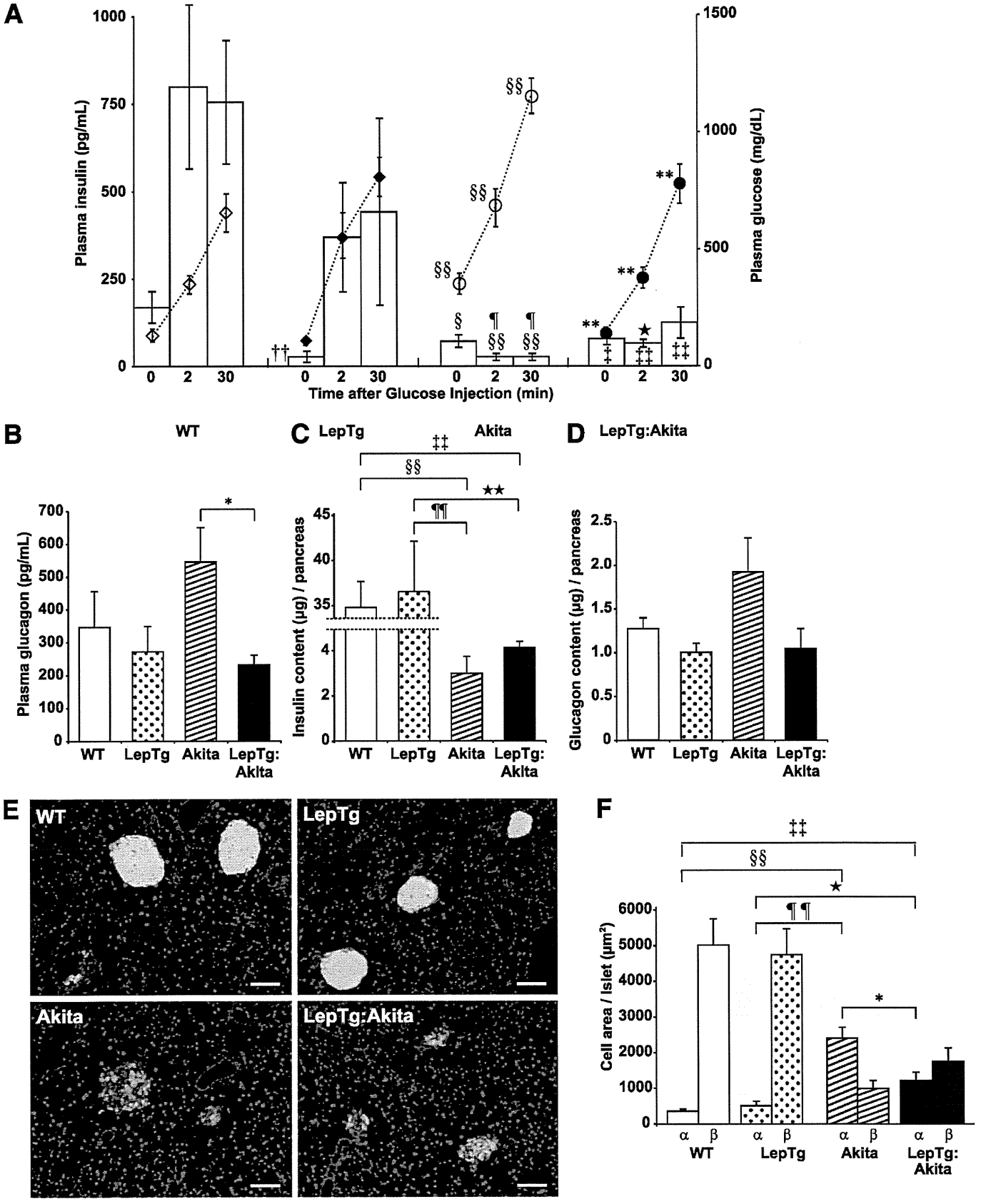

FIG. 3. Glucose-stimulated insulin secretion, plasma glucagon levels, and pancreatic hormone contents. *A*: Plasma insulin (open bars) and glucose (black lines) concentrations after glucose (3 g/kg i.p.) injection in WT, LepTg, Akita, and LepTg:Akita mice at 8 weeks of age ($n \geq 4$ in each group). *B*: Plasma glucagon concentration in ad libitum–fed WT, LepTg, Akita, and LepTg:Akita mice at 22 weeks of age ($n \geq 4$ in each group). *C* and *D*: Pancreatic insulin (*C*) and glucagon (*D*) content measured in acid-ethanol extracts of homogenized pancreas from WT, LepTg, Akita, and LepTg:Akita mice at 18 weeks of age ($n \geq 5$ in each group). *E*: Double immunofluorescent stainings against insulin (green) and glucagon (red) in pancreatic sections from WT, LepTg, Akita, and LepTg:Akita mice at the age of 18 weeks. Scale bar indicates 50 μm. *F*: α-Cell and β-cell areas per islet

Akita mice were elevated compared with WT mice; however, they remained >4% lower than those of Akita mice, even at 40 weeks of age (Fig. 1*D*). Average HbA_{1c} levels were 11.4 ± 0.9% in Akita, 7.5 ± 1.1% in LepTg:Akita, 3.9 ± 0.3% in LepTg, and 4.1 ± 0.9% in WT mice from 5 to 40 weeks of age.

Time course of body weight and food intake. The body weight of all groups of mice increased gradually from birth (Fig. 1*E*). LepTg:Akita mice weighed significantly less than did Akita mice from 5 to 20 weeks of age. Akita mice lost body weight after 20 weeks of age. Percent body fat at 18 weeks of age was 27.6 ± 1.9, 29.9 ± 0.5, 2.1 ± 0.5, and 1.0 ± 0.4% for WT, LepTg, Akita, and LepTg:Akita mice, respectively.

The food intake of Akita mice was nearly double that of the other three groups of mice during the observation period (Fig. 1*F*). Food intake did not differ significantly between the WT, LepTg, and LepTg:Akita mice at any time during the study.

Pair-feeding experiments. To investigate whether leptin's ability to decrease food intake is the main reason for its efficacy in improving diabetes, Akita mice were pair fed to achieve the ad libitum food intake of LepTg:Akita mice for from 3 weeks to 8 weeks of age. Although pair feeding reduced the body weight of Akita mice to that of LepTg:Akita mice (Fig. 1*G*), it did not significantly improve the blood glucose levels in the Akita mice (Fig. 1*H*).

Glucose tolerance and insulin sensitivity. GTTs were performed to evaluate glucose metabolism further (Fig. 2*A*). At 8 weeks of age, Akita mice had reduced glucose tolerance; however, LepTg:Akita mice exhibited normal glucose tolerance similar to that of the LepTg and WT mice of the same age. At 16 weeks of age, Akita mice developed more severe glucose intolerance than that at 8 weeks of age. Although glucose tolerance was impaired in 16-week-old LepTg:Akita mice compared with that of LepTg and WT mice, it was better than that of 8- and 16-week-old Akita mice. These results indicate that hyperleptinemia significantly improved glucose tolerance during the progressive course of diabetes in Akita mice.

ITTs were performed to determine whether the improved glucose tolerance observed in LepTg:Akita mice was associated with insulin sensitivity (Fig. 2*B*). At 8 weeks of age, both LepTg and LepTg:Akita mice showed similar, exaggerated hypoglycemic responses to insulin relative to WT and Akita mice (Fig. 2*C*). At 18 weeks of age, the effect of insulin was blunted in LepTg mice compared with that in LepTg:Akita mice and was comparable with those in WT and Akita mice. At 28 weeks of age, glucose responses to insulin in LepTg and WT mice were severely impaired compared with those in Akita and LepTg:Akita mice. Insulin sensitivities in LepTg and WT mice deteriorated in parallel with advancing age and increasing body weight. In contrast, insulin sensitivities in Akita mice did not deteriorate with age, and the enhanced sensitivity in LepTg:Akita mice did not change at all during the course of our study.

Secretion and production of insulin and glucagon. Insulin secretion in response to a maximal glucose challenge was assessed. Plasma insulin levels were measured after an injection of glucose (3 g/kg body wt i.p.) (Fig. 3*A*). The fasting insulin levels were similar in LepTg, Akita, and LepTg:Akita mice at 8 weeks of age, and all were significantly lower than in WT mice at the same age.

However, the fasting plasma insulin-to-glucose ratio was about three times higher in LepTg:Akita mice than in Akita mice. Both WT and LepTg mice showed an acute insulin response to glucose, Akita mice had virtually no response, and LepTg:Akita mice maintained a slow and slight response.

Plasma glucagon concentration was measured in ad libitum–fed mice at 22 weeks of age (Fig. 3*B*). Despite marked hyperglycemia, the glucagon level in Akita mice was nearly twice that in WT and LepTg mice. By contrast, LepTg:Akita mice had a normal plasma glucagon level, equivalent to that in the WT and LepTg mice.

Total pancreatic insulin contents of the Akita and LepTg:Akita mice decreased similarly to about one-tenth of those in the WT and LepTg mice (Fig. 3*C*). The pancreatic glucagon content of the Akita mice was twice that of the WT and LepTg mice; the glucagon content of the LepTg:Akita was half that in Akita mice (Fig. 3*D*).

Immunohistochemical examination of the pancreas revealed that Akita mice had profoundly abnormal islet histology with few active β-cells and a higher proportion of α-cells compared with WT and LepTg mice (Fig. 3*E* and *F*). LepTg:Akita mice had fewer β-cells, but α-cell hyperplasia was suppressed relative to Akita mice (Fig. 3*E* and *F*). These characteristics agree with the plasma hormone levels (Fig. 3*A* and *B*) and pancreatic hormone contents (Fig. 3*C* and *D*).

Lipids and ketones. Transgenic leptinemia did not significantly affect plasma levels of TG, NEFA, and β-hydroxybutyrate in WT mice (Fig. 4*A–C*). Akita mice had lower levels of both NEFA and β-hydroxybutyrate, possibly reflecting their lower adipose mass (Fig. 4*B* and *C*). None of the Akita mice developed ketonuria as determined by a urine ketone dipstick test (data not shown). In the Akita mice, leptin significantly decreased plasma TG levels by half (LepTg:Akita 37.4 ± 11.6 vs. Akita 89.4 ± 13.6 mg/dL; $P < 0.05$) (Fig. 4*A*) but did not change plasma NEFA or β-hydroxybutyrate levels (Fig. 4*B* and *C*).

Systemic oxidative stress. The plasma level of TBARS was examined as a marker of systemic oxidative stress (Fig. 4*D*). Akita mice exhibited the highest TBARS levels (35.1 ± 4.9 μmol/L) of the four genotypes at 18 weeks of age; the plasma TBARS levels were similar in WT, LepTg, and LepTg:Akita mice (WT 12.7 ± 1.3, LepTg 16.3 ± 1.8, and LepTg:Akita 17.8 ± 2.3 μmol/L).

Diabetic nephropathy. The renoprotective effects of leptin were investigated in Akita mice (Fig. 5*A*). Akita mice developed overt albuminuria at 12 weeks of age, and urinary albumin excretion was >200 μg/day during the follow-up period. By contrast, the increase in albuminuria was largely attenuated in LepTg:Akita mice throughout the follow-up period.

The increase in mesangial matrix (defined as mesangial area) observed in Akita mice was prevented completely in LepTg:Akita mice at 22 weeks of age (Fig. 5*B* and Table 1). Systolic and diastolic blood pressure and heart rates did not differ significantly between the four groups of mice (Table 1).

in WT, LepTg, Akita, and LepTg:Akita mice at 18 weeks of age ($n \geq 4$in each group). Data are expressed as means ± SE. ††$P < 0.01$ for WT vs. LepTg, §§$P < 0.01$ for WT vs. Akita, ‡$P < 0.05$, ‡‡$P < 0.01$ for WT vs. LepTg:Akita, ¶$P < 0.05$, ¶¶$P < 0.01$ for LepTg vs. Akita, ★$P < 0.05$, ★★$P < 0.01$ for LepTg vs. LepTg:Akita, *$P < 0.05$, and **$P < 0.01$ for Akita vs. LepTg:Akita. (A high-quality digital representation of this figure is available in the online issue.)

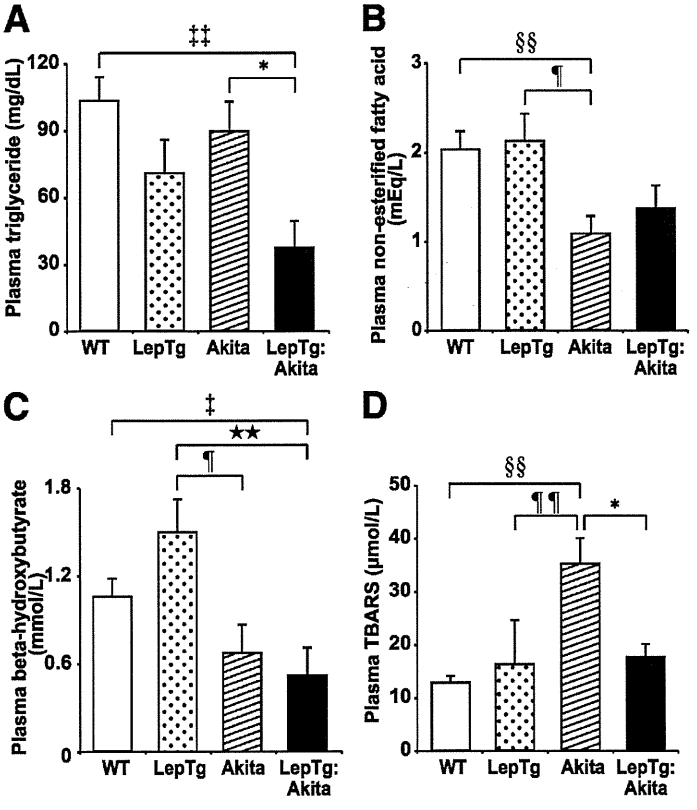

FIG. 4. Plasma levels of TG, NEFA, β-hydroxybutyrate, and TBARS. *A* and *B*: Fasting plasma levels of TG (*A*) and NEFA (*B*) concentrations in WT, LepTg, Akita, and LepTg:Akita mice at 18 weeks of age ($n \geq 4$ in each group). *C*: Fasting plasma levels of β-hydroxybutyrate concentrations in WT, LepTg, Akita, and LepTg:Akita mice at 11 weeks of age ($n \geq 4$ in each group). *D*: Fasting plasma levels of TBARS concentrations in WT, LepTg, Akita, and LepTg:Akita mice at 18 weeks of age ($n \geq 4$ in each group). Data are expressed as means ± SE. §§$P < 0.01$ for WT vs. Akita, ‡$P < 0.05$, ‡‡$P < 0.01$ for WT vs. LepTg:Akita, ¶$P < 0.05$, ¶¶$P < 0.01$ for LepTg vs. Akita, ★★$P < 0.01$ for LepTg vs. LepTg:Akita, and *$P < 0.05$ for Akita vs. LepTg:Akita.

Survival rate. As shown in Fig. 6, the first Akita mouse died at 27 weeks of age, and half of the mice died within 40 weeks. Before death, Akita mice exhibited rough coats and decreased activity. Blood analysis showed liver and kidney dysfunction and extreme hyperglycemia ~1,000 mg/dL without overt ketone body production.

By contrast, the life span was significantly longer in LepTg:Akita mice than in Akita mice ($n \geq 14$, $P < 0.01$), and none of the LepTg:Akita mice died during the observation period of 1 year (Fig. 6). Considering that the median survival time of male C57BL/6 mice is ~120 weeks (The Jackson Laboratory, Bar Harbor, ME), the survival rate did not appear to be affected significantly in LepTg:Akita mice.

DISCUSSION

The current study demonstrates that transgenic expression of leptin raised its plasma concentration to the level observed in morbidly obese individuals, markedly reduced mortality, and prolonged the survival time in Akita mice. The extension of life span in LepTg:Akita mice was accompanied by various beneficial effects on the course of diabetes.

We have pursued the therapeutic potential of leptin as an antidiabetic agent using transgenic skinny mice and propose that leptin could be used therapeutically in the treatment of diabetes of different etiology and pathophysiology (2,5–8,11–13). Leptin effectively improves glucose and lipid metabolism in streptozotocin (STZ)-induced insulinopenic diabetic mice (7) and in mildly obese mice fed a high-fat diet and administered low-dose STZ (6). Transgenic overexpression of leptin can delay the onset of insulin resistance and diabetes in KKAy mice at younger ages, when they are of normal weight (8). These findings suggest that leptin alone is effective in treating diabetes without obesity-induced leptin resistance. We also found that transgenic overexpression of leptin can rescue insulin resistance and diabetes in a mouse model of lipoatrophic diabetes, showing that leptin should be effective in the treatment of lipoatrophic diabetes (5). Therapeutic leptinemia can be achieved clinically by subcutaneous injection of recombinant human leptin (11,12). Leptin has been used in the treatment of human diabetes in patients with leptin deficiency and lipodystrophy (11–14).

Leptin delayed the onset and progression of diabetes in Akita mice. Hyperglycemia after a 16-h fast was prevented completely for >10 months in LepTg:Akita mice. The onset of the increase in the 6-h fasting blood glucose and HbA_{1c} levels was also delayed for at least several weeks. Good metabolic control was achieved after the onset of diabetes in LepTg:Akita mice. There are several possible explanations for this glucose-lowering effect of leptin. This study demonstrated that the constitutive hyperleptinemia (approximately 10 times higher than control) strongly and stably increased insulin sensitivity in Akita mice. Leptin increases the effects of insulin in suppressing hepatic glucose production and stimulating muscular glucose uptake (2). The mechanisms through which leptin regulates insulin signaling are not understood completely, although reduction in ectopic fat deposition, especially in the liver and muscle, and activation of AMP-activated protein kinase in the peripheral tissues via stimulation of the hypothalamic-autonomic nervous system are important components (6,15,16). Increased mitochondrial biogenesis and oxygen consumption in skeletal muscle and adipose tissue may also contribute to the increase in glucose disposal independent of insulin action (15,17,18). Previous studies also demonstrated the antidiabetic effects of leptin in insulin-deficient diabetic rodents (19,20).

This study showed no attenuation of the biological effects of leptin (increase in insulin sensitivity and decrease in food intake) in Akita mice throughout the long follow-up. Most of the obese patients have elevated plasma leptin levels (21,22), implying leptin resistance for weight control (23). The basis for such leptin resistance is not understood enough, although such resistance coexists with hyperinsulinemia and insulin resistance (24). This context suggests that leptin has potent therapeutic effects on insulin-deficient diabetes with minimum insulin intervention (7). We and others reported that exogenously administered leptin can normalize hyperglycemia in STZ-induced diabetes, when fed plasma insulin levels were >0.10 ng/mL (7,25). The glycemic control of LepTg:Akita mice worsened gradually when the plasma insulin levels became extremely low (<0.10 ng/mL). These results show that the threshold of plasma insulin level is ~0.10 ng/mL, above which leptin can prevent hyperglycemia.

Akita mice, which have low plasma insulin and leptin levels, have increased food intake. Transgenic hyperleptinemia prevented hyperphagia in Akita mice. Although short-term food restriction did not affect hyperglycemia in Akita mice, continuous reduction in food intake might

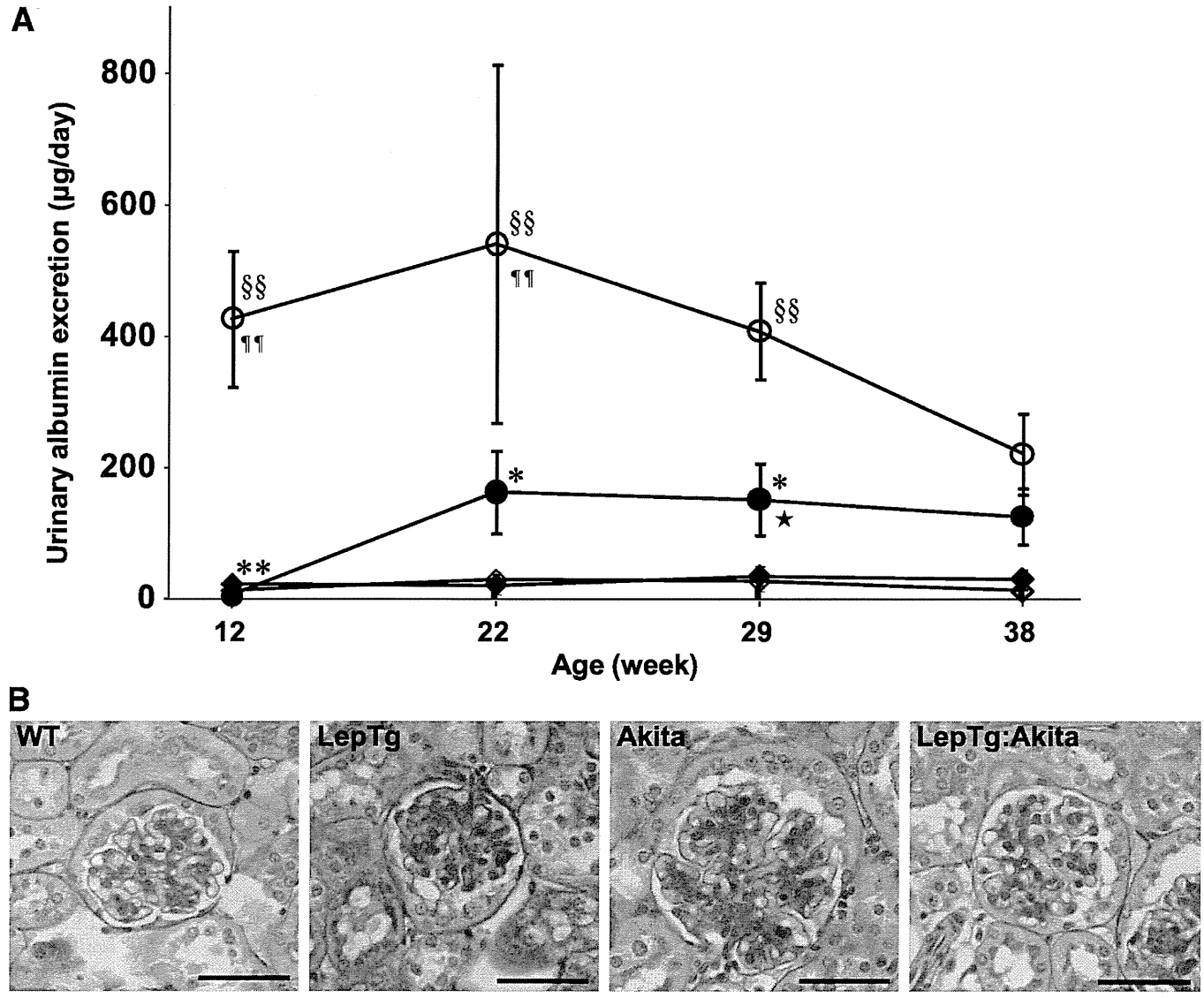

FIG. 5. Urinary albumin excretion and histology of glomeruli. *A*: Time course of urinary albumin excretion of WT (◇), LepTg (◆), Akita (○), and LepTg:Akita (●). Data are expressed as means ± SE ($n \geq 4$ in each group). §§$P < 0.01$ for WT vs. Akita, ¶¶$P < 0.01$ for LepTg vs. Akita, ★$P < 0.01$ for LepTg vs. LepTg:Akita, *$P < 0.05$, and **$P < 0.01$ for Akita vs. LepTg:Akita. *B*: PAS-staining of representative glomeruli from WT, LepTg, Akita, and LepTg:Akita mice at 22 weeks of age. Scale bar indicates 50 μm. (A high-quality color representation of this figure is available in the online issue.)

play a role in the antidiabetic effect of leptin. In insulin-deficient diabetic animals, adiposity and plasma leptin levels decrease and food intake increases concomitantly (26). In our previous report, leptin administration reversed hyperphagia by correcting the imbalance in the hypothalamic neuropeptide expression in STZ-administered mice (7). Upregulation of orexigenic neuropeptides (neuropeptide Y and agouti-related peptide) and downregulation of anorexigenic neuropeptides (proopiomelanocortin) were observed in the hypothalamus of insulin-deficient diabetic mice (7,27). Leptin and insulin are crucial signals that convey "adiposity negative feedback" information to the hypothalamus. Our previous and present results indicate that leptin is useful for preventing diabetic hyperphagia.

Glucose-stimulated insulin secretion and pancreatic insulin content were markedly lower in both LepTg:Akita and Akita mice compared with WT and LepTg mice; however, both the plasma concentration and pancreatic content of glucagon decreased to the normal level in LepTg:Akita mice. Leptin is reported to suppress the synthesis and secretion of insulin by pancreatic β-cells (28,29). Our results did not reveal any negative effect of leptin on β-cell function in Akita mouse but showed that systemic hyperleptinemia plays a role in restoring the insulin-glucagon balance to proper equilibrium, which is lost in Akita mice. Wang et al. (30) reported recently that like insulin, leptin suppresses glucagon secretion in NOD mice. Our current data suggest that the antiglucagon and insulin-sensitizing effects of leptin on glucoregulatory hormones are therapeutically useful actions.

We found that chronic overexpression of leptin effectively prevented the development of diabetic nephropathy in Akita mice, as we have also demonstrated in lipoatrophic diabetic A-ZIP/F1 mice (31). Leptin suppressed the induction of massive albuminuria and the expansion of mesangial matrix in the glomeruli of Akita mice. Increased urinary albumin excretion and mesangial matrix accumulation are well-established features of diabetic nephropathy. Akita mice manifest the typical renal injury observed in human diabetic nephropathy that is associated

TABLE 1
Renal characteristics of 22-week-old F1 mice

	WT	LepTg	Akita	LepTg:Akita
Albuminuria (μg/day)	32.0 ± 9.4	23.5 ± 13.1	544.6 ± 272.7	165.9 ± 62.1*
Urine volume (mL/day)	1.7 ± 0.4	0.9 ± 0.1	27.4 ± 4.6	7.3 ± 2.2**
Mesangial area (μm^2)	1,210 ± 50	1,278 ± 50	2,025 ± 76	1,222 ± 67**
Body weight (g)	37.1 ± 1.7	38.4 ± 1.3	26.2 ± 0.9	25.0 ± 0.5
Kidney weight (g)	0.21 ± 0.03	0.18 ± 0.02	0.26 ± 0.04	0.21 ± 0.01
sBP (mmHg)	96.8 ± 0.3	93.5 ± 1.9	98.5 ± 4.1	107.7 ± 1.3
dBP (mmHg)	44.0 ± 0.5	42.3 ± 3.8	47.6 ± 3.9	52.2 ± 4.7
Heart rate (bpm)	714 ± 18	726 ± 7	611 ± 32	730 ± 30

Values are expressed as the mean ± SE (n = 4). sBP, systolic blood pressure; dBP, diastolic blood pressure. *$P < 0.05$. **$P < 0.01$, LepTg:Akita vs. Akita.

with renal hypertrophy, glomerular hypertrophy, mesangial expansion, and overt proteinuria (32). The nephropathy in Akita mice is more similar to that seen in human patients with diabetes than is the nephropathy in chemically induced diabetic mice (32). Although several reports suggest that leptin exerts profibrotic action in the kidney, which has caused concern about possible pathogenic roles of leptin in obesity-related glomerulopathy (31,33), the present results clearly show that leptin prevented renal injury in Akita mice. It is likely that leptin is beneficial for nephropathy in patients with insulin-dependent diabetes.

Reduced insulin action in diabetes elevates plasma TG levels by decreasing lipoprotein lipase activity and increasing hormone-sensitive lipase activity. We demonstrated previously a significant reduction in plasma VLDL-TG level in LepTg mice (34). Leptin suppresses the activities of liver lipogenic enzymes (35,36). In LepTg:Akita mice, decreased levels of plasma lipids may also result from dwindling body fat stores (orthotopic and ectopic) because of augmented effects of leptin. Since hypertriglyceridemia is reported to be an independent cardiovascular risk factor in patients with glucose intolerance (37), our observation of the TG-lowering effects of leptin may be useful in preventing and treating diabetic cardiovascular complications.

Lipid peroxidation is a well-established mechanism of cellular injury as a diabetes complication and is used as an indicator of oxidative stress. Increased oxidative stress also participates in the development and progression of diabetes and its complications (38). LepTg:Akita mice maintained normal levels of plasma TBARS, in contrast to Akita mice. Increased levels of serum TBARS were reported in patients with peripheral arterial disease, ischemic heart disease, hypertension, and diabetes (39). Our finding that leptin relieved systemic oxidative stress in Akita mice is of interest because TBARS level does not depend only on the blood glucose or lipid level but reflects the complex net redox balance (40,41).

Various interventions have been reported to improve the metabolic profiles in Akita mice (42,43). Targeted disruption of the transcription factor C/EBP homologous protein gene or C/EBP-β gene alleviates endoplasmic reticulum stress in pancreatic β-cells and improves hyperglycemia in Akita mice (42). Intracerebroventricular administration of adeno-associated viral vector expressing leptin also attenuates hyperglycemia in Akita mice (43). However, it is unclear whether those interventions are directly applicable to the human therapeutic settings. Therapeutic leptinemia in LepTg mice is induced by transgenic overexpression. Leptinemia can be achieved clinically by subcutaneous injection of leptin, as shown in leptin-replacement therapy (11,12,44). Whether leptin affects the immunological processes of type 1 diabetes remains to be established. Leptin, a cytokine-like hormone, is suggested to be involved in linking nutritional status and immune response (45). Leptin administration accelerates autoimmune diabetes in NOD mice, and the incidence of diabetes is significantly reduced in NOD mice and BB/Wor rats with Ob-R mutation (46–48). However, another study that assessed NOD mice with defective leptin signaling (*Ay*, *db/db*, and *ob/ob*) has shown that leptin is not essential for the development of autoimmune diabetes (49). Whether the beneficial effects of leptin in Akita mice can be translated to type 1 diabetes in humans will be important to determine.

In conclusion, the current study demonstrates that leptin has a therapeutic impact on the onset and progression of glucose intolerance, diabetes complications, and longevity in a mouse model of insulin-deficient nonobese diabetes. These data offer proof of concept that leptin may be useful as a long-term therapeutic agent for treating human diabetes.

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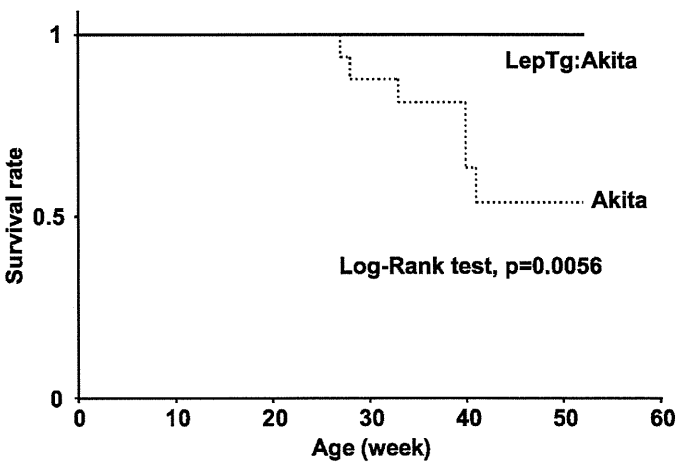

FIG. 6. Survival rates. Survival curves of Akita (dotted line) and LepTg:Akita (solid line) mice (n ≥11 in each group). Survival rate of Akita mice markedly decreased relative to LepTg:Akita mice ($P < 0.01$) and was ~50% at 52 weeks of age.

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M.N. researched data. J.F. researched data and wrote the manuscript. K.E. contributed to discussion. F.M. researched data. H.Y. researched data and contributed to discussion. T.K., Y.Y., C.S., M.M., K.H., and K.N. contributed to discussion.

REFERENCES

1. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. Nature 1998;395:763–770
2. Ogawa Y, Masuzaki H, Hosoda K, et al. Increased glucose metabolism and insulin sensitivity in transgenic skinny mice overexpressing leptin. Diabetes 1999;48:1822–1829
3. Frederich RC, Hamann A, Anderson S, Löllmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med 1995;1:1311–1314
4. Considine RV, Sinha MK, Heiman ML, et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 1996; 334:292–295
5. Ebihara K, Ogawa Y, Masuzaki H, et al. Transgenic overexpression of leptin rescues insulin resistance and diabetes in a mouse model of lipoatrophic diabetes. Diabetes 2001;50:1440–1448
6. Kusakabe T, Tanioka H, Ebihara K, et al. Beneficial effects of leptin on glycaemic and lipid control in a mouse model of type 2 diabetes with increased adiposity induced by streptozotocin and a high-fat diet. Diabetologia 2009;52:675–683
7. Miyanaga F, Ogawa Y, Ebihara K, et al. Leptin as an adjunct of insulin therapy in insulin-deficient diabetes. Diabetologia 2003;46:1329–1337
8. Masuzaki H, Ogawa Y, Aizawa-Abe M, et al. Glucose metabolism and insulin sensitivity in transgenic mice overexpressing leptin with lethal yellow agouti mutation: usefulness of leptin for the treatment of obesity-associated diabetes. Diabetes 1999;48:1615–1622
9. Yoshioka M, Kayo T, Ikeda T, Koizumi A. A novel locus, Mody4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. Diabetes 1997;46:887–894
10. Han BG, Hao CM, Tchekneva EE, et al. Markers of glycemic control in the mouse: comparisons of 6-h- and overnight-fasted blood glucoses to Hb A1c. Am J Physiol Endocrinol Metab 2008;295:E981–E986
11. Ebihara K, Masuzaki H, Nakao K. Long-term leptin-replacement therapy for lipoatrophic diabetes. N Engl J Med 2004;351:615–616
12. Ebihara K, Kusakabe T, Hirata M, et al. Efficacy and safety of leptin-replacement therapy and possible mechanisms of leptin actions in patients with generalized lipodystrophy. J Clin Endocrinol Metab 2007;92:532–541
13. Nakao K, Yasoda A, Ebihara K, Hosoda K, Mukoyama M. Translational research of novel hormones: lessons from animal models and rare human diseases for common human diseases. J Mol Med 2009;87:1029–1039
14. Dardeno TA, Chou SH, Moon HS, Chamberland JP, Fiorenza CG, Mantzoros CS. Leptin in human physiology and therapeutics. Front Neuroendocrinol 2010;31:377–393
15. Tanaka T, Hidaka S, Masuzaki H, et al. Skeletal muscle AMP-activated protein kinase phosphorylation parallels metabolic phenotype in leptin transgenic mice under dietary modification. Diabetes 2005;54:2365–2374
16. Long YC, Zierath JR. AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest 2006;116:1776–1783
17. Kakuma T, Wang ZW, Pan W, Unger RH, Zhou YT. Role of leptin in peroxisome proliferator-activated receptor gamma coactivator-1 expression. Endocrinology 2000;141:4576–4582
18. Li L, Pan R, Li R, et al. Mitochondrial biogenesis and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) deacetylation by physical activity: intact adipocytokine signaling is required. Diabetes 2011; 60:157–167
19. Yu X, Park BH, Wang MY, Wang ZV, Unger RH. Making insulin-deficient type 1 diabetic rodents thrive without insulin. Proc Natl Acad Sci USA 2008;105:14070–14075
20. Fujikawa T, Chuang JC, Sakata I, Ramadori G, Coppari R. Leptin therapy improves insulin-deficient type 1 diabetes by CNS-dependent mechanisms in mice. Proc Natl Acad Sci USA 2010;107:17391–17396
21. Hosoda K, Masuzaki H, Ogawa Y, et al. Development of radioimmunoassay for human leptin. Biochem Biophys Res Commun 1996;221:234–239
22. Maffei M, Halaas J, Ravussin E, et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1995;1:1155–1161
23. Enriori PJ, Evans AE, Sinnayah P, et al. Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons. Cell Metab 2007;5:181–194
24. Knight ZA, Hannan KS, Greenberg ML, Friedman JM. Hyperleptinemia is required for the development of leptin resistance. PLoS ONE 2010;5:e11376
25. Chinookoswong N, Wang JL, Shi ZQ. Leptin restores euglycemia and normalizes glucose turnover in insulin-deficient diabetes in the rat. Diabetes 1999;48:1487–1492
26. Sivitz WI, Walsh S, Morgan D, Donohoue P, Haynes W, Leibel RL. Plasma leptin in diabetic and insulin-treated diabetic and normal rats. Metabolism 1998;47:584–591
27. Asakawa A, Toyoshima M, Inoue K, Koizumi A. Ins2Akita mice exhibit hyperphagia and anxiety behavior via the melanocortin system. Int J Mol Med 2007;19:649–652
28. Seufert J. Leptin effects on pancreatic beta-cell gene expression and function. Diabetes 2004;53(Suppl. 1):S152–S158
29. Kulkarni RN, Wang ZL, Wang RM, et al. Leptin rapidly suppresses insulin release from insulinoma cells, rat and human islets and, in vivo, in mice. J Clin Invest 1997;100:2729–2736
30. Wang MY, Chen L, Clark GO, et al. Leptin therapy in insulin-deficient type I diabetes. Proc Natl Acad Sci USA 2010;107:4813–4819
31. Suganami T, Mukoyama M, Mori K, et al. Prevention and reversal of renal injury by leptin in a new mouse model of diabetic nephropathy. FASEB J 2005;19:127–129
32. Gurley SB, Clare SE, Snow KP, Hu A, Meyer TW, Coffman TM. Impact of genetic background on nephropathy in diabetic mice. Am J Physiol Renal Physiol 2006;290:F214–F222
33. Wolf G, Hamann A, Han DC, et al. Leptin stimulates proliferation and TGF-beta expression in renal glomerular endothelial cells: potential role in glomerulosclerosis [see comments]. Kidney Int 1999;56:860–872
34. Matsuoka N, Ogawa Y, Masuzaki H, et al. Decreased triglyceride-rich lipoproteins in transgenic skinny mice overexpressing leptin. Am J Physiol Endocrinol Metab 2001;280:E334–E339
35. Gallardo N, Bonzón-Kulichenko E, Fernández-Agulló T, et al. Tissue-specific effects of central leptin on the expression of genes involved in lipid metabolism in liver and white adipose tissue. Endocrinology 2007; 148:5604–5610
36. Cohen P, Miyazaki M, Socci ND, et al. Role for stearoyl-CoA desaturase-1 in leptin-mediated weight loss. Science 2002;297:240–243
37. Keech A, Simes RJ, Barter P, et al.; FIELD study investigators. Effects of long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2 diabetes mellitus (the FIELD study): randomised controlled trial. Lancet 2005;366:1849–1861
38. Maritim AC, Sanders RA, Watkins JB 3rd. Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol 2003;17:24–38
39. Oberley LW. Free radicals and diabetes. Free Radic Biol Med 1988;5: 113–124
40. Altomare E, Vendemiale G, Chicco D, Procacci V, Cirelli F. Increased lipid peroxidation in type 2 poorly controlled diabetic patients. Diabete Metab 1992;18:264–271
41. Fujiwara Y, Kondo T, Murakami K, Kawakami Y. Decrease of the inhibition of lipid peroxidation by glutathione-dependent system in erythrocytes of non-insulin dependent diabetics. Klin Wochenschr 1989;67:336–341
42. Oyadomari S, Koizumi A, Takeda K, et al. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. J Clin Invest 2002;109:525–532
43. Ueno N, Inui A, Kalra PS, Kalra SP. Leptin transgene expression in the hypothalamus enforces euglycemia in diabetic, insulin-deficient nonobese Akita mice and leptin-deficient obese ob/ob mice. Peptides 2006;27:2332–2342
44. Ozata M, Ozdemir IC, Licinio J. Human leptin deficiency caused by a missense mutation: multiple endocrine defects, decreased sympathetic tone, and immune system dysfunction indicate new targets for leptin action, greater central than peripheral resistance to the effects of leptin, and spontaneous correction of leptin-mediated defects. J Clin Endocrinol Metab 1999;84:3686–3695
45. Matarese G, Leiter EH, La Cava A. Leptin in autoimmunity: many questions, some answers. Tissue Antigens 2007;70:87–95
46. Matarese G, Sanna V, Lechler RI, et al. Leptin accelerates autoimmune diabetes in female NOD mice. Diabetes 2002;51:1356–1361
47. Lee CH, Reifsnyder PC, Naggert JK, et al. Novel leptin receptor mutation in NOD/LtJ mice suppresses type 1 diabetes progression: I. Pathophysiological analysis. Diabetes 2005;54:2525–2532
48. Guberski DL, Butler L, Manzi SM, Stubbs M, Like AA. The BBZ/Wor rat: clinical characteristics of the diabetic syndrome. Diabetologia 1993;36:912–919
49. Nishimura M, Miyamoto H. Immunopathological influence of the Ay, db, ob and nu genes placed on the inbred NOD background as murine models for human type I diabetes. J Immunogenet 1987;14:127–130

original article

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ORIGINAL ARTICLE

Azilsartan treatment improves insulin sensitivity in obese spontaneously hypertensive Koletsky rats

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Aim: Hypertension often coexists with insulin resistance. However, most metabolic effects of the antihypertensive agents have been investigated in nomotensive animals, in which different conclusions may arise. We investigated the metabolic effects of the new angiotensin II type 1 receptor blocker azilsartan using the obese Koletsky rats superimposed on the background of the spontaneously hypertensive rats.

Methods: Male Koletsky rats were treated with azilsartan (2 mg/kg/day) over 3 weeks. Blood pressure was measured by tail-cuff. Blood biochemical and hormonal parameters were determined by enzymatic or ELISA methods. Gene expression was assessed by RT-PCR.

Results: In Koletsky rats, azilsartan treatment lowered blood pressure, basal plasma insulin concentration and the homeostasis model assessment of insulin resistance index, and inhibited over-increase of plasma glucose and insulin concentrations during oral glucose tolerance test. These effects were accompanied by decreases in both food intake and body weight (BW) increase. Although two treatments showed the same effect on BW gain, insulin sensitivity was higher after azilsartan treatment than pair-feeding. Azilsartan neither affected plasma concentrations of triglyceride and free fatty acids, nor increased adipose mRNA levels of peroxisome proliferator-activated receptor (PPAR)γ and its target genes such as adiponectin, aP2. In addition, azilsartan downregulated 11β-hydroxysteroid dehydrogenase type 1 expression.

Conclusions: These results show the insulin-sensitizing effect of azilsartan in obese Koletsky rats. This effect is independent of decreases in food intake and BW increase or of the activation of adipose PPARγ. Our findings indicate the possible usefulness of azilsartan in the treatment of metabolic syndrome.

Keywords: angiotensin II type 1 receptor, azilsartan, insulin, obesity, peroxisome proliferator-activated receptor

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Introduction

Hypertension is one component of the metabolic syndrome. Hypertension often coexists with insulin resistance, glucose intolerance and hyperlipidaemia. It has been well documented that activation of the renin–angiotensin system is a common feature in patients with the metabolic syndrome [1]. Blockade of the renin–angiotensin system/angiotensin II type 1 receptor (AT1) signalling has been shown in clinical and experimental studies to improve the metabolic syndrome [1]. Some AT1 blockers (angiotensin II type 1 receptor blockers ARBs) have been shown to improve insulin sensitivity in rodents and humans [2–6]. However, most studies of the antihypertensive agents have been performed in nomotensive animals, in which different conclusions from those in hypertensive objectives may arise.

Azilsartan (TAK-536) is a new ARB. A randomized, double-blind, placebo-controlled trial shows that, in patients with stages 1 and 2 hypertension, azilsartan at its maximal dose has superior efficacy to both olmesartan and valsartan at their maximal, approved doses without increasing adverse events, providing higher rates of hypertension control within the ARB class [7]. Radioligand binding and functional studies *in vitro* have shown that azilsartan inhibits human AT1 more potently than olmesartan, telmisartan, valsartan and irbesartan [8]. In nomotensive KK-A^y mice azilsartan has been found to be more effective than candesartan; it reduced plasma concentrations of glucose and fatty acids, decreased adipose tissue weight and adipocyte size and increased adipose expression of peroxisome proliferator-activated receptor (PPAR)γ and its target genes adiponectin and aP2; but did not affect blood pressure and plasma insulin concentrations [9]. The metabolic effects of the antihypertensive agent need to be further investigated in hypertensive objectives.

The Koletsky (fa^k/fa^k) rat carries a nonsense mutation in the leptin receptor and exhibits hyperphagia, obesity, hyperinsulinaemia/insulin resistance and hyperlipidaemia which are superimposed on the spontaneously hypertensive rat (SHR) background [6,10]. In the present study, we investigated the effects of azilsartan on metabolic abnormalities in the obese Koletsky rats.

Methods

Animals, Diet and Experimental Protocol

All animal procedures were in accordance with the 'Principles of laboratory animal care' (http://grants1.nih.gov/grants/olaw/

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references/phspol.htm) and were approved by the Animal Ethics Committee, Kyoto University, Japan.

Male Wistar-Kyoto (WKY) rats, obese Koletsky (fa^k/fa^k) rats and their lean (+/+) littermates (SHR) were generous gifts from Japan SLC, Inc., Shizuoka, Japan. Rats were housed in a temperature controlled facility (21 ± 1°C, 55 ± 5% relative humidity) with a 12-h light/dark cycle (two to three rats per cage). Animals were allowed free access to water and the standard diet (CLEA, Tokyo, Japan) for at least 1 week prior to starting the experiments at 10 weeks of age.

Experiment 1: Optimization of Azilsartan Dosage in Obese Koletsky Rats. Age-matched WKY rats and SHR were used as controls (n = 6 per group). As many as 24 obese Koletsky rats were divided into 4 groups (n = 6 per group): Koletsky azilsartan 0 mg/kg (control), Koletsky azilsartan 1 mg/kg, Koletsky azilsartan 2 mg/kg and Koletsky azilsartan 3 mg/kg. There were no differences in body weight (BW) among the Koletsky groups before treatments commenced. Animals in azilsartan-treated groups were administered azilsartan (1, 2 and 3 mg/kg, a generous gift from Takeda Co., Ltd., Japan, suspended in 5% Gum Arabic) by oral gavage once daily (9:00–10:00 hours) for 5 days. The WKY rats, SHR and Koletsky rats in control group received vehicle (5% Gum Arabic) alone. Rats were weighed once every 3–4 days to determine gavage volumes. To minimize the influence of measurement manipulation-induced stress on metabolic effects determined 2–3 weeks after treatments commenced in experiments 2 and 3, systolic blood pressure (SBP) was measured on day 5.

Experiment 2: Study of Metabolic Effects in Obese Koletsky Rats. In this experiment, 16 obese Koletsky rats were divided into two groups (n = 8 per group): Koletsky control (azilsartan 0 mg/kg) and Koletsky azilsartan (azilsartan 2 mg/kg). Eight lean (+/+) littermates (SHR) were used as controls. BWs were comparable between the Koletsky groups before treatments commenced. Animals in azilsartan group were administered azilsartan 2 mg/kg by oral gavage once daily (9:00–10:00 hours) for 21 days. The SHR and Koletsky control rats received vehicle (5% Gum Arabic) alone. Rats had free access to standard laboratory chow. Food intake was monitored daily and rats were weighed once every 3–4 days to determine gavage volume. SBP was measured on day 5 and blood samples were collected by retroorbital venous puncture under ether anaesthesia after rats had been fasted for 6 h on day 14, for determination of fasted plasma glucose, insulin, corticosterone, triglyceride and non-esterified fatty acids (NEFA) concentrations. Insulin sensitivity was expressed as the homeostasis model assessment of insulin resistance (HOMA-IR) index {[fasted insulin (μIU/ml) × fasted glucose (mM)]/22.5} [6,11,12]. Oral glucose tolerance tests (OGTT) were performed on day 17. Animals were weighed on day 21 and then killed by prompt dislocation of the neck vertebra. Epididymal white adipose tissues (eWAT) were collected and weighed. Segments of eWAT were snap frozen in liquid nitrogen and stored at −80°C for subsequent determination of gene analysis.

Experiment 3: Comparison of the Metabolic Effects of Azilsartan and Pair-feeding Treatments in Obese Koletsky Rats. In this experiment, 10 obese Koletsky rats were divided into two groups (n = 5 per group): Koletsky azilsartan (azilsartan 2 mg/kg) and Koletsky pair-feeding (azilsartan 0 mg/kg). BWs were insignificantly different between the groups before treatments commenced. Rats in azilsartan group had free access to standard laboratory chow. The food consumption in the pair-feeding group was restricted to that in azilsartan-treated animals (chow was provided daily at 10:00 hours). The other procedures were the same as that described in experiment 2.

SBP Measurement

SBP was measured 2–6 h after administration of azilsartan or vehicle in conscious rats by a tail-cuff method (MK-2000ST; Muromachi Kikai Co Ltd, Tokyo, Japan). At least six readings were taken for each measurement.

OGTT

To reduce the difference in plasma insulin concentrations between groups, rats were fasted for 15 h with free access to water before OGTT. Rats received a glucose solution (4 g/kg in 10 ml) by the oral route. Blood samples were collected prior to and 20, 60 and 120 min after administration of glucose solution for determination of plasma glucose and insulin concentrations.

Blood Biochemical Determination

Plasma concentrations of glucose, triglyceride and NEFA were determined using commercial enzymatic methods (kits from Wako, Osaka, Japan). Plasma insulin (kit from Morinaga, Tokyo, Japan) and corticosterone (kit from Cayman Chemical, Ann Arbor, MI, USA) concentrations were determined by ELISA.

Histological Examination

A portion of eWAT was fixed with 10% formalin and embedded in paraffin. Ten micron sections were cut and stained with haematoxylin and eosin for examination of adipose tissue histology (IX-81, Olympus Corporation, Tokyo, Japan). The adipocyte cross-sectional area was measured using an ImageJ 1.43 analysing system.

Gene Expression Analysis

Total RNA was isolated from eWAT of individual rats using TRIzol (Invitrogen, Osaka, Japan). Single-stranded cDNA was synthesized from 1 μg of total RNA using SuperScript III First-Strand Synthesis System for RT-PCR, according to the manufacturer's instructions (Invitrogen, Osaka, Japan). Quantitative RT-PCR was performed with an AB 7300 RT-PCR System using TaqMan (Applied Biosystems, Foster City, CA, USA). The sequences of primers and probes (Sigma-Genosys, Japan) used in the present study were as follows: PPARγ: sense, TGACCAGGGAGTTCCTCAAAA; antisense, AGCAAACTCAAACTTAGGCTCCAT; probe, CC

TGCGGAAGCCCTTTGGTGACT; adiponectin: sense, GGAC CAAGAACACCTGCGTCT; antisense, TCCTGGTCACAATG GGATACC; probe, TTCTCTCCAGGAGTGCCATCTCTGCC; aP2: sense, TCCAGTGAGAACTTCGATGATTACA; antisense, GGCCATACCGGCCACTTT; probe, TGGGAGTTGGCTTCG CCACCAG; 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1): sense, 5′-GCTGAAACAGAGCAATGGCAG-3′; antisense, 5′-GAACCCATCCAGAGCAAACTTG-3′; probe, TGG CTGGGAAAATGACCCAACCTCTGA; 18s: sense, GCAATT ATTCCCCATGAACGA; antisense, CAAAGGGCAGGGACT TAATCAAC; probe, AATTCCCAGTAAGTGCGGGTCATAA GCTTG. Rat mitochondrial subunit 18s rRNA was selected as the endogenous control (housekeeping gene).

Data Analysis

All results are expressed as means ± s.e.m. Data obtained from experiments 1 and 2 were analysed by one-way analysis of variance (ANOVA). If a difference was detected (F-ratio), the Student–Newman–Keuls test was performed to locate the differences between groups. Data obtained from experiment 3 were analysed using the Student's t-test. $p < 0.05$ was considered to be statistically significant.

Results

Optimization of Azilsartan Dosage in Obese Koletsky Rats

SHR and obese Koletsky rats appeared hypertensive compared to normal WKY controls; but there was no significant difference between SHR and obese Koletsky rats (figure 1). Azilsartan was administered by oral gavage (0–3 mg/kg once daily for 5 days); controls received vehicle alone. From the dose–response relationship, azilsartan (2 mg/kg) decreased SBP in obese Koletsky rats to that of normal rats, whereas the 3 mg/kg dose elicited hypotension (figure 1). From these findings azilsartan 2 mg/kg was selected for subsequent experiments.

Effects of Azilsartan Treatment on Glucose Metabolism in Obese Koletsky Rats

In this experiment, azilsartan 2 mg/kg showed similar blood pressure-lowering effect to that of above experiment (data not shown). In fasted obese animals plasma glucose concentrations were unchanged (figure 2A); however, plasma insulin concentrations (figure 2B) and the HOMA-IR index (figure 2C) were over fivefold higher than those in SHR. Azilsartan treatment did not affect basal plasma glucose concentrations, but decreased plasma insulin concentrations. The decline in insulin concentration also contributed to the observed decrease in the HOMA-IR index. Moreover, in the OGTT assessments, azilsartan modulated the abnormal increase in plasma glucose and insulin concentrations (figure 2D, E).

Effects of Azilsartan Treatment on Adiposity-associated Variables in Obese Koletsky Rats

The hyperphagia observed in obese Koletsky rats (an increase in food intake of 34% over SHR control) was attenuated by

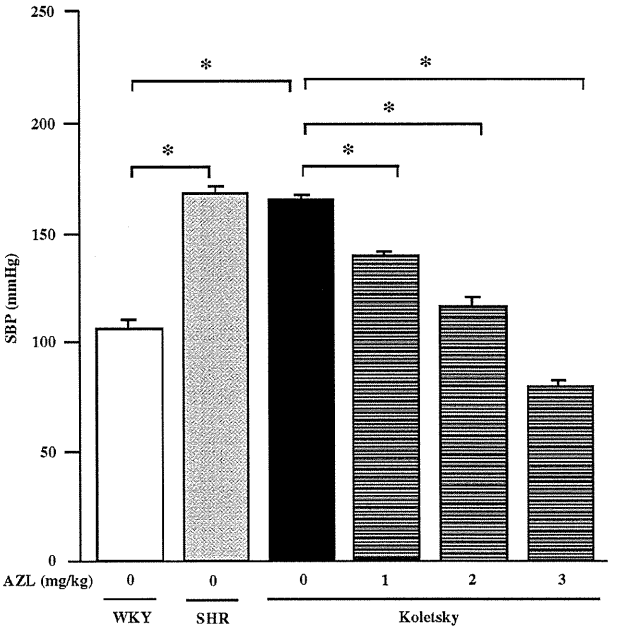

Figure 1. Systolic blood pressure (SBP) in male Wistar-Kyoto (WKY), spontaneously hypertensive rats (SHR) and obese Koletsky rats. Animals received azilsartan at different dosages or vehicle by oral gavage once daily. SBP was measured with a tail-cuff method 2–5 h after the treatment on day 5. Data are means ± s.e.m. (n = 6 each group). *$p < 0.05$ (analysis of variance).

azilsartan treatment (figure 3A). Azilsartan also prevented the excess BW increase that was evident in untreated obese Koletsky rats (figure 3B). However, azilsartan treatment minimally affected the increased eWAT weight (figure 3C) and adipocyte size (figure 3D, E).

Effects of Azilsartan Treatment on Plasma Concentrations of Triglyceride, NEFA and Corticosterone in Obese Koletsky Rats

Compared to lean SHR-littermates, obese Koletsky rats exhibited increased fasted plasma triglyceride concentration (figure 4A), whereas NEFA concentration was unchanged (figure 4B). Treatment of obese Koletsky rats with azilsartan did not affect plasma concentrations of triglyceride or NEFA.

Plasma corticosterone concentrations in obese Koletsky rats were over threefold higher than those in respective SHR control. Azilsartan treatment minimally affected plasma corticosterone concentrations (figure 4C).

Comparison of the Effects Between Azilsartan and Pair-feeding Treatments in Obese Koletsky Rats

The results in above experiments show that azilsartan at 2 mg/kg decreased food intake. To ensure that the actions of azilsartan were independent of effects on food intake, a comparison between azilsartan and pair-feeding experiments was performed. The obese rats in two groups consumed similar amounts of food (data not shown). In contrast with azilsartan treatment, pair-feeding did not affect SBP (pair-feeding: 175 ± 4 vs. azilsartan: 122 ± 2 mmHg, $p < 0.05$); there were

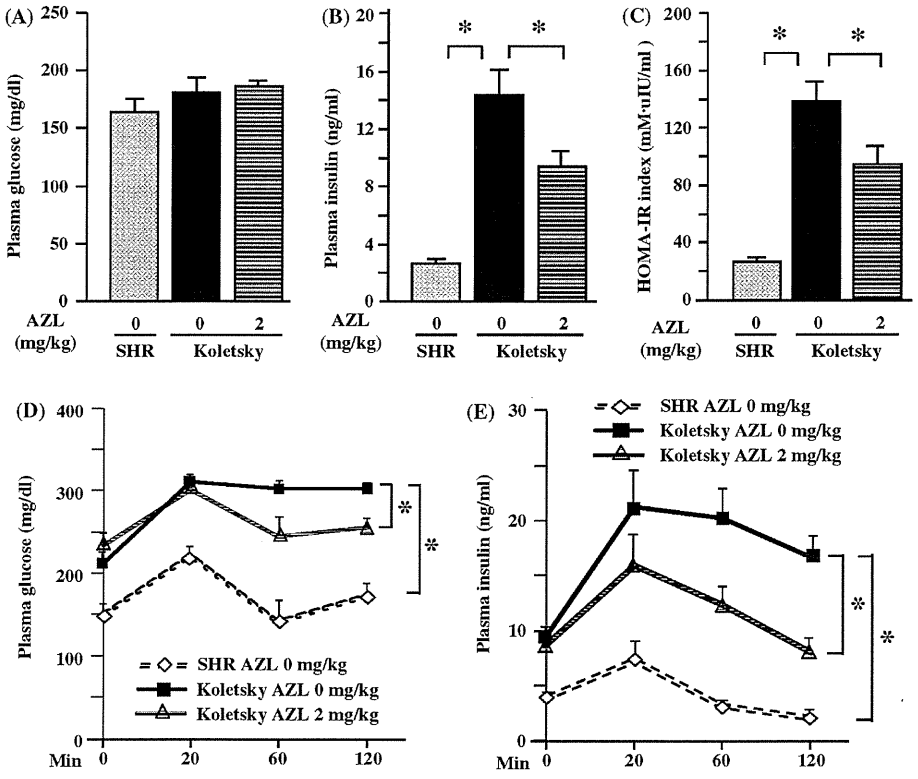

Figure 2. Fasted plasma glucose (A) and insulin (B) concentrations (day 14), the index of the homeostasis model assessment of insulin resistance (C), and glucose (D) and insulin (E) concentrations during oral glucose tolerance testing (day 17, glucose dosage: 4 g/kg) in male obese Koletsky rats and their littermates spontaneously hypertensive rat (SHR). Animals received azilsartan (2 mg/kg) or vehicle daily as described in the legend to figure 1. Data are means ± s.e.m. (n = 8 each group). *$p < 0.05$ (analysis of variance).

no differences in BW, eWAT weight and adipocyte size (data not shown). Although fasted plasma glucose concentrations (figure 5A) between two treatments were not different, fasted plasma insulin concentration (figure 5B), the HOMA-IR index (figure 5C) and plasma glucose concentrations during OGTT (figure 5D) were lower after treatment with azilsartan than pair-feeding.

Gene Expression Profile in Obese Koletsky Rats

In adipose tissue there was no significant difference in PPARγ, aP2 and 11β-HSD1 expression between two genotypes, while adiponectin mRNA level was decreased in obese Koletsky rats than SHR ($p < 0.05$, data not shown). Azilsartan treatment did not significantly affect PPARγ and adiponectin mRNA expression, but downregulated aP2 and 11β-HSD1 expression in obese Koletsky rats ($p < 0.05$, data not shown).

Discussion

The present findings clearly show that, in addition to its potent antihypertensive property, azilsartan treatment also improves insulin sensitivity in obese spontaneously hypertensive Koletsky rats. Azilsartan treatment decreased the hyperinsulinaemia, improved the HOMA-IR index and suppressed the over-increase in plasma glucose and insulin concentrations during OGTT. Thus, azilsartan treatment may diminish the risk of cardiovascular morbidity and mortality as a result of hypertension, especially in the presence of insulin resistance and hyperinsulinaemia in obesity and type 2 diabetes.

As azilsartan treatment attenuated hyperphagia and decreased BW increase in obese Koletsky rats, we evaluated the impact of decreases in both food intake and BW gain on metabolic parameters. Although there was no difference in BW and adiposity variables between azilsartan and pair-feeding treatments, pair-feeding did not mimic the insulin-sensitizing effect of azilsartan. From these considerations it appears that azilsartan treatment improves insulin sensitivity via additional mechanisms.

Adipose PPARγ is the master regulator of adipogenesis and is activated by the thiazolidinediones that are used clinically to stimulate the action of insulin [13]. PPARγ-activating ligands alter fat topography and adipocyte phenotype and activate genes that regulate fatty acid metabolism and triglyceride storage [14]. Thus, the treatment with rosiglitazone decreased plasma concentrations of glucose, triglyceride and NEFA, but increased BW in obese Zucker rats [15–17]. Azilsartan (6.58 mg/kg/day delivered orally in chow) has been shown to decrease BW, adipose tissue weight and adipocyte size in KK-A^y mice, which was accompanied by a decrease in NEFA concentrations, as well as enhanced adipose expression of PPARγ and its target genes adiponectin, aP2 [9]. In the present study, however, azilsartan minimally affected eWAT weight, adipocyte size and basal plasma concentrations

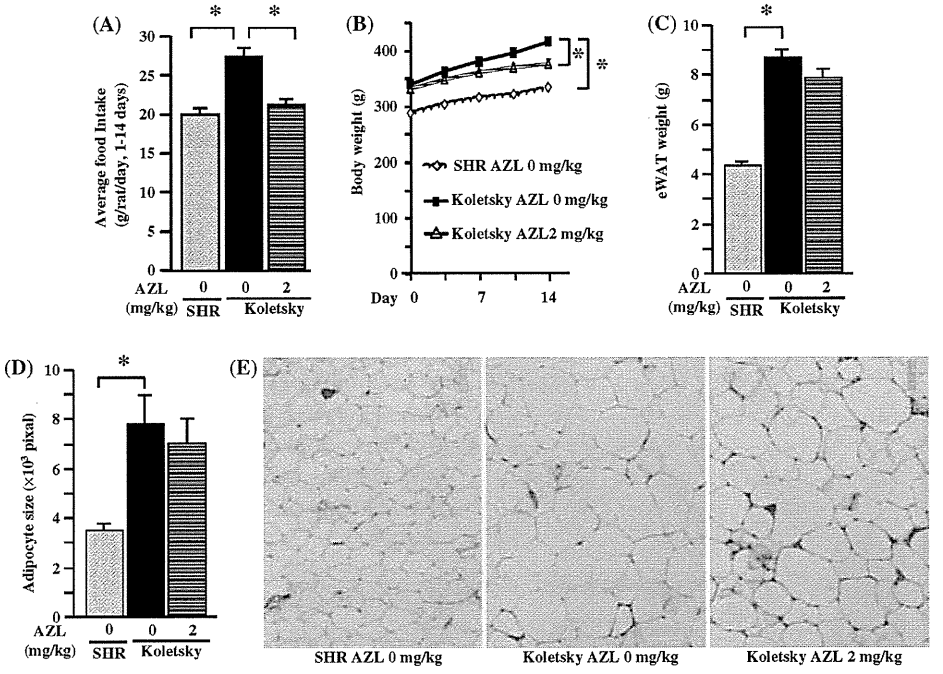

Figure 3. (A) Average food intake, (B) body weight, (C) epididymal white adipose tissue (eWAT) weight, (D) epididymal adipocyte size and (E) representative images showing histology of eWAT (haematoxylin and eosin staining, ×100) in male spontaneously hypertensive rat (SHR) and obese Koletsky rats. Animals received azilsartan (2 mg/kg) or vehicle daily for 21 days as described in the legend to figure 1. Food intake for 24 h was determined over a 14-day period with average food intake calculated accordingly. Data are means ± s.e.m. (n = 8 each group). *p < 0.05 (analysis of variance).

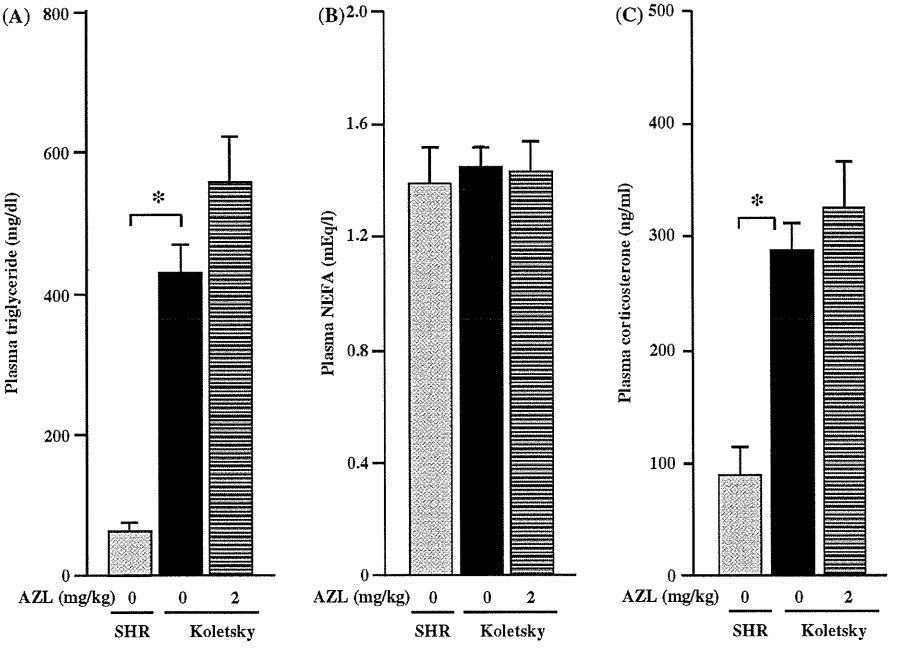

Figure 4. Fasted plasma concentrations of triglyceride (A), non-esterified fatty acids (NEFA) (B) and corticosterone (C) in male spontaneously hypertensive rat (SHR) and obese Koletsky rats on day 14. Animals received azilsartan (2 mg/kg) or vehicle daily as described in the legend to figure 1. Data are means ± s.e.m. (n = 8 each group).

of glucose, triglyceride and NEFA in obese Koletsky rats. Furthermore, azilsartan treatment did not upregulate PPARγ, adiponectin and aP2 expression in adipose tissue. Azilsartan at the concentrations up to 10^{-5} M did not show effect on PPARγ in the reporter gene assay in Chinese hamster ovary cells (data not shown). Thus, the effect of azilsartan on insulin sensitivity unlikely involves PPARγ activation in adipose tissue. Further investigations are now required to

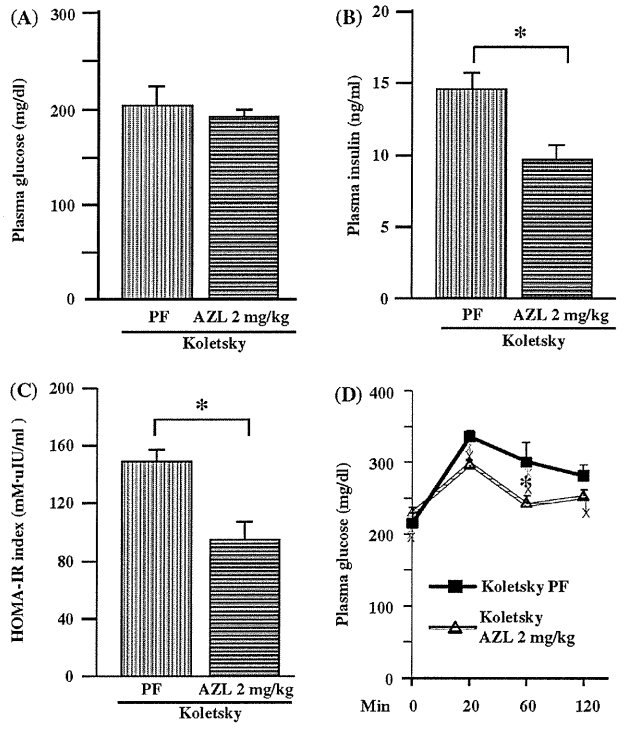

Figure 5. Fasted plasma glucose (A) and insulin (B) concentrations (day 14), the index of the homeostasis model assessment of insulin resistance (HOMA-IR) (C) and glucose concentrations (D) during oral glucose tolerance testing (day 17, glucose dosage: 4 g/kg) in male obese Koletsky rats. Animals received azilsartan (2 mg/kg) or pair-feeding and vehicle daily as described in section Methods. Data are means ± s.e.m. ($n = 5$ each group). *$p < 0.05$ (Student's t-test).

assess whether the discrepancies between previous reports in mice and the present study conducted in obese rats are because of species differences (mouse vs. rat), blood pressure status (normotensive vs. hypertensive) and/or others. As azilsartan treatment showed minor effect on adipose tissue weight, significant attenuation of hepatomegaly, cardiac hypertrophy, renomegaly (our unpublished data) and/or others might mainly contribute to diminishment of BW gain after the treatment in obese Koletsky rats.

The adipose enzyme, 11β-HSD1, which locally converts inactive glucocorticoids into bioactive forms [18], plays an important role in the development of visceral obesity and its associated metabolic disturbances [19–22]. Recently, 11β-HSD1 has been recognized as a potential therapeutic target for the treatment of metabolic syndrome as various 11β-HSD1 inhibitors have been shown to improve insulin sensitivity in ob/ob, db/db, KK-A^{Y} and high fat diet-induced obese mice [23–26]. Differentiation of 3T3-L1 cells caused a strong increase in 11β-HSD1 protein levels; combination of dexamethasone and insulin induced 11β-HSD1 expression [27]. In contrast, an 11β-HSD1 inhibitor KR-66344 downregulated expression of 11β-HSD1 and adipogenesis-related genes in cortisone-treated 3T3-L1 cells [26]. Recently, we have shown that ceramide, an intracellular lipid second messenger, is a novel regulator of 11β-HSD1 expression and activity in cultured preadipocytes [28]. It is known that angiotensin II interacts with ceramide production via its receptors, in which some cytokines, such as tumour necrosis factor-α, are involved [29]. In the present study, azilsartan treatment significantly downregulated expression of 11β-HSD1, as well as aP2 gene in adipose tissue of obese Koletsky rats. These results raise the possibility that azilsartan-elicited improvement of insulin sensitivity involves regulation of 11β-HSD1 activity. It needs to further investigate whether ceramide pathway is associated with azilsartan-elicited downregulation of adipose 11β-HSD1expression.

Considered together, the present results show that treatment with the new ARB azilsartan improves glucose metabolism in obese spontaneously hypertensive Koletsky rats. This insulin-sensitizing effect is independent of decreases in food intake and BW increase or of the activation of PPARγ in adipose tissue. Our findings indicate the possible usefulness of azilsartan in the treatment of the metabolic syndrome.

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Conflict of Interest

The authors declared that they have no conflict of interest. M. Z. conducted data collection and contributed to writing the manuscript. Y. L. was involved in the design, analysis and writing of manuscript. J. W. conducted data collection and analysis. K. E., X. R., K. H., T. T., and K. N. contributed to the analysis.

References

1. Prasad A, Quyyumi AA. Renin-angiotensin system and angiotensin receptor blockers in the metabolic syndrome. Circulation 2004; **110**: 1507–1512.
2. Benson SC, Pershadsingh HA, Ho CI et al. Identification of telmisartan as a unique angiotensin II receptor antagonist with selective PPARgamma-modulating activity. Hypertension 2004; **43**: 993–1002.
3. Negro R, Formoso G, Hassan H. The effects of irbesartan and telmisartan on metabolic parameters and blood pressure in obese, insulin resistant, hypertensive patients. J Endocrinol Invest 2006; **29**: 957–961.
4. Aksnes TA, Seljeflot I, Torjesen PA, Höieggen A, Moan A, Kjeldsen SE. Improved insulin sensitivity by the angiotensin II-receptor blocker losartan is not explained by adipokines, inflammatory markers, or whole blood viscosity. Metabolism 2007; **56**: 1470–1477.
5. Nishimura H, Sanaka T, Tanihata Y, Naito T, Higuchi C, Otsuka K. Losartan elevates the serum high-molecular weight-adiponectin isoform and concurrently improves insulin sensitivity in patients with impaired glucose metabolism. Hypertens Res 2008; **31**: 1611–1618.
6. Rong X, Li Y, Ebihara K et al. Irbesartan treatment up-regulates hepatic expression of PPARalpha and its target genes in obese Koletsky (fak/fak) rats: a link to amelioration of hypertriglyceridaemia. Br J Pharmacol 2010; **160**: 1796–1807.

7. White WB, Weber MA, Sica D et al. Effects of the Angiotensin receptor blocker azilsartan medoxomil versus olmesartan and valsartan on ambulatory and clinic blood pressure in patients with stages 1 and 2 hypertension. Hypertension 2011; **57**: 413–420.
8. Ojima M, Igata H, Tanaka M et al. In vitro antagonistic properties of a new angiotensin type 1 receptor blocker, azilsartan, in receptor binding and function studies. J Pharmacol Exp Ther 2010; **336**: 801–808.
9. Iwai M, Chen R, Imura Y, Horiuchi M. TAK-536, a new AT1 receptor blocker, improves glucose intolerance and adipocyte differentiation. Am J Hypertens 2007; **20**: 579–586.
10. Takaya K, Ogawa Y, Hiraoka J et al. Nonsense mutation of leptin receptor in the obese spontaneously hypertensive Koletsky rat. Nat Genet 1996; **14**: 130–131.
11. Rong X, Li Y, Ebihara K et al. An adipose tissue-independent insulin-sensitizing action of telmisartan: a study in lipodystrophic mice. J Pharmacol Exp Ther 2009; **331**: 1096–1103.
12. Rong X, Li Y, Ebihara K et al. Angiotensin II type 1 receptor-independent beneficial effects of telmisartan on dietary-induced obesity, insulin resistance and fatty liver in mice. Diabetologia 2010; **53**: 1727–1731.
13. Evans RM, Barish GD, Wang YX. PPARs and the complex journey to obesity. Nat Med 2004; **10**: 355–361.
14. Sharma AM, Staels B. Peroxisome proliferator-activated receptor gamma and adipose tissue–understanding obesity-related changes in regulation of lipid and glucose metabolism. J Clin Endocrinol Metab 2007; **92**: 386–395.
15. Cai XJ, Lister CA, Buckingham RE et al. Down-regulation of orexin gene expression by severe obesity in the rats: studies in Zucker fatty and Zucker diabetic fatty rats and effects of rosiglitazone. Brain Res Mol Brain Res 2000; **77**: 131–137.
16. Finegood DT, McArthur MD, Kojwang D et al. Beta-cell mass dynamics in Zucker diabetic fatty rats. Rosiglitazone prevents the rise in net cell death. Diabetes 2001; **50**: 1021–1029.
17. Reifel-Miller A, Otto K, Hawkins E et al. A peroxisome proliferator-activated receptor alpha/gamma dual agonist with a unique in vitro profile and potent glucose and lipid effects in rodent models of type 2 diabetes and dyslipidemia. Mol Endocrinol 2005; **19**: 1593–1605.
18. Seckl JR, Walker BR. Minireview: 11β-hydroxysteroid dehydrogenase type 1: a tissue-specific amplifier of glucocorticoid action. Endocrinology 2001; **142**: 1371–1376.
19. Livingstone DE, Jones GC, Smith K et al. Understanding the role of glucocorticoids in obesity: tissue-specific alterations of corticosterone metabolism in obese Zucker rats. Endocrinology 2000; **141**: 560–563.
20. Masuzaki H, Paterson J, Shinyama H et al. A transgenic model of visceral obesity and the metabolic syndrome. Science 2001; **294**: 2166–2170.
21. Paulmyer-Lacroix O, Boullu S, Oliver C, Alessi MC, Grino M. Expression of the mRNA coding for 11β-hydroxysteroid dehydrogenase type 1 in adipose tissue from obese patients: an in situ hybridization study. J Clin Endocrinol Metab 2002; **87**: 2701–2705.
22. Tomlinson JW, Walker EA, Bujalska IJ et al. 11β-Hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. Endocr Rev 2004; **25**: 831–866.
23. Alberts P, Engblom L, Edling N et al. Selective inhibition of 11beta-hydroxysteroid dehydrogenase type 1 decreases blood glucose concentrations in hyperglycaemic mice. Diabetologia 2002; **45**: 1528–1532.
24. Alberts P, Nilsson C, Selen G et al. Selective inhibition of 11 beta-hydroxysteroid dehydrogenase type 1 improves hepatic insulin sensitivity in hyperglycemic mice strains. Endocrinology 2003; **144**: 4755–4762.
25. Wang SJ, Birtles S, de Schoolmeester J et al. Inhibition of 11beta-hydroxysteroid dehydrogenase type 1 reduces food intake and weight gain but maintains energy expenditure in diet-induced obese mice. Diabetologia 2006; **49**: 1333–1337.
26. Park JS, Rhee SD, Kang NS et al. Anti-diabetic and anti-adipogenic effects of a novel selective 11β-hydroxysteroid dehydrogenase type 1 inhibitor, 2-(3-benzoyl)-4-hydroxy-1,1-dioxo-2H-1,2-benzothiazine-2-yl-1-phenylethanone (KR-66344). Biochem Pharmacol 2011; **81**: 1028–1035.
27. Kim J, Temple KA, Jones SA, Meredith KN, Basko JL, Brady MJ. Differential modulation of 3T3-L1 adipogenesis mediated by 11beta-hydroxysteroid dehydrogenase-1 levels. J Biol Chem 2007; **282**: 11038–11046.
28. Arai N, Masuzaki H, Tanaka T et al. Ceramide and adenosine 5'-monophosphate-activated protein kinase are two novel regulators of 11beta-hydroxysteroid dehydrogenase type 1 expression and activity in cultured preadipocytes. Endocrinology 2007; **148**: 5268–5277.
29. Berry C, Touyz R, Dominiczak AF, Webb RC, Johns DG. Angiotensin receptors: signaling, vascular pathophysiology, and interactions with ceramide. Am J Physiol Heart Circ Physiol 2001; **281**: H2337–2365.

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ORIGINAL ARTICLE

Genetic variations in the *CYP17A1* and *NT5C2* genes are associated with a reduction in visceral and subcutaneous fat areas in Japanese women

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Visceral fat accumulation has an important role in increasing the morbidity and mortality rates, by increasing the risk of developing several metabolic disorders, such as type 2 diabetes, dyslipidemia and hypertension. New genetic loci that are associated with increased systolic and diastolic blood pressures have been identified by genome-wide association studies in Caucasian populations. This study investigates whether single nucleotide polymorphisms (SNPs) that confer susceptibility to high blood pressure are also associated with visceral fat obesity. We genotyped 1279 Japanese subjects (556 men and 723 women) who underwent computed tomography for measuring the visceral fat area (VFA) and subcutaneous fat area (SFA) at the following SNPs: *FGF5* rs16998073, *CACNB2* rs11014166, *C10orf107* rs1530440, *CYP17A1* rs1004467, *NT5C2* rs11191548, *PLEKHA7* rs381815, *ATP2B1* rs2681472 and rs2681492, *ARID3B* rs6495112, *CSK* rs1378942, *PLCD3* rs12946454, and *ZNF652* rs16948048. In an additive model, risk alleles of the *CYP17A1* rs1004467 and *NT5C2* rs11191548 were found to be significantly associated with reduced SFA (P=0.00011 and 0.0016, respectively). When the analysis was performed separately in men and women, significant associations of rs1004467 (additive model) and rs11191548 (recessive model) with reduced VFA (P=0.0018 and 0.0022, respectively) and SFA (P=0.00039 and 0.00059, respectively) were observed in women, but not in men. Our results suggest that polymorphisms in the *CYP17A1* and *NT5C2* genes influence a reduction in both visceral and subcutaneous fat mass in Japanese women.
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INTRODUCTION

Metabolic syndrome is a combination of multiple risk factors, including central obesity, impaired glucose tolerance, dyslipidemia and hypertension, which increases cardiovascular disease morbidity and mortality.[1] Several studies have indicated that the intra-abdominal adipose tissue has a central role in metabolic syndrome, as the

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accumulated visceral adipose tissue leads to alterations in the plasma levels of adipocytokines, resulting in the development of dyslipidemia, hypertension and insulin resistance.[2,3] Intra-abdominal fat accumulation (central adiposity) is determined by waist circumference, waist–hip ratio, biological impedance or the visceral fat area (VFA) measured using computed tomography.[1,4,5] There is abundant evidence that body fat distribution is influenced by genetic loci.[6–8] Individual variation in waist–hip ratio is heritable, with heritability estimates ranging from 22 to 61%. Recent genome-wide association studies (GWAS) showed that genetic loci were associated with waist circumference and waist–hip ratio in the Caucasian population.[9,10] We previously reported that the rs1558902 and rs1421085 genotypes of the fat mass- and obesity-associated gene (*FTO*) were significantly associated with VFA, as well as with the subcutaneous fat area (SFA) and body mass index (BMI) in the Japanese population.[11]

Recent progress in GWAS has increased the number of known genetic susceptibility loci for obesity.[12–16] We investigated the association between the single nucleotide polymorphisms (SNPs) underlying susceptibility to obesity and fat distribution (as determined by computed tomography), and found that rs7498665 in the SH2B adaptor protein 1 (*SH2B1*) gene was associated with VFA, uncovering the genetic background of central obesity.[17]

GWAS, and meta-analysis of GWAS, have identified various disease-associated genetic variations.[18] Hypertension is one of the risk factors of metabolic syndrome and is considerably related to central obesity. Obesity-associated allele of rs1558902 and rs1421085 in the *FTO* gene were associated with hypertension, but not that of rs7498665 in the *SH2B1* gene in the Japanese population.[19] The genetic variations associated with hypertension have been identified by GWAS.[20,21] In this study, we investigate whether the recently reported hypertension-related loci are also associated with VFA, which is another important factor responsible for metabolic syndrome.

MATERIALS AND METHODS

Study subjects

We enrolled 1279 Japanese subjects from outpatient clinics; these patients agreed to undergo computed tomography testing (in the supine position) to determine VFA and SFA values at the umbilical level (L4–L5), as previously reported.[17] Both VFA and SFA values were calculated using the FatScan software program (N2system, Osaka, Japan).[22] The patients visited the hospitals to undergo treatment for obesity and/or metabolic abnormalities, such as hypertension, dyslipidemia and type 2 diabetes. Patients with secondary obesity and obesity-related hereditary disorders were excluded from this study. Patients with disease (such as cancer, and renal, heart and hepatic failure), or under treatment (such as corticosteroid and chemotherapy) that strongly affects body weight, were also excluded. Athletes were also excluded from this study. Clinical data were recorded at the first visit to the hospital. The clinical characteristics of the subjects are summarized in Table 1. Metabolic syndrome and metabolic abnormalities were diagnosed according to the criteria released by the Japanese Committee for the Diagnostic Criteria of Metabolic Syndrome in April 2005.[4,5] Written informed consent was obtained from each subject, and the protocol was approved by the ethics committee of each institution and by that of Kyoto University.

DNA extraction and SNP genotyping

Genomic DNA was extracted from the blood samples collected from each subject using the Genomix kit (Talent Srl, Trieste, Italy). We selected 12 SNPs that were previously identified as susceptibility loci for hypertension by GWAS in Caucasian populations,[20,21] and constructed Invader probes (Third Wave Technologies, Madison, WI, USA) for each. The 12 selected SNPs were as follows: rs16998073 in the fibroblast growth factor 5 (*FGF5*) gene; rs11014166 in the calcium channel, voltage-dependent, β-2 subunit (*CACNB2*) gene; rs1530440 in the chromosome 10 open reading frame 107 (*C10orf107*) gene; rs1004467 in the cytochrome P450, family 17, subfamily A, polypeptide 1 (*CYP17A1*) gene; rs11191548 in the 5′-nucleotidase, cytosolic II (*NT5C2*) gene; rs381815 in the pleckstrin homology domain containing, family A member 7 (*PLEKHA7*) gene; rs2681472 and rs2681492 in the ATPase, Ca^{2+} transporting, plasma membrane 1 (*ATP2B1*) gene; rs6495112 in the AT-rich interactive domain 3B (BRIGHT-like) (*ARID3B*) gene; rs1378942 in the c-src tyrosine kinase (*CSK*) gene; rs12946454 in the phospholipase C, delta 3 (*PLCD3*) gene; and rs16948048 in the zinc finger protein 652 (*ZNF652*) gene. The SNPs were genotyped using Invader assays, as previously described.[23] The success rate of these assays was >99.0%.

Table 1 Clinical characteristics of the subjects

	Men	*Women*	*Total*
n	556	723	1279
Age (years)	49.4±12.2	52.2±11.3	51.0±11.8
BMI (kg m^{-2})	30.2±6.1	28.1±5.3	29.0±5.8
VFA (cm^2)	155.3±67.7	99.8±53.6	123.9±66.1
SFA (cm^2)	206.7±108.6	241.6±97.2	226.5±103.7
Waist circumference (cm)	97.5±11.3	91.8±10.3	94.2±11.1
Prevalence of metabolic disease			
Dyslipidemia	293 (53%)	244 (34%)	537 (42%)
Hypertension	379 (68%)	452 (63%)	831 (65%)
Impaired fasting glucose	177 (32%)	176 (24%)	353 (28%)
Metabolic syndrome	248 (45%)	162 (22%)	410 (32%)

Abbreviations: BMI, body mass index; SFA, subcutaneous fat area; VFA, visceral fat area.
Data are represented as mean±s.d.

Statistical analysis

For the additive model, we coded the genotypes as 0, 1 or 2 depending on the number of copies of the risk alleles. For the recessive model, homozygosity with the risk allele was coded as 1 and the others were coded as 0. Risk alleles refer to the hypertension-associated alleles, according to previous reports.[20,21] Multiple linear regression analyses were performed to test the independent effect of the risk alleles on BMI, VFA and SFA, by taking into account the effects of other variables (that is, age and gender) that were assumed to be independent of the effect of each SNP. The values of BMI, VFA and SFA were logarithmically transformed before performing the multiple linear regression analysis. Differences in the quantities of anthropometric parameters among the different genotypes were assessed by the analysis of covariance, by taking into account the effects of other variables (that is, age and/or institute). Hardy–Weinberg equilibrium was assessed using the χ^2-test.[24] To test SNP×SNP epistasis, we used a linear regression model for each SNP1 and SNP2, and fit the model in the form of $Y=\beta_0+\beta_1\times\mathrm{SNP1}+\beta_2\times\mathrm{SNP2}+\beta_3\times\mathrm{SNP1}\times\mathrm{SNP2}+\beta_4\times\mathrm{age}+\beta_5\times\mathrm{gender}$. Although we collected the samples at the region of Hondo (Kanto, Kinki, Chugoku and Kyushu; Supplementary Table 1), we performed Wright's F-statistics[25] to evaluate the difference in the population structures of our sample using randomly selected 31 SNPs. We divided our samples into two groups (SFA >208 cm^2 and ⩽208 cm^2). Median of SFA (208 cm^2) was used as a cut-off value. The results indicated that the population structure of the two groups were almost the same in view of a very small F_{ST} value between both the groups (mean F_{ST}=0.00023). Statistical analysis was performed using R software (http://www.r-project.org/). *P*-values were assessed with a Bonferroni correction and $P<0.0042$ (0.05/12) was considered statistically significant.

RESULTS

The clinical characteristics and genotypes of the subjects are shown in Tables 1 and 2, respectively. All the SNPs were in Hardy–Weinberg equilibrium and the minor allele frequencies did not diverge from those reported in the HapMap database. The BMI, VFA and SFA values for each SNP genotype are reported in Table 3. Multiple linear regression analyses of the anthropometric parameters with respect to the 12 analyzed SNPs are shown in Table 4. The A-allele of rs1004467