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

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Leptin acts as an adipocyte-derived blood-borne satiety factor that can increase glucose metabolism. To elucidate the therapeutic implications of leptin for obesity-associated diabetes, we crossed transgenic skinny mice overexpressing leptin (Tg/+), which we have developed recently, and lethal *yellow* KKA^y mice (A^y/+), a genetic model for obesity-diabetes syndrome, and examined the metabolic phenotypes of F_1 animals. At 6 weeks of age, plasma leptin concentrations in Tg/+ mice with the A^y allele (Tg/+:A^y/+) were significantly higher than those in A^y/+ mice. Although no significant differences in body weight were noted among Tg/+:A^y/+ mice, A^y/+ mice, and their wild-type lean littermates (+/+), glucose and insulin tolerance tests revealed increased glucose tolerance and insulin sensitivity in Tg/+:A^y/+ compared with A^y/+ mice. However, at 12 weeks of age, when plasma leptin concentrations in A^y/+ mice were comparable to those in Tg/+:A^y/+ mice, Tg/+:A^y/+ mice developed obesity-diabetes syndrome similar to that of A^y/+ mice. Body weights of 12-week-old Tg/+:A^y/+ and A^y/+ mice were reduced to those of +/+ mice by a 3-week food restriction; when plasma leptin concentrations remained high in Tg/+:A^y/+ mice but were markedly reduced in A^y/+ and +/+ mice, glucose tolerance and insulin sensitivity in Tg/+:A^y/+ mice were markedly improved as compared with A^y/+ and +/+ mice. The present study demonstrates that hyperleptinemia can delay the onset of impaired glucose metabolism and accelerate the recovery from diabetes during caloric restriction in Tg/+:A^y/+ mice, thereby suggesting the potential usefulness of leptin in combination with a long-term caloric restriction for the treatment of obesity-associated diabetes. *Diabetes* 48:1615–1622, 1999

Leptin is an adipocyte-derived blood-borne satiety factor that decreases food intake and increases energy expenditure, thereby leading to a marked reduction in body weight (1–5). Because of its potent biological effects, the potential usefulness of leptin for the treatment of obesity and related metabolic disorders has attracted the interest of many investigators. However, a number of studies have demonstrated that plasma leptin concentrations are elevated in several models of rodent and human obesity in proportion to the degree of adiposity, suggesting a state of "leptin resistance" in obesity (6–11).

Evidence has accumulated indicating that leptin can stimulate glucose metabolism independent of body weight changes via central as well as peripheral mechanisms (12–14). Several studies have shown that exogenously administered leptin not only enhances glucose metabolism in normal-weight nondiabetic rodents (11–15) but also ameliorates impaired glucose metabolism in obese-diabetic rodent models with a reduced amount of leptin or leptin deficiency (16–18). However, the therapeutic usefulness of leptin in obesity-associated diabetes, which is often accompanied by hyperleptinemia, has not been fully elucidated.

For the treatment of obesity-associated diabetes, it is universally accepted that dietary management is initially used with specific emphasis on weight reduction, because weight reduction leads to improvement in deteriorated glucose metabolism with a concomitant decrease in other metabolic and cardiovascular risk factors (19). Taking the beneficial effects of leptin on glucose homeostasis into account, a rapid fall in plasma leptin concentrations during caloric restriction or fasting (20–22) might be disadvantageous to therapeutic efficacy for diabetes. Accordingly, we hypothesized that persistent hyperleptinemia during caloric restriction is profitable to improve impaired glucose metabolism in obesity-associated diabetes.

We have recently created transgenic mice on a C57BL/6J background with overexpression of leptin under the control of the liver-specific promoter and demonstrated that chronic overexpression of leptin results in complete disappearance of white and brown adipose tissue in mice (23). We have,

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ANOVA, analysis of variance; GTT, glucose tolerance test; ITT, insulin tolerance test; RIA, radioimmunoassay.

therefore, called these animals transgenic skinny mice overexpressing leptin (Tg/+). Tg/+ mice exhibit increased glucose tolerance and insulin sensitivity accompanied by an activation of insulin signaling in the skeletal muscle and liver (23). Accordingly, Tg/+ mice may serve as a useful model system with which to assess the antidiabetic mechanism of action of leptin.

In the present study, to explore the therapeutic implications of leptin for obesity-associated diabetes, we performed genetic crosses between Tg/+ and lethal *yellow* obese mice (A^y/+), a well-characterized experimental model for obesity-associated diabetes (24,25), and we examined the metabolic phenotypes of F_1 mice with four genotypes: Tg/+ with the A^y/+ allele (Tg/+:A^y/+), Tg/+, A^y/+, and wild-type lean littermates (+/+). We here analyzed F_1 mice at 6 weeks of age, when Tg/+:A^y/+ and A^y/+ mice were of normal weight, and at 12 weeks of age, when Tg/+:A^y/+ and A^y/+ mice developed marked obesity. Furthermore, to elucidate the effect of hyperleptinemia on glucose metabolism during caloric restriction, we also analyzed 12-week-old +/+, A^y/+, and Tg/+:A^y/+ mice after a 3-week food restriction.

RESEARCH DESIGN AND METHODS

Animals. Generation of Tg/+ mice on a C57BL/6J background has been reported elsewhere (23). The 8-week-old male KKA^y mice (A^y/+), in which the A^y gene is transferred into the original Japanese KK strain (26), were purchased from Japan CLEA (Tokyo). We crossed female Tg/+ mice with male A^y/+ mice to obtain F_1 animals because female A^y/+ mice are infertile (24,27). Four genotypes of F_1 mice—Tg/+:A^y/+, Tg/+, A^y/+, and +/+ (on the C57BL/6J and KK hybrid background)—were determined by Southern blot analysis of tail DNAs using the mouse leptin cDNA fragment as a probe (1,28,29). Male F_1 mice with four genotypes were used in the present study. Mice were housed in a temperature-, humidity-, and light-controlled room (12-h light/12-h dark cycle) and allowed free access to water and standard rat food (CE-2, 352 kcal/100 g, Japan CLEA). All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Measurements of body weight and cumulative food intake. Body weight was measured daily, beginning at 4 weeks of age. Cumulative food intake was measured daily over a 2-week period using 6- and 12-week-old male F_1 mice maintained in individual metabolic cages.

Plasma leptin, glucose, and insulin concentrations. Blood was sampled from the retro-orbital sinus of mice when fed ad libitum at 9:00 A.M. Plasma leptin concentrations were determined using the radioimmunoassay (RIA) for mouse leptin (Linco Research Immunoassay; St. Louis, MO). Plasma glucose and insulin concentrations were determined by the glucose oxidase method with a reflectance glucometer (One Touch II; Lifescan, Milpitas, CA), and the RIA was determined with rat insulin standards (Linco).

Glucose and insulin tolerance tests. For the glucose tolerance test (GTT), after an 8-h fast, male F_1 mice were injected with 1.0 mg/g glucose i.p. For the insulin tolerance test (ITT), after a 2-h fast, mice were injected with 0.5 mU/g human regular insulin i.p. (Nobolin R; Novo Nordisk, Bagsvaerd, Denmark). Blood was sampled from the tail vein before and 15, 30, 60, and 90 min after the injection. Plasma glucose concentrations were determined as described above.

Food restriction experiments. Based on the daily food intake of F_1 animals at 12 weeks of age, they were provided with 60% of the amount of food consumed regularly. During a 3-week food restriction, body weight change was measured. At the end of the experiments, blood was sampled and plasma leptin, glucose, and insulin concentrations were determined. Glucose and ITTs were also performed as described above.

Statistical analysis. All data were expressed as means ± SE. Statistical significance of differences was assessed by analysis of variance (ANOVA) with repeated measures analysis (Statview 4.01; Abacus Concepts, Berkeley, CA), ANOVA with the Bonferroni-Dunn test, and Student's *t* test, where applicable.

RESULTS

Body weight changes in F_1 mice. We examined the time course of body weight changes in F_1 animals with four genotypes (Tg/+:A^y/+, Tg/+, A^y/+, and +/+), from 4 to 30 weeks of age, that are produced by genetic crosses between Tg/+ and A^y/+ mice (Fig. 1*A*). No significant differences in body weight were noted among genotypes at birth. At 4 weeks of age, Tg/+:A^y/+ and Tg/+ mice developed no apparent adipose tissue throughout the body. At 6 weeks of age or thereafter, Tg/+ mice gained ~20 to 30% less weight than +/+ mice. However, by 6 weeks of age, Tg/+:A^y/+ mice gradually developed adiposity compared with Tg/+ mice. No significant differences in body weight were observed among +/+, A^y/+, and Tg/+:A^y/+ mice at 6 weeks of age. Tg/+:A^y/+ mice developed

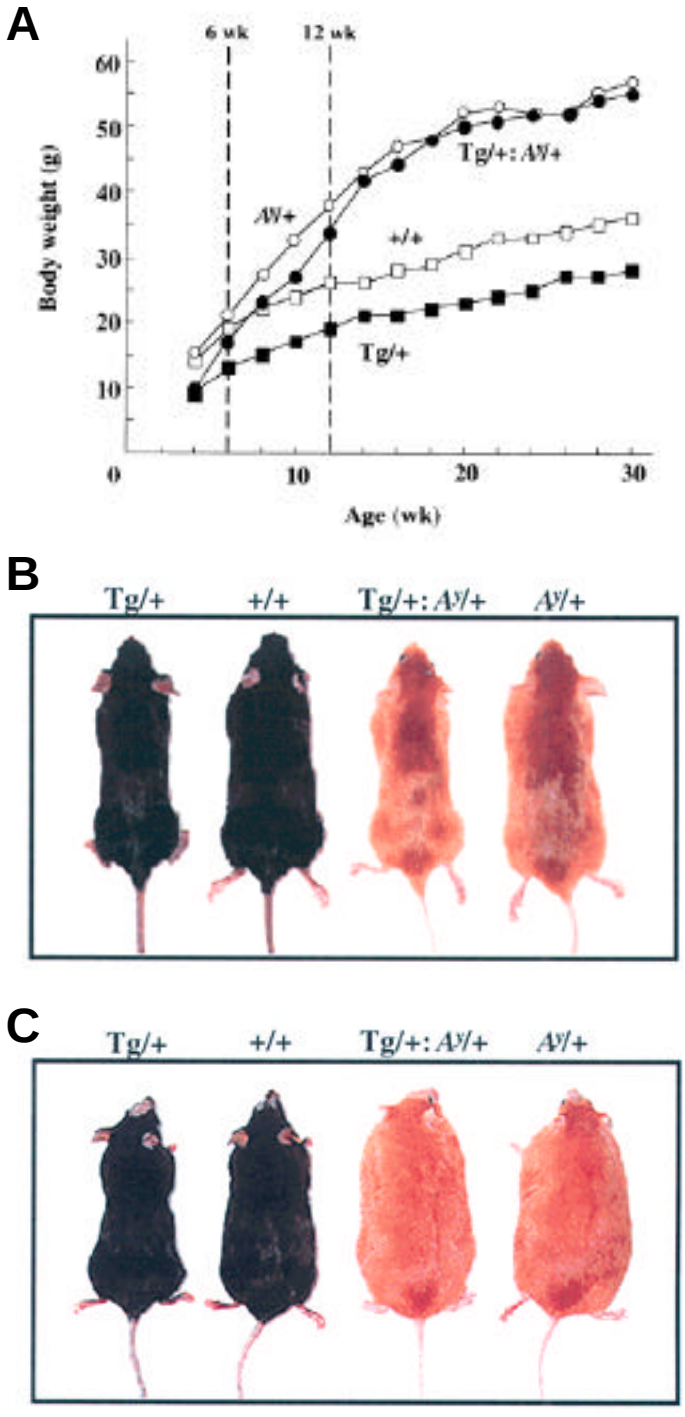

FIG. 1. Body weight changes and gross appearance in male F_1 mice. *A*: Time course of body weight changes of +/+ (□), Tg/+ (■), A^y/+ (○), and Tg/+:A^y/+ (●) mice from 4 to 30 weeks of age. Data are expressed as the mean values of 10 mice in each genotype. *B*: Gross appearance of 6-week-old male F_1 mice. *C*: Gross appearance of 12-week-old male F_1 mice.

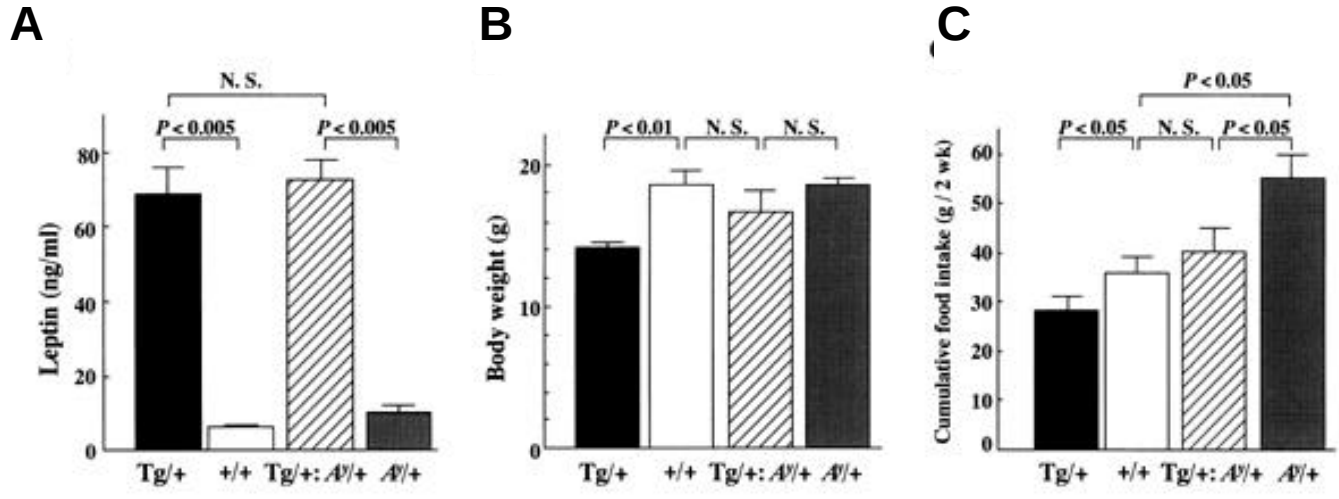

FIG. 2. Plasma leptin concentrations, body weight change, and food intake in 6-week-old male Tg/+ (■), +/+ (□), Tg/+:A^y/+ (▨), and A^y/+ (■) mice. Values are expressed as means ± SE (n = 10 in each genotype). Statistical significance of differences is assessed by ANOVA with the Bonferroni-Dunn test. NS, not significant. *A*: Plasma leptin concentrations in 6-week-old F_1 mice. *B*: Body weight in 6-week-old F_1 mice. *C*: Cumulative food intake in 6-week-old F_1 mice for 2 weeks.

obesity similar to that of A^y/+ mice by 12 weeks of age, and thereafter, both genotypes remained markedly obese compared with +/+ and Tg/+ mice. In the present study, we analyzed a series of metabolic phenotypes of F_1 animals at 6 weeks of age, when Tg/+:A^y/+ and A^y/+ mice were of normal weight (Fig. 1*B*), and at 12 weeks of age, when both genotypes were overweight compared with +/+ mice (Fig. 1*C*).

Plasma leptin concentrations, body weight change, and food intake in 6-week-old F_1 mice. Plasma leptin concentrations in 6-week-old Tg/+ mice were 70.3 ± 6.1 ng/ml; these levels were ~12-fold higher than those in +/+ mice (6.0 ± 0.8 ng/ml) (n = 10, P < 0.005) (Fig. 2*A*). Plasma leptin concentrations in A^y/+ mice (9.8 ± 1.4 ng/ml) were roughly equivalent to those in +/+ mice, whereas those in Tg/+:A^y/+ mice (74.5 ± 4.8 ng/ml) were approximately eightfold higher than those in A^y/+ mice (P < 0.005). There was no statistical difference in plasma concentrations between Tg/+ and Tg/+:A^y/+ mice. At 6 weeks of age, no significant differences in body weight were observed among +/+, A^y/+, and Tg/+:A^y/+ genotypes (18.6 ± 1.0, 18.5 ± 0.6, and 16.8 ± 1.5 g, respectively) (n = 10) (Fig. 2*B*). Tg/+ mice gained 23% less weight than did +/+ mice (P < 0.01). Cumulative food intake was measured using 6-week-old F_1 animals over a 2-week period. Food intake was reduced significantly in 6-week-old Tg/+ mice compared with +/+ mice (Fig. 2*C*). No significant differences in food intake were noted between +/+ and Tg/+:A^y/+ mice. However, cumulative food intake of A^y/+ mice was increased by 50% relative to +/+ mice (P < 0.05), whereas that of Tg/+:A^y/+ mice was reduced significantly compared with A^y/+ mice (P < 0.05).

Glucose metabolism in 6-week-old F_1 mice. We determined plasma glucose and insulin concentrations in 6-week-old F_1 animals (Fig. 3*A*). No significant differences in plasma glucose concentrations were noted among genotypes at 6 weeks of age (Tg/+, +/+, Tg/+:A^y/+, and A^y/+: 9.4 ± 0.4, 8.7 ± 0.8, 9.0 ± 0.3, and 8.8 ± 0.7 mmol/l, respectively) (n = 10). Plasma insulin concentrations were decreased significantly in 6-week-old Tg/+ mice compared with +/+ mice (91 ± 12 vs. 209 ± 17 pmol/l) (P < 0.01). Plasma insulin concentrations in Tg/+:A^y/+ mice tended to be higher than those in +/+ mice

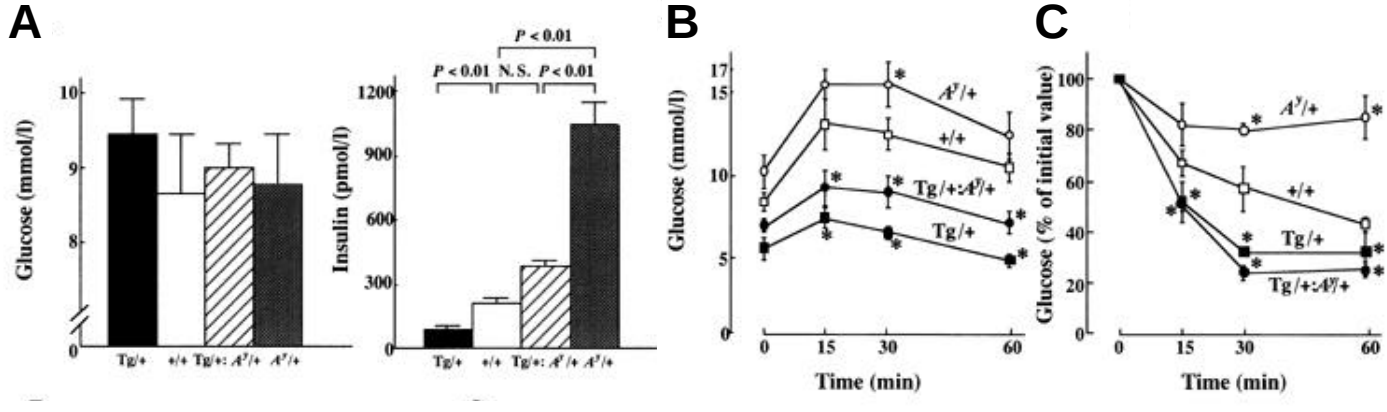

FIG. 3. Glucose metabolism in 6-week-old male F_1 mice. *A*: Plasma glucose and insulin concentrations in 6-week-old Tg/+ (■), +/+ (□), Tg/+:A^y/+ (▨), and A^y/+ (■) mice. Values are expressed as means ± SE (n = 10 in each genotype). Statistical significance of differences is assessed by ANOVA with the Bonferroni-Dunn test. NS, not significant. *B*: GTT in 6-week-old male +/+ (□), Tg/+ (■), A^y/+ (○), and Tg/+:A^y/+ (●) mice. *P < 0.05 vs. +/+ mice assessed by ANOVA with repeated measures analysis. *C*: ITT in 6-week-old male F_1 mice. Plasma glucose concentrations were expressed as a percentage of the initial values in each genotype. *P < 0.05 vs. +/+ mice assessed by ANOVA with repeated measures analysis.

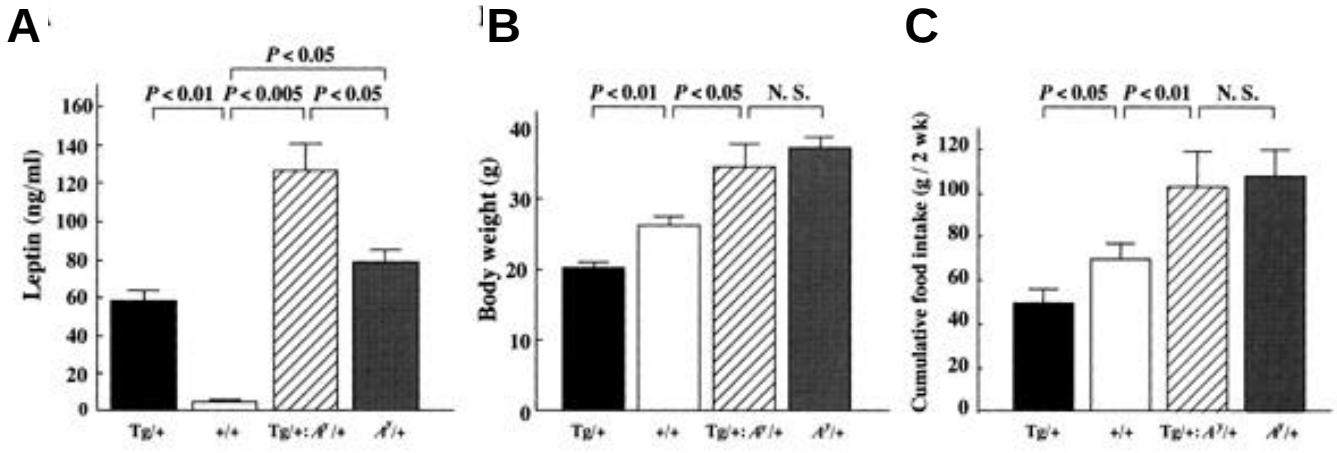

FIG. 4. Plasma leptin concentrations, body weight change, and food intake in 12-week-old male Tg/+ (■), +/+ (□), Tg/+:A^y/+ (▨), and A^y/+ (■) mice. Values are expressed as means ± SE (n = 10 in each genotype). Statistical significance of differences was assessed by ANOVA with the Bonferroni-Dunn test. NS, not significant. *A*: Plasma leptin concentrations in 12-week-old F_1 mice. *B*: Body weight in 12-week-old F_1 mice. *C*: Cumulative food intake in 12-week-old F_1 mice for 2 weeks.

(315 ± 44 vs. 209 ± 17 pmol/l); however, no significant differences were observed between these genotypes. A^y/+ mice exhibited marked hyperinsulinemia (1,046 ± 109 pmol/l) compared with +/+ and Tg/+:A^y/+ mice ($P < 0.01$). To further evaluate glucose homeostasis in 6-week-old F_1 animals, we performed GTTs and ITTs. GTTs revealed that plasma glucose elevation is blunted significantly in Tg/+ compared with +/+ animals ($P < 0.05$) (Fig. 3*B*). When injected intraperitoneally with glucose, Tg/+:A^y/+ animals also showed a response as good as that of Tg/+ animals. However, plasma glucose concentrations 30 min after the injection were increased significantly in A^y/+ compared with +/+ animals ($P < 0.05$). ITTs showed that the hypoglycemic response is exaggerated in Tg/+ and Tg/+:A^y/+ compared with +/+ mice ($P < 0.05$) (Fig. 3*C*). No significant changes in plasma glucose concentrations were noted in A^y/+ mice during the observation period.

Plasma leptin concentrations, body weight change, and food intake in 12-week-old F_1 mice. Plasma leptin concentrations in 12-week-old Tg/+ mice were 58.0 ± 3.1 ng/ml; these levels were approximately ninefold higher than those in +/+ mice (6.3 ± 0.5 ng/ml) (n = 10, $P < 0.01$) (Fig. 4*A*). Plasma leptin concentrations in Tg/+:A^y/+ mice were significantly higher than those in A^y/+ mice (122.2 ± 13.5 vs. 77.0 ± 2.9 ng/ml) ($P < 0.05$). Plasma leptin concentrations in both genotypes were significantly higher than those in the +/+ genotype ($P < 0.005$). At 12 weeks of age, body weight of Tg/+ mice was ~77% of that of +/+ mice (20.3 ± 0.6 vs. 26.2 ± 1.3 g) (n = 10, $P < 0.01$) (Fig. 4*B*). Body weights of Tg/+:A^y/+ and A^y/+ mice (34.5 ± 3.2 and 37.0 ± 1.5 g, respectively) were significantly greater than that of +/+ mice ($P < 0.05$). No significant differences in body weight were observed between A^y/+ and Tg/+:A^y/+ genotypes. Cumulative food intake was also reduced significantly in 12-week-old Tg/+ mice compared with +/+ mice (Fig. 4*C*). A^y/+ and Tg/+:A^y/+ mice were significantly hyperphagic compared with +/+ mice ($P < 0.01$). No significant differences in food intake were noted between A^y/+ and Tg/+:A^y/+ mice.

Glucose metabolism in 12-week-old F_1 mice. No significant differences in plasma glucose concentrations were noted between Tg/+ and +/+ mice (8.8 ± 0.4 and 8.1 ± 0.7 mmol/l, respectively) (n = 10) (Fig. 5*A*). Plasma glucose concentrations were elevated significantly in 12-week-old

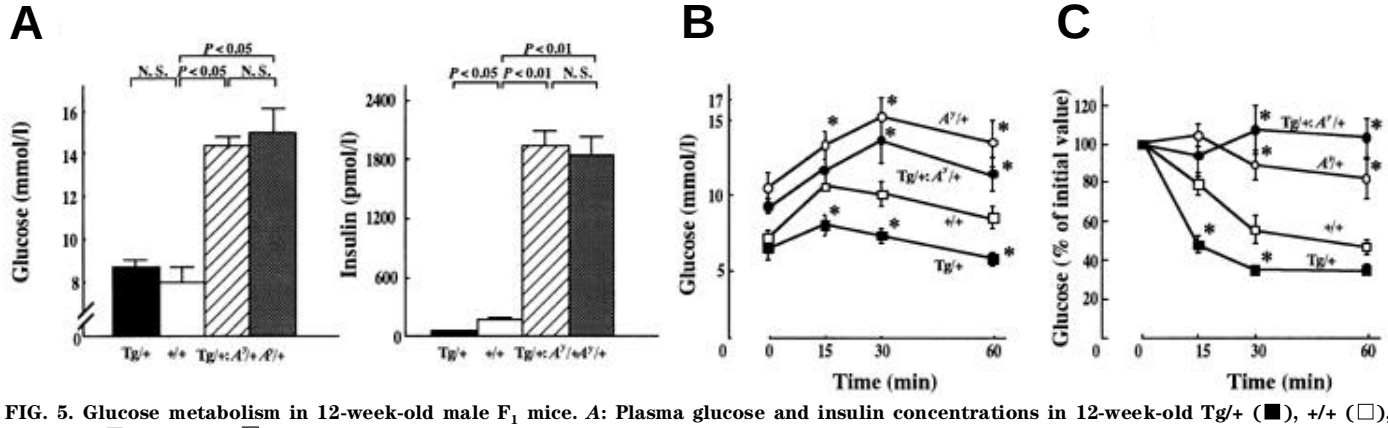

FIG. 5. Glucose metabolism in 12-week-old male F_1 mice. *A*: Plasma glucose and insulin concentrations in 12-week-old Tg/+ (■), +/+ (□), Tg/+:A^y/+ (▨), and A^y/+ (■) mice. Values are expressed as means ± SE (n = 10 in each genotype). Statistical significance of differences is assessed by ANOVA with the Bonferroni-Dunn test. NS, not significant. *B*: GTT in 12-week-old male +/+ (□), Tg/+ (■), A^y/+ (○), and Tg/+:A^y/+ (●) mice. *$P < 0.05$ vs. +/+ mice assessed by ANOVA with repeated measures analysis. *C*: ITT in 12-week-old male F_1 mice. Plasma glucose concentrations were expressed as a percentage of the initial values in each genotype. *$P < 0.05$ vs. +/+ mice assessed by ANOVA with repeated measures analysis.

A^y/+ and Tg/+:A^y/+ (15.0 ± 1.2 and 14.4 ± 0.5 mg/dl, respectively) compared with +/+ mice ($P < 0.05$). No significant differences in plasma glucose concentrations were noted between A^y/+ and Tg/+:A^y/+ mice. Plasma insulin concentrations were decreased significantly in Tg/+ compared with +/+ mice (67 ± 8 vs. 182 ± 12 pmol/l) ($P < 0.05$). Plasma insulin concentrations were elevated significantly in A^y/+ and Tg/+:A^y/+ (1,840 ± 194 and 1,942 ± 138 pmol/l, respectively) compared with +/+ mice ($n = 10$, $P < 0.01$). No significant differences in plasma insulin concentrations were observed between A^y/+ and Tg/+:A^y/+ mice. GTTs revealed that plasma glucose elevation is blunted significantly in Tg/+ compared with +/+ mice ($P < 0.05$) (Fig. 5*B*). Plasma glucose concentrations 30 and 60 min after the injection were increased significantly in A^y/+ and Tg/+:A^y/+ compared with +/+ and Tg/+ mice ($P < 0.01$). No significant differences were noted between A^y/+ and Tg/+:A^y/+ mice. When injected with insulin, the hypoglycemic response was also exaggerated in Tg/+ compared with +/+ mice (Fig. 5*C*). No hypoglycemic responses were noted in A^y/+ and Tg/+:A^y/+ mice during the ITT.

Body weight changes and plasma leptin concentrations in 12-week-old F_1 mice after food restriction. To assess the impact of body weight differences on glucose metabolism of 12-week-old F_1 animals, we examined their glucose metabolism after body weight adjustment by food restriction. We measured the time course of body weights of F_1 animals given ~60% of the amount of food they had eaten when fed ad libitum (Fig. 6*A*). After a 2-week food restriction, body weights of Tg/+ and +/+ mice were 17 and 12% less, respectively, than those before the experiment. Body weights of A^y/+ and Tg/+:A^y/+ mice after a 2-week food restriction were also decreased significantly (26 ± 5 and 24 ± 5%, respectively) relative to those before the food restriction. After the food restriction, no significant differences in body weight were noted among +/+, A^y/+, and Tg/+:A^y/+ genotypes. Plasma leptin concentrations in +/+, Tg/+, A^y/+, and Tg/+:A^y/+ genotypes after the food restriction were 2.1 ± 0.4, 42.3 ± 5.2, 10.0 ± 1.9, and 49.7 ± 8.2 ng/ml, respectively ($n = 5$) (Fig. 6*B*). Plasma leptin concentrations in Tg/+ mice were significantly higher than those in +/+ mice ($P < 0.01$). Plasma concentra-

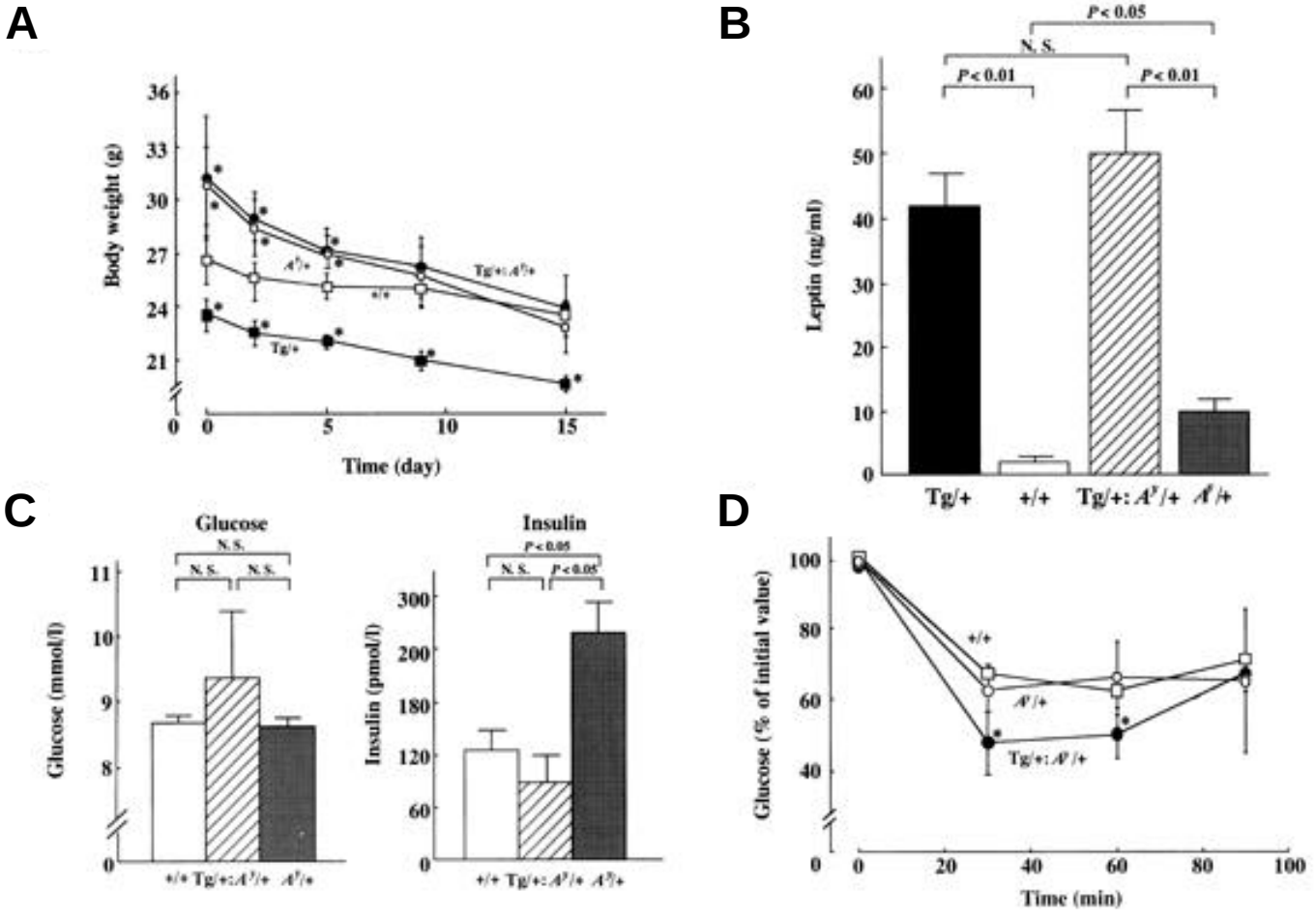

FIG. 6. Body weight changes, plasma leptin concentrations, and glucose homeostasis in 12-week-old F_1 mice during and after food restriction. *A*: Time course of body weights of +/+ (□), Tg/+ (■), A^y/+ (○), and Tg/+:A^y/+ (●) mice that received ~60% of the amount of food as that consumed when fed ad libitum. Values are expressed as means ± SE ($n = 5$ in each genotype). Statistical significance of differences is assessed by ANOVA with repeated measures analysis. *$P < 0.05$ vs. +/+ mice assessed by ANOVA with repeated measures analysis. *B*: Plasma leptin concentrations in Tg/+ (■), +/+ (□), Tg/+:A^y/+ (▨), and A^y/+ (■) mice after food restriction for 3 weeks. Statistical significance of differences is assessed by ANOVA with the Bonferroni-Dunn test. *C*: Plasma glucose and insulin concentrations in +/+ (□), Tg/+:A^y/+ (▨), and A^y/+ (■) mice after food restriction. Values are expressed as means ± SE ($n = 5$ in each genotype). Statistical significance of differences is assessed by ANOVA with the Bonferroni-Dunn test. *D*: ITT in +/+ (□), A^y/+ (○), and Tg/+:A^y/+ (●) mice after food restriction. Plasma glucose concentrations are expressed as a percentage of the initial values in mice with each genotype. *$P < 0.05$ vs. +/+ mice assessed by ANOVA with repeated measures analysis.

tions in Tg/+:A^y/+ mice were significantly higher than those in A^y/+ mice ($P < 0.01$). There were no statistical differences in plasma leptin concentrations between Tg/+ and Tg/+:A^y/+ and between +/+ and A^y/+ genotypes.

Glucose metabolism in 12-week-old F_1 mice after food restriction. After a 3-week food restriction, no significant differences in plasma glucose concentrations were noted among +/+, A^y/+, and Tg/+:A^y/+ mice (8.7 ± 0.1, 9.4 ± 1.0, and 8.7 ± 0.1 mmol/l, respectively) ($n = 5$). However, plasma insulin concentrations in A^y/+ mice (254 ± 32) were significantly higher than those in +/+ and Tg/+:A^y/+ mice (126 ± 26 and 91 ± 28 pmol/l, respectively) ($n = 5$, $P < 0.05$). No significant differences in plasma insulin concentrations were noted between Tg/+:A^y/+ and +/+ mice (Fig. 6*C*). When injected with insulin, the hypoglycemic response in A^y/+ mice was increased to an extent such as that in +/+ mice. The hypoglycemic response in Tg/+:A^y/+ mice 30 and 60 min after the injection was significantly increased compared with +/+ and A^y/+ mice ($P < 0.05$) (Fig. 6*D*).

DISCUSSION

The aim of the present study is to explore the potential usefulness of leptin for the treatment of obesity-associated diabetes. Several studies with crosses between genetically defined animal models have provided fresh insights into the pathophysiology of obesity and diabetes (16,30,31). Our fundamental strategy is to examine the effects of chronic hyperleptinemia on glucose metabolism using Tg/+:A^y/+, Tg/+, A^y/+, and +/+ mice, which are produced by genetic crosses between Tg/+ and A^y/+ mice. In mice with the Tg allele, such as Tg/+ and Tg/+:A^y/+, hepatic overexpression of leptin is constitutive irrespective of the change in caloric intake (H.M., M.A.-A., unpublished observations), while endogenous leptin production in the adipose tissue is regulated by the peripheral nutritional status (20–22). A^y/+ mice develop a maturity-onset obesity and diabetes syndrome due to the antagonism of hypothalamic melanocortin receptor-4 by ectopic expression of the agouti protein (25,32–35). At the stage of established obesity, A^y/+ mice exhibit marked hyperleptinemia accompanied by resistance to exogenously administered leptin (24,36).

The present study demonstrates that glucose tolerance and insulin sensitivity are increased in Tg/+:A^y/+ mice as they are in Tg/+ mice at 6 weeks of age, while A^y/+ mice have already developed moderate insulin resistance compared with +/+ mice. At 6 weeks of age, plasma leptin concentrations in Tg/+:A^y/+ mice are higher than those in A^y/+ mice, which are roughly equivalent to those in +/+ mice, although no significant differences in body weight are noted among these genotypes. These results indicate that overproduction of leptin can delay the onset of impaired glucose metabolism in normal-weight Tg/+:A^y/+ mice at 6 weeks of age, while endogenous leptin cannot do so in A^y/+ mice. These findings suggest that leptin can exert its antidiabetic effect in normal-weight animals.

At 12 weeks of age, Tg/+:A^y/+ mice with elevated plasma leptin concentrations develop obesity and diabetes in a manner similar to that of A^y/+ mice. These results suggest that the satiety effect of leptin is mediated chronically through the hypothalamic melanocortin system, which is consistent with previous reports that the satiety effect of leptin is antagonized acutely by SHU9119, a potent synthetic antagonist of α-melanocyte–stimulating hormone (37,38). A recent work demonstrated that exogenous leptin can suppress appetite in nonobese young *Mc4r*$^{-/-}$ (melanocortin receptor-4–deficient) mice and nonobese adult *Mc4r*$^{-/-}$ mice in which the development of obesity was attenuated compulsively by caloric restriction (39). These findings suggest that, at least without obesity, hypothalamic melanocortin signaling is not exclusive for the satiety effect of leptin. In the present study, leptin can stimulate glucose metabolism in nonobese Tg/+:A^y/+ mice, but it is also possible that the antidiabetic action of leptin is mediated by signaling pathways other than the hypothalamic melanocortin system in nonobese animals.

The mechanisms by which the antidiabetic action of leptin is abolished in 12-week-old Tg/+:A^y/+ mice require further investigations. At 12 weeks of age, Tg/+:A^y/+ mice might develop resistance to the antidiabetic action of leptin through the antagonism of the hypothalamic melanocortin system by the agouti protein. In this context, we have recently observed that a single intracerebroventricular administration of SHU9119 deteriorates acutely glucose tolerance in Tg/+ mice (N.S., H.M., M.A.-A., unpublished observations). It has been demonstrated that the agouti protein is expressed in various peripheral tissues that might also contribute to diabetes in A^y/+ mice (35). Furthermore, leptin acts directly on peripheral tissues, where the leptin receptor is expressed (4,12,22). Therefore, in 12-week-old Tg/+:A^y/+ mice, the antidiabetic effect of leptin might also be antagonized by the agouti protein in peripheral tissues. Alternatively, obesity-linked diabetogenic effects, including glucose toxicity (40), lipotoxicity (41), and tumor necrosis factor-α derived from hypertrophied adipocytes (22), might surpass the antidiabetic action of leptin in 12-week-old obese Tg/+:A^y/+ mice.

Treatment of obesity-associated diabetes is one of the most formidable tasks, and a long-term caloric restriction regimen is generally used as a therapeutic strategy, sometimes in vain (19,20). In view of the beneficial effects of leptin on glucose homeostasis, a substantial decline in plasma leptin concentrations during caloric restriction (20–22) might be responsible for such a failure. This is reminiscent of the notion that leptin deficiency plays a role in starvation-induced suppression of neuroendocrine and immune functions (21,42). We have, therefore, hypothesized that exogenous administration of leptin during caloric restriction can improve impaired glucose metabolism in obesity-associated diabetes. To test this hypothesis, we examined the glucose metabolism in 12-week-old +/+, A^y/+, and Tg/+:A^y/+ mice after a 3-week food restriction, when they did not differ in body weight. When body weights of Tg/+:A^y/+ and A^y/+ mice are reduced to those of +/+ mice by food restriction, plasma leptin concentrations have remained high in Tg/+:A^y/+ mice with hepatic overproduction of leptin, while those in A^y/+ mice are markedly decreased. In the present study, glucose metabolism is improved, to some extent, in A^y/+ mice after a long-term body weight reduction, which is consistent with previous reports that weight reduction considerably restores glucose tolerance and insulin sensitivity in A^y/+ and *ob*/*ob* mice and mice homozygous for the diabetes (*db*) mutation (26,27,43). Noteworthy is that glucose metabolism is markedly improved in Tg/+:A^y/+ as compared with A^y/+ and +/+ mice after a 3-week food restriction. These findings indicate the unmasking of the antidiabetic action of leptin in 12-week-old Tg/+:A^y/+ mice after weight reduction, suggest-

ing that persistent hyperleptinemia can enhance glucose metabolism in 12-week-old Tg/+:A^y/+ mice when body weight is normalized. Moreover, our results suggest that caloric restriction in combination with leptin administration should be more effective as compared with food restriction alone for the treatment of obesity-associated diabetes. Recent studies have shown that exogenous leptin treatment can ameliorate severe diabetes in obese rodent models with leptin deficiency, such as *ob*/*ob* with the A^y/+ allele (16), *ob*/*ob* (17), and *ob*/*ob* transgenic mice expressing leptin at a low dose (18), suggesting that leptin can exert its antidiabetic action in obesity-associated diabetic patients with a reduced amount of leptin during caloric restriction. In this regard, Tg/+:A^y/+ mice obtained in the present study provide the unique experimental model system with which to assess the effect of leptin on glucose metabolism and insulin sensitivity during fasting or food restriction. Although A^y/+ mice are one of the representative experimental models for obesity-associated diabetes, the etiology of their phenotypes, ectopic overproduction of the agouti protein, is uncommon in human obesity (12,24). Therefore, it will be necessary to assess the antidiabetic action of leptin using other obese-diabetic rodent models.

In summary, the present study demonstrates that persistent hyperleptinemia can delay the onset of impaired glucose metabolism and accelerate the recovery from diabetes in A^y/+ mice during the course of caloric restriction, thereby suggesting the potential usefulness of leptin for the treatment of obesity-associated diabetes. The information obtained and the strategy used in the present study should help us better understand the pathophysiological and therapeutic implications of leptin in obesity-associated diabetes.

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REFERENCES

1. Zhang Y, Proenca R, Maffei M, Barone, M, Leopold L, Friedman JM: Positional cloning of the mouse *obese* gene and its human homologue. *Nature* 372: 425–432, 1994
2. Pelleymounter MA, Cullen MJ, Bake MBR, Hecht R, Winters D, Boone T, Collins F: Effects of the *obese* gene product on body weight regulation in *ob*/*ob* mice. *Science* 269:540–543, 1995
3. Halaas JL, Gajiwata KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM: Weight-reducing effects of the plasma protein encoded by the *obese* gene. *Science* 269:543–546, 1995
4. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P: Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 269:546–549, 1995
5. Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays CG, Woolf EA, Monroe CA, Tepper RI: Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83:1263–1271, 1995
6. Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS: Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nature Med* 1:1311–1314, 1995
7. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA, Friedman JM: Leptin levels in human and rodent: measurement of plasma leptin and *ob* RNA in obese and weight-reduced subjects. *Nature Med* 1:1155–1161, 1995
8. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, Mckee LJ, Bauer TL, Caro JF: Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 334:292–295, 1996
9. Caro JF, Kolaczynsk JW, Nyce MR, Ohanenesian JP, Opentanova I, Goldman WH, Lynn RB, Zhang PI, Sinha MK, Considine RV: Decreased cerebrospinal fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. *Lancet* 348:159–161, 1996
10. Schwartz MW, Peskind E, Raskind M, Boyko EJ, Porte DJ: Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. *Nature Med* 2:589–593, 1996
11. Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, Friedman JM: Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc Natl Acad Sci U S A* 94:8878–8883, 1997
12. Auwerx J, Staels B: Leptin. *Lancet* 351:737–742, 1998
13. Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ: Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature* 389:374–377, 1997
14. Cusin I, Zakrzewska KE, Boss O, Muzzin P, Giacobino JP, Ricquier D, Jeanrenaud B, Jeanrenaud FR: Chronic central leptin infusion enhances insulin-stimulated glucose metabolism and favors the expression of uncoupling proteins. *Diabetes* 47:1014–1019, 1998
15. Shi ZQ, Nelson A, Whitcomb L, Wang J, Cohen AM: Intracerebroventricular administration of leptin markedly enhances insulin sensitivity and systemic glucose utilization in conscious rats. *Metabolism* 47:1274–1280, 1998
16. Boston BA, Blaydon KM, Varnerin J, Cone RD: Independent and additive effects of central POMC and leptin pathways on murine obesity. *Science* 278: 1641–1644, 1997
17. Murphy JE, Zhou S, Giese K, Williams LT, Escobedo JA, Dwadki VJ: Long-term correction of obesity and diabetes in genetically obese mice by a single intramuscular injection of recombinant adeno-associated virus encoding mouse leptin. *Proc Natl Acad Sci U S A* 94:13921–13926, 1997
18. Ioffe E, Moon B, Connolly E, Friedman JM: Abnormal regulation of the *leptin* gene in the pathogenesis of obesity. *Proc Natl Acad Sci U S A* 95: 11852–11857, 1998
19. Amatruda JM, Richeson JF, Welle SL, Brodows RG, Lockwood DH: The safety and efficacy of a controlled low-energy (very-low-calorie) diet in the treatment of non-insulin-dependent diabetes and obesity. *Arch Intern Med* 148:873–877, 1988
20. Campfield LA, Smith FJ, Burn P: Strategies and potential molecular targets for obesity treatment. *Science* 280:1383–1387, 1998
21. Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E, Flier JS: Role of leptin in the neuroendocrine response to fasting. *Nature* 382: 250–252, 1996
22. Spiegelman BM, Flier JS: Adipogenesis and obesity: rounding out the big picture. *Cell* 87:377–389, 1996
23. Ogawa Y, Masuzaki H, Hosoda K, Aizawa-Abe M, Suga J, Suda M, Ebihara K, Iwai H, Matsuoka N, Satoh N, Odaka H, Kasuga M, Fujisawa Y, Inoue G, Nishimura H, Yoshimasa Y, Nakao K: Increased glucose metabolism and insulin sensitivity in transgenic skinny mice overexpressing leptin. *Diabetes*. In press
24. Leibel RL, Chung WK, Chua SC: The molecular genetics of rodent single gene obesities. *J Biol Chem* 272:31937–31940, 1997
25. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD: Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 385: 165–168, 1997
26. Hoffmann C, Lorenz K, Braitwaite SS, Colca JR, Palazuk BJ, Hotamisligil GS, Spiegelman BM: Altered gene expression for tumor necrosis factor alpha and its receptors during drug and dietary modulation of insulin resistance. *Endocrinology* 134:264–270, 1994
27. Herberg L, Coleman DL: Laboratory animals exhibiting obesity and diabetes syndromes. *Metabolism* 26:59–87, 1997
28. Ogawa Y, Masuzaki H, Isse N, Okazaki T, Mori K, Shigemoto M, Satoh N, Tamura N, Hosoda K, Yoshimasa Y, Jingami H, Kawada T, Nakao K: Molecular cloning of rat obese cDNA and augmented gene expression in genetically obese Zucker fatty (*fa*/*fa*) rats. *J Clin Invest* 96:1647–1652, 1995
29. Masuzaki H, Ogawa Y, Isse N, Satoh N, Okazaki T, Shigemoto M, Mori K, Tamura N, Hosoda K, Yoshimasa Y, Jingami H, Kawada T, Nakao K: Human *obese* gene expression: adipocyte-specific expression and regional differ-

ences in the adipose tissue. *Diabetes* 44:855–858, 1995
30. Erickson JC, Hollopeter G, Palmiter RD: Attenuation of the obesity syndrome of *ob/ob* mice by the loss of neuropeptide Y. *Science* 274:1704–1707, 1996
31. Terauchi Y, Iwamoto K, Tamemoto H, Komeda K, Ishii C, Kanazawa Y, Asanuma N, Aizawa T, Akanuma Y, Yasuda K, Kodama T, Tobe K, Yazaki Y, Kadowaki T: Development of non-insulin-dependent diabetes mellitus in the double knockout mice with disruption of insulin receptor substrate-1 and β-cell glucokinase genes: genetic reconstitution of diabetes as a polygenic disease. *J Clin Invest* 99:861–866, 1997
32. Miller MW, Duhl DMJ, Vrieling H, Cordes SP, Ollmann MM, Winkes BM, Barsh GS: Cloning of the mouse *agouti* gene predicts a secreted protein ubiquitously expressed in mice carrying the lethal *yellow* mutation. *Gene Dev* 7:454–467, 1993
33. Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO, Cone RD: Agouti protein is an antagonist of the melanocyte-stimulating hormone receptor. *Nature* 371:799–802, 1994
34. Millar SE, Miller MW, Stevens ME, Barsh GS: Expression and transgenic studies of the mouse *agouti* gene provide insight into the mechanisms by which mammalian coat color patterns are generated. *Development* 121:3223–3232, 1995
35. Manne J, Argeson AC, Siracusa LD: Mechanisms for the pleiotropic effects of the *agouti* gene. *Proc Natl Acad Sci U S A* 92:4721–4724, 1995
36. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeir LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F: Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88:131–141, 1997
37. Seeley RJ, Yagaloff KA, Fisher SL, Burn P, Thiele TE, van Dijk G, Baskin DG, Schwartz MW: Melanocortin receptors in leptin effects (Letter). *Nature* 390:349, 1997
38. Satoh N, Ogawa Y, Katsuura G, Numata Y, Masuzaki H, Yoshimasa Y, Nakao K: Satiety effect of and sympathetic activation of leptin are mediated by hypothalamic melanocortin system. *Neurosci Lett* 249:107–110, 1998
39. Marsh DJ, Hollopeter G, Huszar D, Laufer R, Yagaloff KA, Fisher SL, Burn P, Palmiter RD: Response of melanocrtin-4 receptor–deficient mice to anorectic and orexigenic peptides. *Nature Genet* 21:119–122, 1999
40. Hawkins M, Barzilai N, Liu R, Hu M, Chen W, Rossetti L: Role of the glucosamine pathway in fat-induced insulin resistance. *J Clin Invest* 99: 2173–2182, 1997
41. Shimabukuro M, Koyama K, Chen G, Wang MY, Trieu F, Lee Y, Newgard CB, Unger RH: Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc Natl Acad Sci U S A* 94:4637–4641, 1997
42. Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI: Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* 394:897–901, 1998
43. Chlouverakis C, White PA: Obesity and insulin resistance in the obese-hyperglycemic mouse (*ob/ob*). *Metabolism* 18:998–1006, 1969