Transplantation of Adipose Tissue Lacking Leptin Is Unable to Reverse the Metabolic Abnormalities Associated With Lipoatrophy

Carlo Colombo,1 Jaime J. Cutson,1 Toshimasa Yamauchi,2 Charles Vinson,3 Takashi Kadowaki,2 Oksana Gavrilova,1 and Marc L. Reitman1

Severe adipose tissue deficiency (lipoatrophy) causes insulin-resistant diabetes, elevated serum triglyceride and fatty acid levels, and massive triglyceride deposition in the liver. In lipoatrophic A-ZIP/F-1 mice, transplantation of normal adipose tissue greatly improved these parameters, whereas 1 week of leptin infusion had more modest effects. In contrast, leptin infusion was strikingly more effective in the aP2-n sterol response element binding protein 1 lipoatrophic mouse. Here we show that a longer duration of leptin infusion further improves the metabolic status of the A-ZIP/F-1 mouse and that genetic background does not make a major contribution to the effect of leptin on glucose and insulin levels. Adipose transplantation using leptin-deficient ob/ob fat had no effect on the phenotype of the A-ZIP/F-1 mice. Moreover, the presence of ob/ob adipose tissue did not enhance the effects of leptin infusion. Serum adiponectin levels were 2% of control levels in the A-ZIP/F-1 mouse and increased only twofold with adipose transplantation and not at all after leptin infusion, suggesting that adiponectin deficiency is not a major contributor to the diabetic phenotype. Taken together, these results suggest that sequestration of triglycerides into fat may not be enough to restore a nondiabetic phenotype and that leptin deficiency plays a major role in causing the metabolic complications of lipoatrophy. Diabetes 51:2727–2733, 2002

White adipose tissue (WAT) participates in energy metabolism and glucose and lipid homeostasis as both a triglyceride storage site and a secretory endocrine tissue. Hormones produced by WAT (leptin, adiponectin, and others) regulate food intake, metabolic efficiency, and energy expenditure. Leptin, the best-studied adipocyte hormone, reduces food intake and stimulates energy metabolism (1). Leptin deficiency (ob/ob mice) or resistance to leptin action (db/db mice, fa/fa rats) causes increased food intake, obesity, enlarged fatty liver, insulin resistance, and diabetes. Leptin treatment of ob/ob mice improves insulin sensitivity even before significantly reducing body weight.

The lipodystrophies are a heterogeneous group of rare diseases characterized by partial or total loss of WAT (2,3). Insulin-resistant diabetes is a major consequence of severe lipoatrophy. We have been studying the transgenic A-ZIP/F-1 mouse model of lipoatrophy, which was produced by adipose-specific expression of a dominant-negative protein (4). This protein prevents the DNA binding of certain bZIP transcription factors that are important in adipose tissue differentiation, resulting in near-complete ablation of WAT. The lack of WAT in the A-ZIP/F-1 mice causes insulin resistance, hyperglycemia and hyperinsulinemia, elevated serum triglycerides and serum free fatty acids, and hepatic steatosis. Lipoatrophic mice have also been produced by other transgenic approaches (5–7). In general, the severity of the metabolic phenotype correlates with the degree of WAT ablation. It is clear that the lack of WAT causes the metabolic abnormalities of lipoatrophic diabetes, because surgical implantation of WAT reverses the phenotype (8).

How does WAT prevent the syndrome? In the aP2-n sterol response element binding protein (SREBP) 1c mouse, leptin infusion for 12 days at near physiological levels produced a dramatic normalization of the glucose and insulin levels (9). In contrast, in the A-ZIP/F-1 mouse, leptin infusion for 6 days at a higher dose produced a modest reduction in glucose and insulin levels (10). These transgenes were studied on different genetic backgrounds, and we have found a major effect of background on the phenotype of the A-ZIP/F-1 mouse (C. Colombo, M. Hahzuk, J.J. Cutson, K.R. Dietz, B. Marcus-Samuels, C. Vinson, O. Gavrilova, and M.L. Reitman, manuscript in preparation). Recently, pharmacological levels of leptin were shown to normalize the A-ZIP/F-1 phenotype (11). These data indicate that pharmacological doses of leptin are sufficient to prevent diabetes but raise the question of what other factors are important when leptin is present at physiological levels.

In this article, we investigated the role of leptin in lipoatrophic diabetes in detail, exploring the contributions of the duration of leptin treatment and the genetic background of the mice. We also studied the efficacy of ob/ob adipose tissue grafts, which are unable to produce leptin, at normalizing the A-ZIP/F-1 phenotype. The data support

From the 1Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland; the 2Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; and the 3Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

Address correspondence and reprint requests to Marc Reitman, Merck Research Laboratories, P.O. Box 2000, RY90M-213, Rahway, NJ 07065-0900. E-mail: marc_reitman@merck.com.

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PPAR, peroxisome proliferator–activated receptor; SREBP-1, sterol response element binding protein 1; WAT, white adipose tissue.
a major role for leptin deficiency in contributing to the metabolic complications of lipoatropy.

RESEARCH DESIGN AND METHODS

Mice. A-ZIP/F-1 mice were studied as hemizygotes, produced by breeding A-ZIP/F-1 males with wild-type females (4). C57BL/6J A-ZIP/F-1 mice were obtained by at least 13 generations of successive backcrossing A-ZIP/F-1 males with C57BL/6J females (The Jackson Laboratories, Bar Harbor, ME). C. Colombo, M. Haluzik, J.J. Cutson, K.R. Dietz, B. Marcus-Samuels, C. Vinson, O. Gavrilova, and M.L. Reitman, manuscript in preparation). FVB/N mice were obtained from Veterinary Resources Program, National Institutes of Health and Harlan Sprague Dawley, and B6.V-Lep^+/+ (hereafter, ob/ob) mice were obtained from The Jackson Laboratories. Mice were individually housed, kept on a 12-h light/dark cycle (0600–1800), and fed NIH-07 rodent diet (12.9 kcal % fat; Zeigler Brothers, Gardners, PA) and water ad libitum. At the end of each experiment, mice were anesthetized with pentobarbital (5 mg/ml, using 0.01 ml/g body wt) and bled via the orbital plexus. For leptin infusion, an Alzet mini-osmotic pump (model 2001; Alza Corporation, Palo Alto, CA) was implanted subcutaneously, delivering saline or 30 μg/day recombinant leptin (R&D Systems).

WAT transplantation. Eight-week-old A-ZIP/F-1 FVB/N × C57BL/6J F1 mice of both sexes were equally distributed between the groups and used as recipients. Two-week-old wild-type B6 and littermate ob/ob mice were used as fat donors. Donors and recipient were sex matched. Donors were PCR genotyped (12), and WAT implantation was performed as described previously (8). Briefly, mice were anesthetized with ketamine (100 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (10 mg/kg; Phoenix Scientific, St. Joseph, MO), and ~500 mg (four pieces, 100–150 mg each) of subcutaneous fat was implanted per mouse. In the second transplantation experiment, 4 weeks after transplantation, osmotic pumps containing leptin or saline were implanted and 7 days later the exhausted pumps were replaced to continue the infusion. Another 7 days later, mice were anesthetized with pentobarbital and killed by cervical dislocation.

Biochemical assays. Samples were obtained in the nonfasting state generally between 0900 and 1200. For live bleed, blood was obtained by tail bleed from unanesthetized mice and immediately analyzed for glucose concentration. For serum analysis, blood was collected into Micro-Hematocrit Capillary tubes (22-362-574; Fisher, Pittsburgh, PA). Glucose was measured using a Glucometer Elite (Bayer, Elkhart, IN). Triglycerides (339-11; Sigma-Aldrich, St. Louis, MO), insulin (SRI-13K; Linco Research, St. Charles, MO), and adiponectin (MADP-60HK; Linco Research) were assayed according to the supplier’s procedures. Adiponectin was also assayed by Western blotting (13).

Statistical analysis. Results are reported as mean ± SE. Data were analyzed using SigmaStat (SPSS, Chicago, IL) by 1 test or two-way ANOVA followed by Tukey test for pairwise multiple comparisons.

RESULTS

Effect of the genetic background on leptin responsiveness. Genetic background influences the phenotype of A-ZIP/F-1 mice, with the C57BL/6J (hereafter B6) A-ZIP/F-1 mice having milder diabetes and greater survival but worse hepatic steatosis than the FVB/N (hereafter FVB) A-ZIP/F-1 mice (C. Colombo, M. Haluzik, J.J. Cutson, K.R. Dietz, B. Marcus-Samuels, C. Vinson, O. Gavrilova, and M.L. Reitman, manuscript in preparation). For determining whether genetic background contributes to the effectiveness of leptin replacement, the phenotype of the A-ZIP/F-1 transgene on three different genetic backgrounds B6, FVB, and B6 × FVB hybrids (hereafter F1) was examined (Fig. 1). One week of leptin infusion at 30 μg/day raised serum leptin to ~5.6 ng/ml, slightly above wild-type levels (~3–4 ng/ml). In the control, leptin-deficient ob/ob mice on the B6 background, leptin infusion normalized blood glucose, serum insulin, and liver weight and reduced food intake and body weight.

The improvement of the A-ZIP/F-1 mice on this treatment regimen was clear but more modest than in the ob/ob mice (10) (Fig. 1). Leptin reduced the insulin levels, but they remained elevated compared with both leptin-treated ob/ob and wild-type mice. Similarly, whereas leptin reduced glucose levels to normal in the ob/ob mice, it reduced glucose levels only modestly in the A-ZIP/F-1 mice. Leptin treatment also reduced the food intake, weight gain, serum fatty acids and triglycerides, and liver weight (Fig. 1). The background genotype (B6 versus FVB) is known to affect serum triglyceride levels, and this difference remained after leptin treatment of A-ZIP/F-1 mice. Liver size is a surrogate for liver triglyceride content, and the leptin-induced reduction in liver size was greater in the B6 (51%) than in the FVB (23%) mice. No background genotype effect was detected on the glucose or insulin levels. These data suggest two conclusions. First, 1 week of leptin treatment has a more profound effect on the phenotype of the ob/ob than the A-ZIP/F-1 mice, even when both are on the B6 background. Second, although genetic background influences the A-ZIP/F-1 phenotype, its effect on the change in glucose and insulin levels caused by leptin treatment is not major and probably does not

![FIG. 1. Effect of continuous subcutaneous leptin infusion in A-ZIP/F-1 mice and ob/ob controls. Within each genotype, the left bar is the saline-infused group and the right bar is the leptin-infused group. Mice are females 9–11 weeks old. Food intake was measured between the third and the fifth treatment days, and mice were killed on the seventh day. Data are mean ± SE, n = 4–8/group (except n = 3 for the ob/ob groups). Significant differences between treated and control mice of the same genetic background are indicated by *P < 0.05, **P < 0.005.](image-url)
explain the different response to leptin of the A-ZIP/F-1 and aP2-nSREBP-1c mice. ob/ob WAT transplantation. We showed previously that WAT transplantation reverses the diabetes of A-ZIP/F-1 mice (8). Here, we used ob/ob WAT to test whether leptin is required for the beneficial effects of WAT. Initial transplantation experiments with donor WAT from 8-week-old ob/ob mice were not successful because of inflammation and necrosis of the grafts, probably as a result of fragility of the enlarged ob/ob donor adipocytes (Fig. 2C and D). This problem was avoided by using WAT from 2-week-old donors. At this age, ob/ob and wild-type mice and their WAT are indistinguishable by gross and microscopic examination (Fig. 2A and B).

We confirmed the technical success of the ob/ob grafts in a number of ways. After transplantation, the grafts were soft and mobile, unlike infected or inflamed grafts. During the 6 weeks, the ob/ob grafts increased 2.6-fold in weight, similar to the 2.3-fold of the wild-type grafts (Fig. 3). The ob/ob grafts also had a normal histological appearance (Fig. 2E and F). Taken together, these data demonstrate that the ob/ob transplantation was technically successful.

Effect of leptin infusion combined with ob/ob WAT transplantation. For determining whether leptin infusion had an additive or synergistic effect when combined with ob/ob WAT transplantation, leptin (or saline) was infused for 2 weeks into sham-operated mice and mice that received ob/ob transplant, starting 4 weeks after transplantation. In the absence of leptin infusion, serum leptin levels in A-ZIP/F-1 mice were undetectable. After leptin infusion, leptin levels were comparable to those of wild-type controls; wild-type WAT transplantation yielded slightly lower values. Sham-operated mice and A-ZIP/F-1 mice that received ob/ob transplant remained with undetectable levels (Fig. 5). Quantitatively similar results were observed in an independent ob/ob WAT transplantation experiment (data not shown). These data demonstrate that transplantation of 500 mg of ob/ob WAT has no major effect on the A-ZIP/F-1 phenotype.
weeks to wild-type A-ZIP/F-1 mice that received a transplant. (Detailed analysis of the triglyceride curves is difficult because of biological variability and an age-dependent decrease in triglyceride levels [C. Colombo, M. Haluzik, J.J. Cutson, K.R. Dietz, B. Marcus-Samuels, C. Vinson, O. Gavrilova, and M.L. Reitman, manuscript in preparation]). Other parameters that were improved by leptin treatment were food intake, body weight gain, and liver weight (Fig. 5) and liver histology (not shown). No significant differences were found between leptin treatment alone and leptin plus ob/ob WAT. Taken together, the data show that leptin treatment was equally efficacious in sham mice as in those carrying ob/ob WAT grafts.

The only measure in which leptin treatment seemed to differ from wild-type WAT transplantation was the change in body weight during the last 2 weeks of the experiment (Fig. 5). Sham-operated mice and A-ZIP/F-1 mice that received ob/ob transplant gained 1.2 and 1.5 g, respectively. Wild-type mice that received a transplant showed no change in weight, whereas leptin-treated sham-operated and A-ZIP/F-1 mice that received ob/ob transplant lost 2.2 and 4.2 g, respectively. Presumably this reflects the difference between continuing treatment (wild-type grafts) compared with the addition of an effective treatment (leptin). It may also reflect the slightly different leptin levels attained.

The group that received ob/ob WAT transplant and was treated with leptin showed less weight gain in the transplanted adipose tissue (1.3-fold), and these grafts had less triglyceride per cell (Fig. 2G). This suggests that the exogenous leptin depleted the triglycerides of the transplanted WAT. Note that at 6 weeks after transplantation, graft adipocyte size was larger than in the endogenous WAT of wild-type mice of the same age and was equally enlarged in the wild-type and ob/ob transplants.

**Contribution of adiponectin/Acrp30.** Adiponectin/Acrp30 is an adipose hormone that increases fatty acid oxidation and insulin sensitivity (13–15) and circulates in inverse proportion to WAT mass (16,17). A-ZIP/F-1 mice have serum adiponectin levels that are 2% of the level of wild-type controls, as measured by radioimmunoassay (Fig. 4). Adiponectin levels in A-ZIP/F-1 mice were also undetectable by Western blotting (data not shown). Transplantation of wild-type WAT increased adiponectin levels only about twofold. Leptin treatment did not significantly increase adiponectin levels (Fig. 4). In the other transplantation experiment, adiponectin levels were 28.8 ± 1006 4.4, 0.37 ± 1006 0.04, 0.62 ± 1006 0.06, and 0.56 ± 0.08 µg/ml in the wild-type mice and A-ZIP/F-1 mice that received sham, wild-type, or ob/ob WAT transplant, respectively.

**FIG. 4.** Effect of WAT transplantation and leptin infusion on blood glucose and serum insulin, triglyceride, and adiponectin levels. Transplantation into A-ZIP/F-1 mice using wild-type (○) or ob/ob (□) WAT (500 mg) was performed at 8 weeks of age. Sham-operated A-ZIP/F-1 (▲) and wild-type (●) controls were also studied. At 4 weeks after transplantation (indicated by the vertical dashed line), the sham-operated mice and A-ZIP/F-1 mice that received ob/ob transplant were split, with half getting saline (solid lines) and the other half getting leptin (30 µg/day, dotted lines) infusions. Note that the insulin axis is logarithmic and there is a break in the adiponectin axis. The week 6 samples are retro-orbital bleeds from anesthetized mice, whereas the rest are tail-vein bleeds from unanesthetized mice. Data are mean ± SE. The experimental groups were as follows: wild-type mice (n = 8, six females, two males), A-ZIP/F-1 mice that received wild-type WAT transplant (n = 6, four females, two males), A-ZIP/F-1 mice that received ob/ob WAT transplant (saline infused: n = 6, four females, two males; leptin infused: n = 6, five females, one male), and A-ZIP/F-1 sham-operated mice (saline infused: n = 7, four females, three males; leptin infused: n = 8, five females, three males).
DISCUSSION

Our goal is to understand how WAT deficiency causes insulin resistance, hypertriglyceridemia, and hepatic steatosis. This article demonstrates that transplantation of ob/ob WAT (adipose tissue without leptin) has no measurable effect on the lipoatrophic phenotype. However, leptin without adipose tissue has a major effect. Thus, leptin is necessary to prevent the metabolic complications caused by a lack of WAT.

Previously, physiological leptin replacement produced different degrees of response in the A-ZIP/F-1 (10) and the less severe aP2-nSREBP-1c (9) mice. Possible explanations include the severity of the adipose deficiency; the specific transgenes used to produce the lipoatrophy model; the animals’ sex, age, diet, and genetic background; and the leptin treatment details (potency, dose, and duration). It is difficult to test for transgene-specific effects, but it is likely that phenotype severity significantly influences the response to leptin. Sex, age, diet, and genetic background all influence the A-ZIP/F-1 phenotype (4; C. Colombo, M. Hahuzik, J.J. Cutson, K.R. Dietz, B. Marcus-Samuels, C. Vinson, O. Gavrilova, and M L. Reitman, manuscript in preparation) and thus probably also contribute. The leptin treatment details are also likely to be very important. Shimomura et al. (9) used 5 μg/day Amgen leptin for 12 days in B6 × SJL F2 female aP2-nSREBP-1c mice. Gavrilova et al. (10) used 5 μg/day for 28 days and 30 μg/day for 6 days of R&D Systems leptin in male FVB A-ZIP/F-1 mice, and in this article we used 30 μg/day for 7 or 14 days in A-ZIP/F-1 mice. Pharmacological doses of leptin, when present in A-ZIP/F-1 mice endogenously from birth, prevented development of insulin resistance, hypertriglyceridemia, and hepatic steatosis (11). When pharmacological doses were infused for 6 days (~140 μg/day Genzyme/Technne leptin), it lessened the metabolic abnormalities of the A-ZIP/F-1 mice (11). In this article, we show that 2 weeks’ therapy is more effective than 1 week, which may be due to the time needed for sufficient reduction of tissue triglyceride levels. Taken together, these observations suggest that the nuances of the therapy regimen are important.

The more modest response to leptin of A-ZIP/F-1 mice as compared with ob/ob mice may have a number of explanations. The ob/ob mice are more leptin deficient and thus possibly more poised to respond to replacement. Although not directly supported by the transplantation experiments, it remains possible that leptin deficiency is made worse by the lack of adipose tissue. The A-ZIP/F-1 mice may also be missing other factors produced by WAT that are present in ob/ob mice.

FIG. 5. Effect of WAT transplantation and leptin infusion on serum leptin, serum fatty acids, food intake, weight gain, liver weight, and liver triglyceride. Experimental details are given in Fig. 4. Food intake was measured between the fourth and seventh days of leptin treatment. Weight gain is during the last 2 weeks of the experiment. Significant differences (t test) between mice that received ob/ob transplant and were treated and untreated and between sham-operated treated and untreated mice are indicated by *P < 0.05 and **P < 0.005. Significant differences (t test) between mice that received ob/ob transplant and were treated and wild-type mice, between sham-operated treated and wild-type mice, and between wild-type mice that received a transplant and wild-type mice are indicated by #P < 0.05 and ##P < 0.005. No significant differences between leptin-treated mice that received ob/ob transplant and leptin-treated sham-operated mice were found in any of these parameters.
Where is leptin acting to reverse the metabolic features of lipoatrophic diabetes? Leptin’s metabolic and insulin-sensitizing effects are largely mediated via the hypothalamus (18–20), but there may also be direct actions on peripheral tissues such as muscle (21) and liver (22,23). Leptin’s hypothalamically mediated reduction of food intake is not the main mechanism, because pair feeding does not reproduce the effects of leptin therapy (9,11). To date, no direct comparison of central versus peripheral leptin administration has been performed in a lipoatrophic model.

One clue to leptin’s mechanism may be the long time needed to achieve maximum effectiveness. This slow pace is consistent with the unloading of triglyceride from non-adipose sites, such as liver, to reverse “lipotoxicity” (24,25). A therapeutic trial of leptin in humans with severe lipoatrophy recently showed remarkable reductions in appetite, insulin resistance, diabetes, hepatic triglyceridermia, and hepatic steatosis (26). The improvement was seen 1 month into therapy, the earliest time point, and was greater after 2 and 4 months of treatment. In the A-ZIP/F-1 mice, lipoatrophy causes an increase in liver peroxisome proliferator–activated receptor (PPAR)-γ levels (27), and the PPAR-γ contributes to the fatty liver (O. Gavrilova, unpublished observations). Treatment of A-ZIP/F-1 mice with a PPAR-α agonist induced lipolysis, reduced liver and muscle triglyceride levels, and increased insulin sensitivity (28). This occurred without reducing the elevated liver PPAR-γ levels, supporting the hypothesis that lipid unloading is important in ameliorating the insulin resistance.

Leptin treatment of A-ZIP/F-1 mice reduces the insulin levels before the glucose levels. Adipose tissue transplantation also does this (8). These results support the notion that leptin had two activities, increasing insulin sensitization (discussed above) and reducing insulin secretion, as documented previously (29,30).

How much of the metabolic syndrome of lipoatrophic diabetes is due to leptin deficiency and how much to other factors, either other hormones or the adipocytes’ ability to store triglyceride safely? In the Hx531-treated PPAR-γ−/−mouse model of lipodystrophy, adiponectin/Acrp30 deficiency seems to contribute to the metabolic phenotype (13). Our data argue against a principal role for adiponectin in the A-ZIP/F-1 mouse, despite its adiponectin deficiency. Specifically, WAT transplantation reversed the metabolic disarray but only increased the adiponectin levels twofold to ~4% of wild-type levels. Also, leptin infusion improved the metabolic situation without any measured effect on adiponectin levels. Additional studies are needed to illuminate the role of adiponectin deficiency in the cause of the remaining hyperinsulinemia in the leptin-treated A-ZIP/F-1 mouse. Our belief is that leptin is unlikely to be the sole factor.

The serum levels of adiponectin achieved by WAT transplantation were very low. It is not clear why adipose tissue transplantation to a level ~25% of wild-type WAT weight and producing nearly wild-type levels of leptin gave adiponectin levels only ~4% of wild-type. Presumably the grafts are secreting less adiponectin, possibly because their adipocytes become engorged with triglyceride (16,17). Another possibility is that the A-ZIP/F-1 mice clear adiponectin from the circulation more rapidly.

The A-ZIP/F-1 mice should also be deficient in other proteins secreted by adipocytes, including tumor necrosis factor α, resistin, plasminogen activator inhibitor 1, angiotensinogen, and others. If so, then why did the ob/ob WAT transplantation not produce any measurable metabolic improvement? Possibly the ob/ob WAT is not producing other factors in addition to leptin. For example, the ob/ob grafts did not greatly increase the circulating adiponectin levels. It is not known whether the ob/ob WAT grafts are poor secretors of other adipocyte proteins. It is also possible that transplantation of more adipose tissue would have produced a measurable effect.

In conclusion, leptin is a necessary hormone of major importance in the regulation of glucose and triglyceride metabolism, and leptin deficiency is a prime contributor to the metabolic disarray in severe lipoatrophy. It seems likely that deficiency of other adipocyte hormones will also contribute, but the details remain to be elucidated.

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