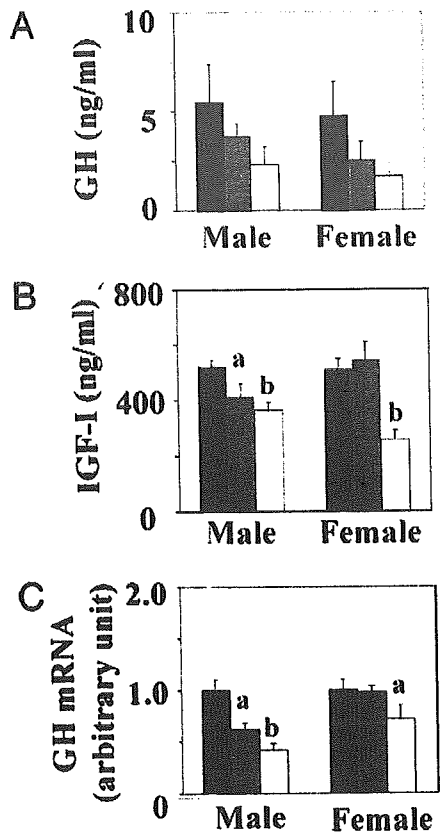

FIG. 4. Serum GH, IGF-I, and pituitary GH mRNA levels in 8-wk-old control (*closed bars*), Tg 9–2 (*shaded bars*), and Tg 10–1 (*open bars*) mice (n = 8/group). A, Serum GH levels. B, Serum IGF-I levels. C, Pituitary GH mRNA levels. a, $P < 0.05$; b, $P < 0.01$ (*vs.* control mice).

5. No significant difference was noted in the levels between control and transgenic mice.

Hematoxylin eosin and immunohistochemical staining for GH of the pituitary

Hematoxylin eosin staining is shown in Fig. 5, A and B. The pituitary morphology of Tg 10–1 mice was not different from that of the control mice. Immunohistochemical staining for GH is shown in Fig. 5, C and D. The distribution of GH-immunoreactive cells in the pituitary of Tg mice was similar to that of control mice.

Effects of GHRH and ghrelin on GH release

Control and Tg 10–1 mice were used. Serum GH levels after GHRH administration in male and female Tg 10–1 mice were similar to those of control mice throughout the course of the experiment (Fig. 6A). There was no significant difference in serum GH level at each time point between both male and female Tg-10 and control mice. Serum GH levels 10 min after ghrelin administration in male Tg10–1 and control mice were 63.1 ± 6.8 and 72.6 ± 12.0 ng/ml, respectively (Fig. 6B, *left panel*). The difference was not significant. Serum GH levels 20 min after ghrelin administration in male Tg10–1 and control mice were 30.2 ± 6.7 and 61.2 ± 15.5 ng/ml, respectively, and levels after 30 min were 11.8 ± 1.4 and 21.9 ± 4.1 ng/ml, respectively (Fig. 6B, *left panel*). Both differences were significant ($P < 0.01$). Serum GH levels 10 min after ghrelin administration in female Tg10–1 and control mice were 8.7 ± 3.7 and 52.8 ± 8.2 ng/ml, respectively, and those after 20 min were 29.8 ± 6.3 and 78.5 ± 14.3 ng/ml, respectively (Fig. 6B, *right panel*). Both differences were significant ($P < 0.01$). Serum GH levels 30 min after ghrelin administration in female Tg10–1 and control mice were 22.8 ± 6.3 and 22.3 ± 8.8 ng/ml, respectively (Fig. 6B, *right panel*). The difference was not significant.

TABLE 5. Plasma ACTH, serum TSH, LH, and FSH levels of 8-wk-old control and transgenic mice (n = 8/group)

	Control	Tg 9-2	Tg 10-1
ACTH (pg/ml)	135 ± 35	144 ± 32	123 ± 44
TSH (ng/ml)	3.31 ± 0.06	3.37 ± 0.11	3.49 ± 0.13
LH (ng/ml)	31.5 ± 2.1	30.8 ± 1.7	27.7 ± 2.1
FSH (ng/ml)	268.4 ± 21.8	221.1 ± 43.9	253.5 ± 24.6

Values are given as the mean ± SEM.

Expression of GHS-R in the pituitary

GHS-R mRNA levels of male control, Tg 9–2, and Tg 10–1 mice were 1.00, 1.56, and 3.46 AU, respectively (Fig. 7). The difference between control and Tg 10–1 mice was significant ($P < 0.01$).

Expression of hypothalamic neuropeptides that regulate GH secretion

GHRH mRNA levels of male control, Tg 9–2, and Tg 10–1 mice were 1.00, 0.88, and, 0.80 AU, respectively (Fig. 8A). The differences between control and Tg 9–2 mice and control and Tg 10–1 mice were not significant. Somatostatin mRNA levels of male control, Tg 9–2, and Tg 10–1 mice were 1.00, 1.08, and 0.97 AU, respectively (Fig. 8B). The differences between control and Tg 9–2 mice and control and Tg 10–1 mice were not significant.

Effects of continuous infusion of des-acyl ghrelin on GH-IGF-I axis and body weights

Male and female C57BL/6 mice were used. Serum GH levels after 10 d treatment with saline and des-acyl ghrelin in male mice were 5.8 ± 1.1 and 7.5 ± 2.0 ng/ml, respectively. The difference was not significant. Those with saline and des-acyl ghrelin in female mice were 9.2 ± 2.2 and 9.5 ± 1.8 ng/ml, respectively. The difference was not significant either. Serum IGF-I levels after 10 d treatment with saline and des-acyl ghrelin in male mice were 769.3 ± 16.6 and 768.7 ± 21.6 ng/ml, respectively. The difference was not significant. Those with saline and des-acyl ghrelin in female mice were 766.2 ± 13.4 and 719.4 ± 49.1 ng/ml, respectively. The difference was not significant either. Body weights and lengths in des-acyl ghrelin-injected mice were not significantly different from those in saline-injected mice in either males or females (data not shown).

Discussion

We have generated transgenic mouse lines that overexpress preproghrelin mRNA in a wide variety of tissues. The wide tissue distribution of preproghrelin mRNA in trans-

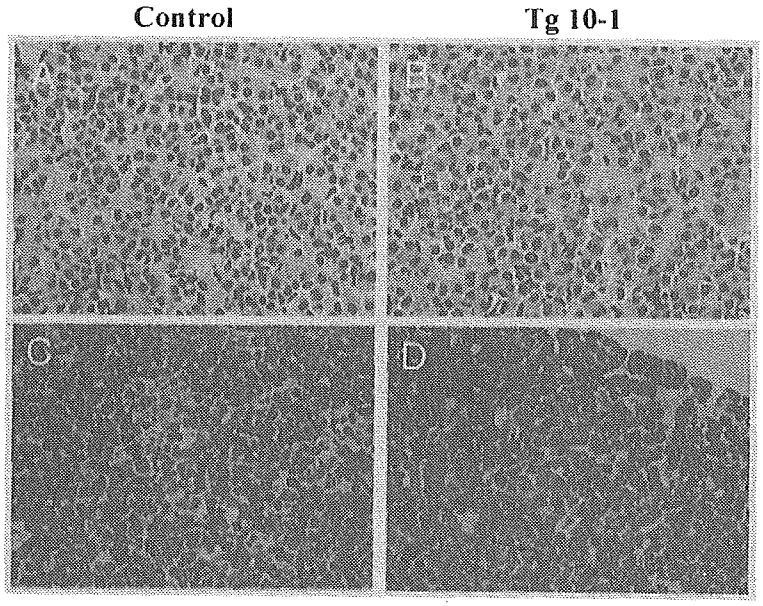

FIG. 5. Morphology of the pituitary and the localization of GH-immunoreactive cells in the pituitary of 8-wk-old male control (A and C) and Tg 10–1 (B and D) mice. A and B, Hematoxylin eosin (HE) staining. C and D, The localization of total and GH-immunoreactive cells in the pituitary. Original magnification, ×40. The immunoreactive cells are stained *brown* by the avidin-biotin complex methods.

genic mice was consistent with previous reports on transgenic mice using the CAG promoter (33, 34). Preproghrelin mRNA expression was increased, especially in Tg 10–1 mice, and its amount in the stomach reached 52-fold of that in control mice. Consistent with the elevated mRNA expression, peptide levels of total ghrelin (des-acyl plus acylated ghrelin) in various tissues were also elevated in transgenic mice. Plasma total ghrelin levels in transgenic mice showed marked results. Those in transgenic mice showed 10- and 44-fold of those in control mice. We originally intended to generate mice overexpressing biologically active ghrelin. Unexpectedly, acylated ghrelin levels were not changed in all tissues examined and plasma of transgenic mice, compared with those of control mice, indicating that transgenic mice overexpress only des-acyl ghrelin. The expression of acylated ghrelin has been reported in a small number of tissues, such as the stomach (X/A cells), duodenum, hypothalamus, and pancreatic α-cells (1, 31, 39, 40). These reports and our present data suggest that only a limited number of cell lineages may able to process proghrelin or acylate ghrelin. The underlying mechanism by which ghrelin is acylated is unknown to date. Further study is needed to clarify the mechanism of the acylation.

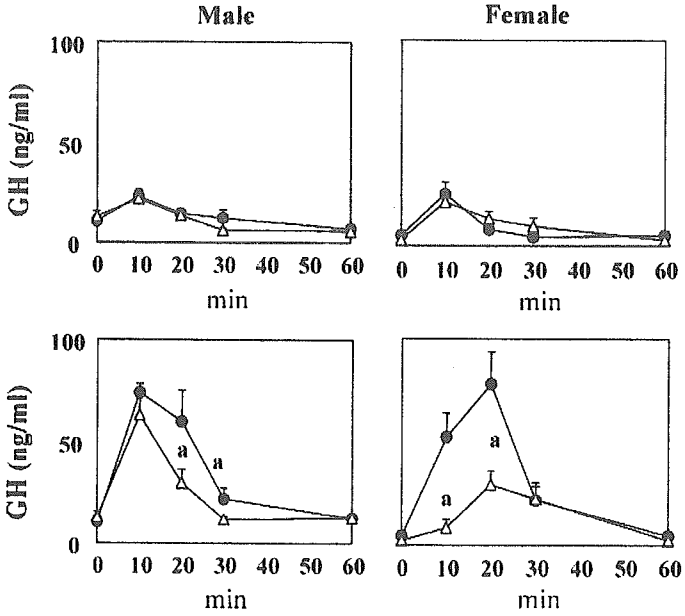

FIG. 6. The responses of GH to GHRH and ghrelin in 8-wk-old control (*closed circles*) and Tg 10–1 (*open triangles*) mice. A, Time course of serum GH levels after iv injection of 60 μg/kg GHRH (n = 8/each point). B, Time course of serum GH levels after iv injection of 40 μg/kg ghrelin (n = 8/each point). a, $P < 0.01$ (*vs.* control mice).

The acylation of ghrelin is assumed to be essential for its actions, and des-acyl ghrelin, which lacks the modification, is devoid of endocrine actions, based on previous studies (1, 41). However, recent studies indicated that des-acyl ghrelin may have some actions. Des-acyl ghrelin as well as acylated ghrelin causes a significant inhibition of cell proliferation in human breast carcinoma cell lines (29) and inhibits cell death in cardiomyocytes and endothelial cells through ERK1/2 and phosphatidylinositol 3-kinase/AKT (30). In addition, one study (42) reported that acylated and des-acyl ghrelin promote adipogenesis directly *in vivo* by a mechanism independent of known GHS-Rs. Moreover, another study (28) indicated that des-acyl ghrelin may offset the action of acylated ghrelin on insulin secretion. Ghrelin has been shown to induce a reduction in serum insulin levels. In the study, coadministration of acylated plus des-acyl ghrelin did not re-

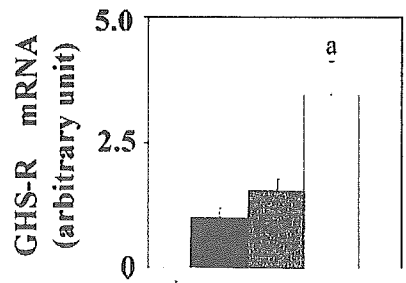

FIG. 7. Pituitary GHS-R mRNA levels in 8-wk-old control (*closed bars*), Tg 9–2 (*shaded bars*), and Tg 10–1 (*open bars*) mice quantified by real-time PCR analysis (n = 8/group). a, $P < 0.01$ (*vs.* control mice).

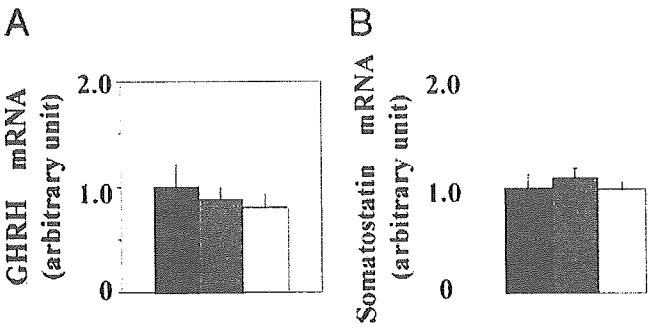

FIG. 8. Hypothalamic GHRH and somatostatin mRNA levels in 8-wk-old control (*closed bars*), Tg 9–2 (*shaded bars*), and Tg 10–1 (*open bars*) mice quantified by real-time PCR analysis. A, GHRH mRNA levels (n = 8/group). B, Somatostatin mRNA levels (n = 8/group).

sult in any changes in serum insulin levels in humans, suggesting that ghrelin action on insulin is modulated by des-acyl ghrelin.

The present study indicates that transgenic mice overexpressing des-acyl ghrelin show small phenotype. Longitudinal growth was the most reduced in female Tg 10–1 mice (20% reduction from control mice). The phenotype was not associated with changes in BMIs. These mice did not show decreased food intake or decreased body fat mass. In addition, they showed normal nutritional condition, based on their biochemical parameters, including blood glucose, serum total protein, and total cholesterol levels. These data indicate that the small phenotype of transgenic mice is not attributed to poor nutritional condition.

Serum IGF-I levels were significantly reduced in male and female transgenic mice, compared with control mice. Female Tg 10–1 mice had no less than 50% reduction in serum IGF-I levels, compared with control mice. Although the differences in serum GH levels between control and transgenic mice were not statistically significant, probably because of the pulsatile character of GH secretion, the levels tended to be reduced in transgenic mice, compared with control mice, and the mean GH level of Tg10–1 mice was only 50% of that of control mice. It should be emphasized that Tg 10–1 mice showed lower serum GH levels than Tg 9–2 mice. Body weights and lengths of the former were more reduced than the latter. It should be also noted that the former showed higher des-acyl ghrelin expression than the latter. Reduced pituitary GH mRNA levels in transgenic mice support the observation. The GH-IGF-I axis-specific alteration in transgenic mice was also indicated by the measurement of other anterior pituitary hormones than GH. Plasma ACTH, serum TSH, LH, and FSH levels were not altered.

The size and morphology of the pituitary including the somatotrope populations of transgenic mice were similar to those of control mice. These data indicated that there is no apparent change, suggesting developmental problems in the pituitary of transgenic mice.

Responses of GH to GHRH and ghrelin in transgenic mice exhibited intriguing results. Transgenic mice showed normal response of GH to GHRH. Alternatively, if we consider that the basal GH levels are lower in transgenic mice, the similar maximal response might indicate that they are hyperresponsive to GHRH. It is not likely that an insufficient dose of GHRH induced submaximal response of GH in both control and transgenic mice, judging from previous reports (43). On the other hand, the responses of GH to ghrelin were reduced in transgenic mice. It is noteworthy that the reduction was much greater in female transgenic mice than in male mice, if we take their serum IGF-I levels into account. Taken together our results and these reports indicate that overexpression of des-acyl ghrelin in our mice may result in reduction of GH response to endogenous ghrelin, and it may result in the reduced serum IGF-I levels in transgenic mice.

The reduced GH response to ghrelin in transgenic mice could be due to down-regulated the GHS-R. However, the pituitary GHS-R mRNA levels in the transgenic mice were rather elevated. It is not likely that overexpressed des-acyl ghrelin acts as a blocking agent to the GHS-R because ^{125}I-labeled acylated ghrelin bound to the GHS-R cannot be displaced by des-acyl ghrelin (20). Overexpressed des-acyl ghrelin may have some effects on endogenous GH secretion, modifying the action of endogenous ghrelin in transgenic mice via, for instance, another receptor or modulation of the signal transduction pathway after the GHS-R.

Previous reports indicated that the hypothalamus plays a critical role in the stimulatory effect of ghrelin on GH secretion as well as the pituitary (21, 22, 23). Because GH secretion is regulated chiefly by two hypothalamic hormones, GHRH and somatostatin, the expression of these hormones could be altered in transgenic mice. We could not find any significant difference in either GHRH or somatostatin mRNA levels between control and transgenic mice. These data might suggest that overexpressed des-acyl ghrelin acts on not only the pituitary but also the hypothalamus in the transgenic mice, judging from the fact that hypothalamus GHRH mRNA were not elevated, and somatostatin mRNA levels were not decreased despite the decreased serum GH levels.

We could not show, unfortunately, that continuous ip infusion of des-acyl ghrelin has some effect on serum GH and IGF-I levels or body weights. It should be noted, however, that plasma des-acyl ghrelin levels in transgenic mice reached 10- and 50-fold of those in control mice. Administration of a higher dose of des-acyl ghrelin, or longer administration, might result in alteration in the GH-IGF-I axis. On the other hand, the phenotype of transgenic mice might reflect direct effects of ubiquitous expression of des-acyl ghrelin. It should also be noted that high levels of des-acyl ghrelin were detected in a various tissues, especially in the pituitary, as well as in plasma of transgenic mice. The des-acyl ghrelin immunoreactive pituitary cells might play an important role in the mechanism for the altered GH-IGF-I axis in a paracrine or autocrine manner. It should be pointed out that preproghrelin mRNA is reported to be expressed in the normal pituitary (44), as we showed in the present study, suggesting its physiological role in GH secretion. The phenotype of transgenic mice may reflect the role. Further study is needed for this issue.

The mechanism underlying the sexual dimorphism in the responses of GH to ghrelin in transgenic mice is not fully understood. It might be due to the gender difference in the secretory regulation of GH. Female mice have been reported to be different from male mice in that they have noncyclical

and rather low somatostatin output and that GHRH plays a dominant role in it (45). There might be a GHRH-dependent mechanism for the reduced response in transgenic mice. Indeed, one recent report (26) indicated that transgenic rats expressing an antisense GHS-R mRNA in the hypothalamic arcuate nucleus show marked gender difference in GH secretion. Although there was no significant difference in pulse frequency and baseline levels of GH between male control and transgenic rats, female transgenic rats showed lower baseline levels and fewer pulses of GH than female control rats (26).

The 94-amino acid proghrelin is cleaved to yield ghrelin. One previous study (46) demonstrated that C-terminal proghrelin peptides are present in the human circulation. Transgenic mice in the present study would also overexpress these peptides. We have not excluded the possibility that the phenotype of transgenic mice might be due to the effects of these peptides.

In conclusion, the present study demonstrates that transgenic mice overexpressing des-acyl ghrelin show small phenotype and altered GH-IGF-I axis. These observations may indicate a role of des-acyl ghrelin in the regulation of GH secretion.

Acknowledgments

The authors gratefully acknowledge the excellent technical support of Chieko Ishimoto and Hitomi Hiratani.

Received May 17, 2004. Accepted October 1, 2004.

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This work was supported by research grants from the Japanese Ministry of Education, Science, and Culture and the Japanese Ministry of Health, Labor, and Welfare.

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Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

The FASEB Journal express article 10.1096/fj.04-2183fje. Published online October 20, 2004.

Prevention and reversal of renal injury by leptin in a new mouse model of diabetic nephropathy

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ABSTRACT

Diabetic nephropathy is the leading cause of end-stage renal disease, for which effective therapy to prevent the progression at advanced stages remains to be established. There is also a long debate whether diabetic glomerular injury is reversible or not. Lipoatrophic diabetes, a syndrome caused by paucity of adipose tissue, is characterized by severe insulin resistance, dyslipidemia, and fatty liver. Here, we show that a genetic model of lipoatrophic diabetes (A-ZIP/F-1 mice) manifests a typical renal injury observed in human diabetic nephropathy that is associated with glomerular hypertrophy, diffuse and pronounced mesangial widening, accumulation of extracellular matrix proteins, podocyte damage, and overt proteinuria. By crossing A-ZIP/F-1 mice with transgenic mice overexpressing an adipocyte-derived hormone leptin, we also reveal that leptin completely prevents the development of hyperglycemia and nephropathy in A-ZIP/F-1 mice. Furthermore, continuous leptin administration to A-ZIP/F-1 mice by minipump beginning at 40 weeks of age significantly alleviates the glomerular injury and proteinuria. These findings demonstrate the therapeutic usefulness of leptin at least for a certain type of diabetic nephropathy. The model presented here will serve as a novel tool to analyze the molecular mechanism underlying not only the progression but also the regression of diabetic nephropathy.

Key words: lipoatrophic diabetes • TGF-β1 • podocytes • proteinuria • transgenic mice

Diabetic nephropathy is the leading cause of end-stage renal disease in many countries (1). Progressive accumulation of extracellular matrix in glomeruli and tubulointerstitial tissues as well as hypertrophy of glomeruli and tubules is the characteristic feature of

diabetic nephropathy. These histological changes, once established, were considered to be progressive and irreversible (2). A recent study, however, has revealed that glomerular lesions of advanced diabetic nephropathy in patients with type 1 diabetes are reversible by long-term normoglycemia achieved with pancreas transplantation (3). Another report showed that established diabetic nephropathy in human cadaveric kidneys was ameliorated after transplantation into nondiabetic recipients (4). These reports suggest that reversibility is still preserved in the diseased kidney of diabetes and that intensive and long-term glycemic control can reverse the lesions of advanced diabetic nephropathy.

Numerous studies implicate that transforming growth factor-β1 (TGF-β1) plays a central role in the pathogenesis of diabetic nephropathy (5). Expression of TGF-β1 is elevated in clinical and experimental diabetic nephropathy (6). Transgenic mice with increased circulating TGF-β1 levels develop progressive glomerulosclerosis (7). Moreover, treatment with anti-TGF-β1 antibody effectively prevents the progression of mesangial matrix expansion and renal insufficiency in several diabetic models (8, 9). However, proteinuria is not affected by anti-TGF-β1 therapy (8), suggesting the presence of as yet unknown mechanisms of proteinuria in the pathogenesis of diabetic nephropathy. Several recent studies also suggest that podocyte loss or podocyte dysfunction contributes to the progression of diabetic nephropathy (10–13). Disruption of several podocyte-specific genes such as nephrin results in overt proteinuria (14). The molecular mechanisms of podocyte injury in diabetes, however, remain to be elucidated. There have been potential difficulties in approaching such issues, because we have no suitable animal models of diabetic nephropathy closely resembling human diabetic glomerulosclerosis available so far.

Lipoatrophic diabetes is a disorder characterized by paucity of adipose tissue, severe insulin resistance, and elevated triglycerides (15). This disease can be genetically inherited, acquired, or caused as a side effect of protease inhibitor treatment for acquired immunodeficiency (15, 16). As a result of adipose tissue disappearance, plasma concentrations of leptin, an adipocyte-derived hormone, are markedly reduced in patients with lipoatrophic diabetes and in animal models of this disease (17, 18). Leptin plays a major role in the regulation of energy expenditure and food intake (19, 20). Plasma leptin concentrations are elevated in obese patients in proportion to the degree of adiposity. Leptin deficiency causes severe obesity, insulin resistance, and diabetes in humans and animals (19, 21). We have recently generated transgenic mice overexpressing leptin in the liver, which show disappearance of lipid from adipose tissue, increased glucose metabolism, and increased insulin sensitivity (22). We have also revealed that overexpression of leptin rescues insulin resistance and diabetes in a severely adipose-deficient mouse model of lipoatrophic diabetes (A-ZIP/F-1 mice) (23). However, renal involvement of A-ZIP/F-1 mice and therapeutic usefulness of leptin as an antidiabetic nephropathy agent remain to be elucidated.

In the present study, we investigated renal injury in A-ZIP/F-1 mice histologically and functionally and evaluated whether the lesion is consistent with diabetic nephropathy in humans. We next genetically crossed A-ZIP/F-1 mice and leptin transgenic mice to produce double transgenic mice in order to clarify whether the development of renal injury in A-ZIP/F-1 mice is prevented by leptin. Finally, we examined the effects of continuous administration of leptin on renal injury in mice, which have already established glomerular lesions of diabetic nephropathy.

METHODS

Animals

Generation of A-ZIP/F-1 mice (A-ZIPTg/+) on the FVB/N background and leptin transgenic mice (LepTg/+) on the C57BL/6J background has been reported elsewhere (18, 22). In brief, A-ZIPTg/+ are expressing a protein that inactivates basic-zipper transcription factors in adipose tissue under the control of aP2 promoter. LepTg/+ are expressing mouse leptin cDNA in the liver under the control of serum amyloid P component promoter. We crossed male A-ZIPTg/+ with female LepTg/+ to obtain F1 mice of four genotypes; +/+, LepTg/+, A-ZIPTg/+ and LepTg/+:A-ZIPTg/+ (23). Mice were fed on standard chow (CE-2, 352 kcal/100 g, Japan CLEA, Tokyo, Japan) and given water ad libitum. We maintained these animals under alternating 12-h cycles of light and dark. Male mice were used in the studies here. All animal experiments were conducted in accordance with our institutional guidelines for animal research.

Blood and urine parameter measurements

Blood samples were obtained under pentobarbital anesthesia, and serum levels of glucose, total cholesterol, triglyceride, creatinine, and urea nitrogen were measured (24, 25). Serum leptin concentrations were determined by radioimmunoassay (Linco Research Immunoassay, St. Louis, MO) (23). Urinary albumin excretion was assayed with a murine albumin enzyme-linked immunosorbent assay kit (Exocell, Philadelphia, PA) (24).

Histology and morphometric analysis

For light microscopy, kidney sections were fixed by 4% buffered formaldehyde and embedded in paraffin. One-μm sections were stained with periodic acid-Schiff. Measurement of the glomerular cross-sectional area of 20 glomeruli randomly selected in each mouse by scanning the outer cortex was performed with a computer-aided manipulator (KS-400; Carl Zeiss Vision, Munich, Germany) (24). This examination was performed by two investigators without knowledge of the origin of the slides, and the mean values were calculated.

For electron microscopy, small blocks of kidneys were fixed in 2.5% buffered glutaraldehyde and postfixed in 2% osmium tetraoxide. The samples were embedded in epoxy resin and sectioned. The ultra-thin sections were stained with uranyl acetate and lead citrate, and examined in an electron microscope (H-7600, Hitachi, Tokyo, Japan) at 75 kV.

Immunostaining

Immunofluorescence study was performed as described previously (24, 25). In brief, 5-μm-thick cryostat sections were fixed in acetone for 1 min, washed with phosphate-buffered saline (PBS), incubated for 2 h at room temperature with rabbit polyclonal antibodies raised against collagen type IV, collagen type I (Chemicon International, Temecula, CA), fibronectin, connexin43 (Sigma, St. Louis, MO), TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA), and nephrin (26), stained with FITC-labeled goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) and observed with a confocal laser microscopy (LSM5Pascal; Carl Zeiss, Munich, Germany).

Immunohistochemical study was carried out with 5-μm-thick cryostat sections for Wilms' tumor-1 (WT-1) and paraffin-embedded sections for α-smooth muscle actin (αSMA). The sections were washed with PBS and treated with 0.3% H_2O_2 in PBS for 10 min to quench endogenous peroxidase activity. For WT-1 immunostaining, the specimens were incubated for 2 h at room temperature with rabbit polyclonal anti-WT-1 antibody (Sigma). After washing with PBS, the sections were incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat polyclonal anti-rabbit IgG (Jackson Immunoresearch). For immunostaining of αSMA, mouse monoclonal anti-αSMA antibody (DAKO Japan, Kyoto, Japan) was incubated with HRP-conjugated goat polyclonal anti-mouse IgG (EnVision+) for 1 h and thereafter mixed with normal mouse serum for another 1 h. The complex of primary and secondary antibodies was applied to the kidney sections. The sections were developed with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. Nonimmune goat serum was used as a negative control.

Cell culture

An immortalized mouse podocyte cell line, MPC5, was a kind gift from Dr. Peter Mundel (27) and cultured as described previously (28). In brief, podocytes were cultured with RPMI1640 medium (Sigma) supplemented with 10% fetal calf serum (Sanko Junyaku, Tokyo, Japan) and antibiotics on 0.01% collagen type I (Koken, Tokyo, Japan)-coated dish. Before the experiment, cells were differentiated under nonpermissive condition (without interferon-γ in media, at 37°C) for two weeks without passage and cultured with RPMI1640 containing 0.5% bovine serum albumin (Sigma) for the last 24 h.

Western blot analysis

Cultured podocytes were stimulated by 5 ng/ml of recombinant human TGF-β1 (R&D systems, Minneapolis, MN) for 24 h at 37°C. Cells were lysed on ice in solution containing 20 mM Tris-HCl (pH 7.5), 138 mM NaCl, 10% glycerol, 1% Igepal (Sigma), 2 mM ethylenediaminetetraacetic acid and protease inhibitor cocktail (Sigma). Samples (30 μg protein/lane) were separated by 12.5% SDS-PAGE, and Western blotting was performed as described previously (29) using anticonnexin43 antibody (Sigma).

Northern blot analysis

Total RNA from the whole kidney was extracted using the acid guanidinium-phenol-chloroform method. Northern blot analysis was performed as described (24, 25). In brief, 30 μg of total RNA was electrophoresed on a 1.0% agarose gel and transferred to a nylon membrane (Biodyne, Pall BioSupport, Port Washington, NY). The cDNA fragments of mouse α1 (IV) collagen (nucleotides 5808 to 6165), fibronectin (5 to 755) and TGF-β1 (1142 to 1546), which were prepared by reverse-transcription PCR using mouse kidney mRNA, were used as probes.

Leptin injection experiments

Continuous subcutaneous leptin administration was performed as described previously (23). In brief, infusion with recombinant mouse leptin (Genzyme/Techne, Minneapolis, MN) (1 μg/g body weight per day) or vehicle (saline) was begun in 10-month-old A-ZIPTg/+ using an

osmotic minipump (Alzet model 1002; Alza, Palo Alto, CA) on the first day of the experiment. During the 14-day infusion experiment, food intake was calculated in the leptin-treated group, and pair-feeding was performed in the vehicle-treated group. On the 14th day of the experiment, mice were killed, and blood, urine and kidney samples were examined.

Statistical analysis

Data are expressed as the mean ± SE. Statistical analysis was performed using ANOVA followed by Scheffe's test. P value < 0.05 was considered statistically significant.

RESULTS

Characteristics of renal injury in A-ZIP/F-1 mice

Table 1 shows anatomical data and blood and urine parameters of A-ZIPTg/+ and +/+ at 4 and 10 months of age. Ten-month-old A-ZIPTg/+ had higher body weight and kidney weight than +/+. Serum glucose, total cholesterol, and triglyceride levels were much higher in A-ZIPTg/+ than those in +/+ at both time points. Serum creatinine and urea nitrogen levels were comparable between the strains. Urinary albumin excretion in A-ZIPTg/+ was significantly increased at 4 months (14-fold of +/+) and was further increased at 10 months (81-fold of +/+).

Histological analysis revealed marked glomerular hypertrophy and mesangial expansion in A-ZIPTg/+ as compared with +/+ (Fig. 1, *A–C*). These changes were diffuse and global and compatible with clinical diabetic nephropathy. Quantitative analysis revealed that the glomerular cross-sectional area in A-ZIPTg/+ was 1.6-fold higher than that in +/+ at 4 months and 1.9-fold higher at 10 months of age (Fig. 1*D*). No apparent macrolipid deposits were observed in the kidney of A-ZIPTg/+ by Oil Red O staining (Fig. 1*E*), although A-ZIPTg/+ showed remarkably high levels of serum triglyceride and cholesterol. Electron microscopic analysis revealed diffuse thickening of glomerular basement membrane, foot process effacement of podocytes, and marked expansion of mesangial matrix in A-ZIPTg/+ (Fig. 1*F*) as compared with +/+ (Fig. 1*G*). These findings are well compatible with functional and histological features of clinical diabetic nephropathy.

Glomerular extracellular matrix accumulation in A-ZIP/F-1 mice

The up-regulation of TGF-β1 is postulated to play a pivotal role in facilitating extracellular matrix gene activation and subsequent glomerulosclerosis in diabetic glomerular injury (5, 6). We therefore examined the expression of TGF-β1 and extracellular matrix proteins in glomeruli of this model by immunostaining (Fig. 2) and Northern blotting (Fig. 3). Immunofluorescence study revealed the intense staining of collagen type IV (Fig. 2*B*), fibronectin (Fig. 2*D*) and collagen type I (Fig. 2*F*) along with TGF-β1 (Fig. 2*H*) primarily in the expanded mesangial area of 10-month-old A-ZIPTg/+ as compared with +/+ (Fig. 2*A*, *C*, *E* and *G*). The de novo immunostaining of αSMA was observed in the mesangial area of A-ZIPTg/+ (Fig. 2*J*), while the staining was confined to smooth muscle cells of the arteriole in +/+ (Fig. 2*I*). Northern blot analysis revealed significant up-regulation of renal collagen type IV, fibronectin and TGF-β1 gene expression in A-ZIPTg/+ aged 4 and 10 months as compared with +/+ (Fig. 3). These findings are well consistent with the characteristics of diabetic renal hypertrophy described

previously (5, 6), and indicate that A-ZIPTg/+ can serve as a new mouse model of diabetic nephropathy.

Changes of podocyte markers in A-ZIP/F-1 mice

Recent studies have indicated that podocyte loss contributes to the progression of diabetic nephropathy in type II diabetes patients (10, 11). Therefore, in order to elucidate podocyte injury in this diabetic model, we immunostained various podocyte markers. Nephrin is a component of the slit membrane, and linear staining of nephrin along the capillary wall was observed in +/+ (Fig. 4*A*). The staining was apparently weak and sparse in 4-month-old A-ZIPTg/+ (Fig. 4*B*) and only faint staining was observed in 10-month-old A-ZIPTg/+ (Fig. 4*C*). Conversely, punctate staining of connexin43, a gap junction protein, along the capillary wall appeared in 4-month-old A-ZIPTg/+ (Fig. 4*E*), although the staining was observed only in the extraglomerular mesangium in +/+ (Fig. 4*D*). Connexin43 immunostaining was also observed in the intraglomerular mesangial area in 10-month-old A-ZIPTg/+ (Fig. 4*F*). The number of WT-1-positive cells (podocytes) was apparently reduced in glomeruli of A-ZIPTg/+ (Fig. 4*H*) than +/+ (Fig. 4G) at 10 months of age. Quantitative analysis showed significant reduction in podocyte number in A-ZIPTg/+ as compared with +/+ as early as 4 months of age (Fig. 4*I*). In cultured differentiated podocytes, treatment with TGF-β1 stimulates connexin43 expression (Fig. 4*J*). These findings indicate podocyte damages in this diabetic nephropathy model.

Prevention of renal injury in A-ZIP/F-1 mice by crossing leptin transgenic mice

Since we have already reported that administration of leptin almost completely normalized metabolic disorders in A-ZIPTg/+ (23), we crossed A-ZIPTg/+ with LepTg/+ in order to investigate the long-term effects of leptin on the development of renal injury. Mean serum leptin concentrations of F1 mice obtained by crossing A-ZIPTg/+ with LepTg/+ were as follows: +/+, 7.7 ng/ml; A-ZIPTg/+, 1.8 ng/ml; LepTg/+, 31 ng/ml; A-ZIPTg/+:LepTg/+, 52 ng/ml (23). LepTg/+ showed normal renal function (Table 2) and apparently normal histology (Fig. 5*B*) at 10 months of age, comparable to +/+ (Table 2 and Fig. 5*A*). The double transgenic LepTg/+:A-ZIPTg/+ exhibited not only marked inhibition of albuminuria (Table 2), but also complete prevention of glomerular hypertrophy and mesangial expansion compared with A-ZIPTg/+ (Fig. 5*C* and *D*). Loss of podocyte number and up-regulation of extracellular matrix proteins and TGF-β1 gene expression were also completely inhibited (Fig. 5*E–I*). LepTg/+ showed significant up-regulation of collagen-type IV and fibronectin gene expression as compared with +/+, but the extent was not remarkable. These findings indicate that chronic overexpression of leptin effectively prevented the development of renal injury in A-ZIPTg/+.

Reversal of renal injury in A-ZIP/F-1 mice by continuous leptin administration

To further investigate whether advanced diabetic nephropathy is reversible, we examined the effects of continuous leptin administration on renal injury in 10-month-old A-ZIPTg/+. Mean serum leptin concentrations after the 2-week treatment of leptin or saline (vehicle) were as follows: the saline-treated group, 1.3 ng/ml; the leptin-treated group, 40 ng/ml. Metabolic disorders were markedly ameliorated in the leptin-treated group as compared with the saline-treated group (Table 3). Although kidney weight was not changed (Table 3), glomerular hypertrophy was significantly ameliorated by leptin treatment (Fig. 6*A–C*). TGF-β1

immunostaining in glomeruli was markedly suppressed (Fig. 6*D* and *E*), and renal TGF-β1 and fibronectin gene expression was also down-regulated by leptin treatment (Fig. 6*F*). Moreover, the leptin-treated group exhibited 66% reduction of albuminuria as compared with the saline-treated group (Table 3). These findings indicate that leptin treatment can reverse renal injury of A-ZIPTg/+ functionally and histologically, at least partly, even at the advanced stage of diabetic nephropathy.

DISCUSSION

In the present study, A-ZIP/F-1 mice exhibited massive proteinuria as well as diffuse and global glomerular lesions characterized by marked glomerular hypertrophy, mesangial expansion, foot process effacement, and thickening of glomerular basement membrane. TGF-β1 as well as extracellular matrix gene expression and protein were markedly increased in the kidney of A-ZIP/F-1 mice. These findings in A-ZIP/F-1 mice are well consistent with functional and histological features of clinical diabetic nephropathy, and also seem to be more pronounced than those in other diabetic models. These phenotypic changes in A-ZIP/F-1 mice may overcome the strain difference, since A-ZIP/F-1 mice used in the study of Tables 1 and 3 were FVB/N background and those in the study of Table 2 were hybrid of FVB/N and C57BL/6J background. In fact, there were small differences in body weight and several blood parameters between the strains. We therefore propose that A-ZIP/F-1 mice can be a new mouse model of human diabetic nephropathy, although lipoatrophic diabetes is not common with regard to the pathogenesis. Similarly to most other animal models of diabetic nephropathy, there was no typical nodular lesion developed until 10 months of age in this model.

In lipoatrophic diabetes and A-ZIP/F-1 mice, deficiency of adipose tissue causes insulin resistance, hyperglycemia, and dyslipidemia, although the underlying mechanisms are not fully understood. We have already shown that transgenic overexpression of leptin rescues these metabolic disorders in A-ZIP/F-1 mice (23), indicating the role of leptin in the pathophysiology of lipoatrophic diabetes. In the present study, leptin completely prevented the development of renal injury in A-ZIP/F-1 mice throughout the observation period. Moreover, continuous administration of leptin significantly ameliorated not only metabolic disorders but also established renal injuries, that is, glomerular hypertrophy with marked mesangial expansion, up-regulation of TGF-β1 and extracellular matrix, and proteinuria. These findings suggest the potential of leptin to prevent or to treat renal injury in clinical lipoatrophic diabetes. Several previous studies showed the reversibility of established diabetic nephropathy by pancreas transplantation (3, 4, 30) or with anti-TGF-β1 antibody (31). Our data reveal significant reduction of TGF-β1 expression in the glomeruli after the treatment of leptin, suggesting the causative role of TGF-β1 in the progression of renal injury in A-ZIP/F-1 mice as well. Leptin treatment also significantly reduced proteinuria, which was not achieved by the anti-TGF-β1 antibody treatment (8, 31). Leptin treatment against A-ZIP/F-1 mice may mimic pancreas transplantation rather than anti-TGF-β1 antibody since leptin treatment almost completely normalizes metabolic disorders, including hyperglycemia and hyperlipidemia. Further study is needed to explore whether renal injury regresses to normal by the long-term treatment of leptin.

Whether leptin directly acts on the kidney and ameliorates diabetic nephropathy is another issue to be discussed. Wolf et al. have reported that the short form of leptin receptor (Ob-Ra), but not

long form of leptin receptor (Ob-Rb), is expressed in mesangial cells and glomerular endothelial cells (32). They have also reported that high-dose leptin administration to rats up-regulates TGF-β1 and collagen IV expression in the glomeruli and causes mild proteinuria, suggesting a possible pathogenic role of leptin in obesity-related glomerulopathy (33). We analyzed gene expression of Ob-Ra and Ob-Rb by RT-PCR in cultured mesangial cells and podocytes and confirmed that Ob-Ra is a predominant leptin receptor not only in mesangial cells but also in podocytes, without a significant expression of Ob-Rb (data not shown). Although circumferential, it may be unlikely that leptin caused beneficial effects directly on podocytes in the current study. Recent reports indicate that leptin also exerts profibrotic action in the liver (34, 35). Consistent with these reports, LepTg/+ showed statistically significant up-regulation of collagen type IV and fibronectin gene expression at 10 months of age, although no apparent histological and functional alterations were observed in LepTg/+ as compared with the wild type. It is noteworthy that the serum leptin concentrations in LepTg/+ and in mice with continuous leptin administration are within the levels almost comparable to those of the patients with morbid obesity (22). Collectively, leptin seems to be primarily profibrotic on the glomerulus but have potential to prevent/reverse renal damage by normalizing metabolic disorders, including hyperglycemia and hyperlipidemia.

Concerning the clinical application of leptin treatment on diabetic nephropathy, it is important to clarify the effects of leptin on diabetes. We have reported that leptin treatment alone is effective in KKA^{y} mice, a model of type 2 diabetes, at younger ages when they are of normal weight (36). In contrast, hyperleptinemia does not prevent the progression of diabetes in KKA^{y} mice at older ages when they develop obesity, at least partly by reason of obesity-induced resistance to leptin. In calorically restricted KKA^{y} mice, however, leptin can accelerate the recovery from diabetes. It is therefore conceivable that leptin treatment with diet therapy might be effective in renal injury of obese type 2 diabetic patients. Furthermore, we have recently revealed that a combination therapy of leptin and insulin is effective for the treatment of hyperglycemia in streptozotocin-induced diabetic mice, although administration of leptin alone has no effects on blood glucose concentrations (37). Taken together, these data suggest that leptin may be therapeutically useful as an anti-diabetic agent for various types of diabetes.

Blood pressure plays a pivotal role in diabetic nephropathy. It is therefore important to elucidate a relationship between blood pressure and renal damage in A-ZIP/F-1 mice. We have shown that leptin activates sympathetic nervous system and elevates blood pressure (38). Although serum leptin concentration of A-ZIP/F-1 mice is extremely low, our preliminary data showed that A-ZIP/F-1 mice exhibit mild blood pressure elevation (mean systolic blood pressure (SBP) 111 mmHg) as compared with wild-type mice (mean SBP 105 mmHg). Double transgenic mice further showed slightly higher blood pressure (mean SBP 114 mmHg) comparable to the level of leptin transgenic mice (mean SBP 115 mmHg). Therefore, it may be less conceivable that blood pressure elevation does play an important role in the development of renal injury in A-ZIP/F-1 mice, since leptin transgenic mice and double transgenic mice showed no apparent renal injury. The precise mechanisms of mild blood pressure elevation in A-ZIP/F-1 mice are currently unknown.

Consistent with human diabetic nephropathy (10, 11), the number of WT-1-positive podocytes was significantly reduced in A-ZIP/F-1 mice. In parallel with loss of WT-1-positive podocytes,

immunostaining of nephrin was gradually decreased, as previously reported in several proteinuric states, including clinical diabetic nephropathy (12, 13, 26). In contrast, de novo immunostaining of connexin43 was clearly observed along the capillary wall as early as 4 months old. Recently, connexin43 has been reported to be one of the earliest responses against podocyte injury in a nondiabetic proteinuric state (39). The present study indicates that podocyte injury was evident in the early stage of this diabetic nephropathy model.

In summary, we demonstrate that A-ZIP/F-1 mice exhibit typical renal lesions observed in diabetic nephropathy. We also reveal that leptin can prevent and reverse glomerular injuries in A-ZIP/F-1 mice, suggesting the potential therapeutic usefulness of leptin for treating clinical nephropathy in lipoatrophic diabetes. Furthermore, this mouse model will provide us a new tool to analyze the molecular mechanism underlying the progression, as well as reversal, of diabetic nephropathy.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Peter Mundel (Albert Einstein College of Medicine, Bronx, NY) for the immortalized podocyte cell line, Drs. Shunji Nakatsuji, Yuji Oishi and Toshikazu Ogawa (Fujisawa Pharmaceutical, Osaka, Japan) for electron microscopic analysis and immunohistochemistry, Ms. Junko Nakamura, Dr. Minako Kiso and Dr. Kozue Uchio-Yamada for technical assistance, and Ms. Shigeka Doi, Ms. Atsuko Sonoda and Mr. Junichi Nomura for secretarial assistance. This work was supported in part by research grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology, the Japanese Ministry of Health, Labor and Welfare, Takeda Science Foundation, Foundation for Total Health Promotion, Smoking Research Foundation, the Tanabe Medical Frontier Conference, and the Salt Science Research Foundation.

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Received May 6, 2004; accepted September 8, 2004.

Table 1

Body weight, kidney weight, blood, and urine data of A-ZIPTg/+ and +/+

	A-ZIPTg/+		+/+	
Variables	4 months	10 months	4 months	10 months
Body weight (g)	32.5 ± 1.5	41.6 ± 0.9*$^{\#}$	32.4 ± 0.9	37.9 ± 0.3
Kidney weight (g)	0.28 ± 0.02	0.42 ± 0.01**$^{\#\#}$	0.24 ± 0.01	0.24 ± 0.01
Serum glucose (mg/dl)	406 ± 37**	455 ± 24**	154 ± 10	148 ± 6
Serum total CHO	192 ± 28*	247 ± 26*	136 ± 5	139 ± 5
Serum triglyceride	479 ± 63*	548 ± 84*	138 ± 15	118 ± 14
Serum creatinine	0.11 ± 0.02*	0.11 ± 0.01	0.07 ± 0.01	0.10 ± 0.01
Serum UN (mg/dl)	29.1 ± 2.6	26.2 ± 1.8	24.2 ± 1.4	23.2 ± 1.9
Albuminuria (μg/mgCr)	704 ± 105**	3417 ± 437*$^{\#\#}$	50 ± 6	42 ± 5

Values are expressed as the mean ± SE for 4-month-old A-ZIPTg/+ (n=8), 10-month-old A-ZIPTg/+ (n=15), 4-month-old +/+ (n=8), and 10-month-old +/+ (n=6). *$P < 0.05$, **$P < 0.01$ vs. +/+, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. 4-month-old A-ZIPTg/+. CHO; cholesterol, UN; urea nitrogen, Cr; creatinine.

Table 2

Body weight, kidney weight, blood, and urine data of 10-month-old F1 mice

Variables	+/+	LepTg/+	A-ZIPTg/+	LepTg/+: A-ZIPTg/+
Body weight (g)	41.9 ± 2.2	40.7 ± 2.5	48.3 ± 1.9*	37.7 ± 2.4$^{\#}$
Kidney weight (g)	0.25 ± 0.02	0.27 ± 0.03	0.48 ± 0.05**	0.30 ± 0.04$^{\#}$
Serum glucose (mg/dl)	175 ± 8	164 ± 18	473 ± 63**	186 ± 23$^{\#\#}$
Serum total CHO (mg/dl)	112 ± 3	101 ± 5	291 ± 25**	122 ± 11$^{\#\#}$
Serum triglyceride (mg/dl)	87 ± 10	73 ± 20	412 ± 22**	141 ± 20$^{\#\#}$
Serum creatinine (mg/dl)	0.06 ± 0.01	0.08 ± 0.02	0.11 ± 0.02*	0.05 ± 0.01$^{\#}$
Serum UN (mg/dl)	21.1 ± 1.6	23.5 ± 1.2	27.6 ± 0.3*	21.9 ± 1.4$^{\#}$
Albuminuria (μg/mgCr)	52 ± 8	62 ± 7	3074 ± 617**	141 ± 20$^{\#\#}$

F1 mice were obtained by crossing A-ZIPTg/+ with LepTg/+. Values are expressed as the mean ± SE for 10-month-old +/+ (n=9), LepTg/+ (n=5), A-ZIPTg/+ (n=5), and LepTg/+:A-ZIPTg/+ (n=5). *$P < 0.05$, **$P < 0.01$ vs. +/+ and LepTg/+, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. A-ZIPTg/+. CHO; cholesterol, UN; urea nitrogen, Cr; creatinine.

Table 3

Body weight, kidney weight, blood, and urine data of 10-month-old A-ZIPTg/+ treated with either leptin or saline

Variables	Saline	A-ZIPTg/+ Leptin
Body weight (g)	39.8 ± 0.6	40.3 ± 0.7
Kidney weight (g)	0.39 ± 0.01	0.37 ± 0.01
Serum glucose (mg/dl)	434 ± 40	195 ± 23*
Serum total CHO (mg/dl)	220 ± 18	139 ± 15*
Serum triglyceride (mg/dl)	610 ± 80	193 ± 33*
Serum creatinine (mg/dl)	0.11 ± 0.01	0.09 ± 0.01
Serum UN (mg/dl)	25.3 ± 1.3	22.8 ± 1.2
Albuminuria (μg/mgCr)	2072 ± 324	713 ± 128**

Values are expressed as the mean ± SE for leptin-treated A-ZIPTg/+ (n=7) and saline-treated A-ZIPTg/+ (n=7). *P < 0.01 vs. saline-treated A-ZIPTg/+, **P < 0.05. CHO; cholesterol, UN; urea nitrogen, Cr; creatinine.