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Obesity and Elevated Plasma Leptin Concentration in oMT1A-o Growth Hormone Transgenic Mice

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Abstract

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Objective: This study was undertaken to evaluate plasma leptin concentration in the regulatable ovine metallothionein-ovine growth hormone (oMT1a-oGH) transgenic (TG) mouse model of obesity.

Research Methods and Procedures: Transgene stimulus (zinc) was provided at 21 days of age to male and female wild-type (WT) and TG mice. Plasma leptin concentrations were measured by radioimmunoassay at 42, 63, 84, and 105 days of age and from inactivated TG mice at 84 and 105 days.

Results: WT and TG mice did not differ significantly in plasma leptin concentration at any of the ages examined (42, 63, 84, and 105 days), although females showed consistently higher plasma leptin concentrations than males regardless of genotype throughout the duration of the study. Male and female TG mice in which the transgene was inactivated at 63 days had a 1.5-fold to 3.5-fold increase in plasma leptin concentration over WT mice and continuously activated TG mice at 84 and 105 days of age. The elevated plasma leptin concentration seen in the inactivated TG mice at 84 and 105 days of age reflects the >300% increase in white adipose tissue seen in this model and correlated with all adipose depot weights and overall body lipid at these later ages. When plasma leptin was expressed per gram of total body fat, the leptin adjusted for body lipid was significantly higher in WT mice than either continuously activated TG or activated and then inactivated TG groups.

Discussion: The inactivated TG mice in this study had higher plasma leptin levels with increasing total body adiposity, but the relative proportion of circulating leptin, on a total body lipid basis, was reduced when compared with the WT mice. This reduction was also observed in activated TG mice at the older ages. Although the absolute levels of circulating leptin were elevated in the inactivated TG animals, the amount of leptin produced per gram of fat was lowered. With the inactivation of the transgene, the leptin remained depressed after the removal of the elevated growth hormone. This represents a potential explanation for the ensuing hypertrophy of the fat depots and the abnormal phenotypic response of inactivated TG mice to elevated plasma leptin concentrations resulting in the development of obesity.

Key words: leptin, preadipocyte, adipocyte

Introduction

The adipocyte hormone leptin plays a direct role in the control of body fat mass (1–4) by regulating energy intake and expenditure. Growth hormone (GH), which has been studied since the 1920s, is also known to play a role in a variety of physiological growth processes, including fat metabolism (5). Leptin acts as a signal of energy reserves stored as body fat and of recent energy intake via changes in insulin secretion (6,7). In contrast, GH is associated with metabolic processes that act to limit the accumulation of body fat and promote the development of lean body mass.

The transgenic (TG) mice used in this study are a unique in vivo model for adipogenesis (8). They have integrated in their genome an ovine metallothionein promoter-driven ovine GH transgene tandem array (oMT1a-oGH). Overexpression of this transgene construct can be tightly controlled (8,9) such that when transgene expression is activated, circulating ovine GH levels rise to >2000 ng/mL, whereas inactivation of transgene expression results in a marked decrease in circulating GH levels

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to <75 ng/mL (8). Animals under the influence of activated transgene expression grow to mature weights that are $\sim 30\%$ to 40% heavier than control mice. In addition, although male mice normally grow at a faster rate than females, the growth rate is equal under the influence of the transgene. When the transgene is inactivated, both genders exhibit an obese phenotype with white adipose tissue (WAT) depots that are $\sim 270\%$ heavier (expressed as percent of fasted body weight) than control animals and an overall increase in total body lipid of 285% (8,10). Furthermore, obesity in this model is not a transient effect of the cessation of transgene expression because the obesity persists and the increased body fat is reflected in WAT hyperplasia and hypertrophy (8). The obese phenotype occurs even when food intake is restricted, indicating that metabolic changes related to the overexpression of GH via transgene activation are responsible (10). Although glucose concentrations in these animals are unaffected by either transgene activation status or extent of obesity, circulating insulin concentrations are elevated 2-fold to 3-fold with elevated GH and then gradually decline after the withdrawal of the transgene stimulus, although never reaching control animal values (8).

GH does not appear to directly control leptin expression (11), but it may indirectly influence leptin expression through its effect on whole-body metabolism. Leptin may play a permissive role in controlling the release of GH from the pituitary when adipose tissue stores are high. Rising plasma leptin concentrations produce an increase of GH that can be prevented by the administration of anti-leptin antibody to the ventricular space of fed rats (12). Despite these clues, it remains unclear how the elevated expression of GH affects the pathway of adipocyte development and differentiation to produce the obese phenotype in the oMT1a-oGH TG model.

This investigation was undertaken to define further the nature of the interaction between GH and leptin, hormones that have an impact on the physiology of adipocyte development, growth, and function. We wanted to determine whether aberrant production of leptin in adipocytes plays a role in the development of the obese phenotype. We know that the increased GH expression induces adipogenesis in this model. Using samples from a study published previously (8), we designed the present study to determine whether the development of this obesity was due to a dysregulation of leptin. Knowledge of the interaction between leptin and GH activity will aid in the development of therapeutic regimes to modulate expression, receptor sensitivity, and/or transport of these endocrine hormones to treat obesity in both humans and animals.

Research Methods and Procedures

Experimental Design

Wild-type (WT) and TG mice for this experiment were generated using a cross of WT females (C57BL/6 $\times$ CBA)

and male mice hemizygous for the transgene (oMT1a-oGH) (8). The GH transgene is expected to segregate into 50% of the resulting offspring in these crosses. Litters were standardized to seven to nine pups at 1 day of age and all pups were toe-notched at 10 days of age for identification and genotyping.

All mice were housed in a facility approved by the American Association for the Accreditation of Laboratory Animal Care under conditions of constant temperature (21°C), humidity (55%), and light (14:10 hour light:dark cycle). At 21 days, mice were weaned and placed two to a cage by gender. All mice were fed Purina Laboratory Chow no. 5008 (4.2 kcal/g gross energy; Purina Mills, St. Louis, MO). Transgene expression was induced at weaning in the TG animals by supplying 25 mM ZnSO_4 in the drinking water ad libitum; WT controls were also given 25 mM ZnSO_4 in drinking water ad libitum. Treatment groups consisted of male and female groups of TG and WT mice sampled at 42, 63, 84, and 105 days of age. At 63 days of age, four new treatment groups were established by discontinuing ZnSO_4 in a subset of male and female WT and TG animals. These new inactivated treatment groups were also sampled at 84 and 105 days. At each age, six to eight mice from each treatment group were fasted for 12 hours and killed with CO_2 . Intracardial aspiration with a 22-gauge needle was performed immediately after killing, and blood was transferred to EDTA-containing tubes that were centrifuged for plasma collection. Plasma samples from each mouse were stored at -80°C until the leptin assay was performed. All mice were sampled at the same time of day. As described previously for the animals used in the present study, adipose tissue depots, the right inguinal subcutaneous, the mesenteric, and right gonadal depots were individually dissected and weighed, with total body lipid determined by lipid extraction of the carcass (8).

Leptin Assay

Mouse plasma leptin was analyzed with a sensitive and specific radioimmunoassay developed to measure mouse leptin (13). Samples were run in duplicate. Coefficients of variation average 7.1% within runs and 10.0% between runs for this assay.

Statistical Analysis

Statistical analysis was performed with Statview for Windows version 5.0.1 (SAS Institute, Cary, NC) using least squares analysis. Leptin measurements for all ages sampled of WT and activated TG mice were tested for differences observed using a statistical model that included the fixed effects of genotype and sex as well as the interaction between them. The data were then subdivided into juvenile (42 and 63 days) and mature (84 and 105 days) subgroups so that the effects of transgene inactivation could be examined. TG animals that had the transgene activated and then inac-

tivated were only included at 84 and 105 days of age and therefore could not be tested in the model inclusive of data from samples obtained from 42- and 63-day mice. Splitting the data allowed for the examination of factors important to leptin expression between the juvenile and mature data sets. Correlations of circulating leptin concentrations and leptin expressed as a percentage of total body lipid with adipose depot weights and total body lipid were performed using correlation procedures of SAS (1988).

Results

To characterize circulating leptin concentrations as a function of genotype, we first determined whether the zinc supplementation affected leptin levels in WT mice. No difference ($p > 0.1$) in plasma leptin concentration was observed between WT animals and WT animals in which the zinc (transgene) stimulus was withdrawn at any age examined. Therefore, data for zinc-treated and untreated WT mice were pooled for subsequent analyses. In comparing WT and activated TG mice, circulating leptin concentrations were not significantly ($p > 0.1$) different between these groups, with the exception of male animals at 42 days of age (Table 1). However, when compared with either WT or activated TG mice, plasma leptin concentrations from inactivated TG mice were higher at both 84 and 105 days of age (Table 1; $p < 0.05$).

In a multivariate regression model, circulating leptin concentrations over the four ages revealed that gender effects ($p < 0.0001$) and the gender by age interaction ($p < 0.05$) were significant. In comparison with males, plasma leptin levels increased with age in the females. In addition, at each age examined, plasma leptin concentrations in females were higher than in males. This increase was statistically significant in older mice ($p < 0.01$) (Figure 1).

When the mature age group data (84 and 105 days of age) were analyzed inclusive of the inactivated genotype present at these ages, age by gender by genotype interactions were significant ($p < 0.0001$). That is illustrated by the plasma leptin concentrations for inactivated female TG mice. At 84 days and 105 days, female TG mice in which the transgene was inactivated had leptin concentrations that were significantly elevated compared with inactivated male TG mice (Table 1, $p < 0.0001$). More important, female mice in which the transgene was inactivated had significantly higher plasma leptin concentrations than either activated TG or WT genotypes ($p < 0.001$); this was true only at 105 days of age for male TG mice in which the transgene was inactivated. However, circulating leptin concentrations for TG mice were not significantly different from those of WT mice within a given gender ($p > 0.6$ for both genotype comparisons).

In an effort to identify whether a particular fat depot was highly correlated with circulating leptin concentrations,

body composition data for the mice used in this study as published previously (8) were compared with the circulating leptin values reported here. Parameters examined included total body fat (TBF) and the weights of subcutaneous, mesenteric, and gonadal fat pads. In both male and female animals, plasma leptin concentrations were positively correlated with all adipose parameters at 84 and 105 days of age, but not at 42 and 63 days of age ($p < 0.05$, Table 2).

Evidence exists that suggests that leptin concentrations can vary independent of TBF, for example during energy restriction (13,14). To investigate leptin levels adjusted for TBF, circulating leptin concentrations were expressed as per gram of TBF. Analysis of leptin adjusted for body fat revealed a decrease with age (3.0 ± 0.2 , 1.5 ± 0.2 , 1.2 ± 0.2 , and 1.0 ± 0.2 ng/mL per gram for 43, 63, 84, and 105 days, respectively, $p < 0.005$). In addition, females generally had higher adiposity-adjusted leptin levels than males, and perhaps most important, female WT animals had greater amounts of leptin per gram of TBF than either activated TG or inactivated TG females at 84 and 105 days of age; this tendency was also observed for male WT animals (Table 1 and Figure 2).

Discussion

In this study, female mice had consistently higher plasma levels of leptin than male mice regardless of treatment. This result concurs with previous studies on gender differences in plasma leptin concentrations in humans (15,16). The most important result was that animals in which the transgene was no longer actively expressing GH had significantly higher leptin values than continuously activated TG or WT control mice. In both female and male mice in which the transgene was inactivated, plasma leptin concentrations were three times higher than those of WT animals of the same gender. This difference was likely due to the enlarged adipose depots (300% greater than control mice) in these mice after the high expression of the GH transgene ceases (8). While leptin significantly correlated with all measures of fat at 84 and 105 days of age, it remains unexplained why the inactivated TG animals, who gain 300% more adipose tissue than WT control animals, do not adjust physiologically by decreasing food intake or increasing energy expenditure, which might be expected in response to increasing plasma leptin concentrations.

Leptin regulation is more complex than a simple reflection of adipose stores. The response of leptin to nutritional factors such as fatty acids and glucose, its diurnal expression patterns (17), and its diverse functions (6,7) are signs of this complexity. Although leptin itself may exert some autocrine/paracrine effect on isolated adipocytes (18), indirect effects of leptin may be regulated through the hypothalamic-pituitary axis or through activation of autonomic (sympathetic) outflow (19,20). In the present study, plasma

Table 1. Plasma leptin protein concentration and body weight in response to transgene activation status in WT, continuously activated TG, and inactivated TG (TG on/off) male and female mice*

	Weight (g) $\pm$ SEM†		Leptin (ng/mL) $\pm$ SEM		Total body fat (g) $\pm$ SEM		Leptin (ng/mL)/body fat (g)	
	Male	Female	Male	Female	Male	Female	Male	Female
42 days								
WT	19.1 $\pm$.6	15.5 $\pm$.6 ^a	1.3 $\pm$.2 ^a	2.0 $\pm$.2	0.65 $\pm$.15	0.72 $\pm$.15	2.1 $\pm$.7 ^a	3.3 $\pm$.7
TG	19.6 $\pm$.7	19.6 $\pm$.6 ^b	2.0 $\pm$.2 ^b	1.5 $\pm$.2	0.55 $\pm$.16	0.84 $\pm$.15	4.6 $\pm$.7 ^b	2.1 $\pm$.7
63 days								
WT	23.0 $\pm$.6 ^a	16.4 $\pm$.7 ^a	1.2 $\pm$.2	1.8 $\pm$.2	1.30 $\pm$.16	0.90 $\pm$.16 ^a	1.1 $\pm$.7	2.5 $\pm$.7
TG	28.5 $\pm$.6 ^b	26.0 $\pm$.7 ^b	1.2 $\pm$.2	1.7 $\pm$.2	1.42 $\pm$.15	1.46 $\pm$.15 ^b	1.0 $\pm$.7	1.3 $\pm$.7
84 days								
WT	25.0 $\pm$.6 ^a	19.9 $\pm$.5 ^a	1.6 $\pm$.3 ^a	2.1 $\pm$.3 ^a	1.72 $\pm$.27 ^a	1.33 $\pm$.26 ^a	1.2 $\pm$.2 ^b	1.9 $\pm$.17 ^b
TG	33.5 $\pm$.9 ^b	29.3 $\pm$.8 ^b	1.7 $\pm$.4 ^{ab}	1.9 $\pm$.4 ^a	2.86 $\pm$.43 ^a	2.08 $\pm$.40 ^a	0.6 $\pm$.3 ^{ab}	0.9 $\pm$.26 ^a
TG on/off	32.0 $\pm$.8 ^b	28.3 $\pm$.8 ^b	2.6 $\pm$.4 ^b	6.4 $\pm$.4 ^b	6.83 $\pm$.37 ^b	5.75 $\pm$.40 ^b	0.4 $\pm$.2 ^a	1.1 $\pm$.26 ^a
105 days								
WT	25.8 $\pm$.5 ^a	20.3 $\pm$.6 ^a	1.4 $\pm$.3 ^a	2.2 $\pm$.3 ^a	2.41 $\pm$.26 ^a	1.59 $\pm$.28 ^a	0.7 $\pm$.2	1.7 $\pm$.2 ^b
TG	35.0 $\pm$.8 ^c	31.2 $\pm$.8 ^c	1.3 $\pm$.4 ^a	2.2 $\pm$.4 ^a	2.53 $\pm$.37 ^a	2.38 $\pm$.37 ^a	0.6 $\pm$.2	0.9 $\pm$.2 ^a
TG on/off	31.1 $\pm$.8 ^b	27.0 $\pm$.8 ^b	2.4 $\pm$.4 ^b	7.9 $\pm$.4 ^b	7.21 $\pm$.37 ^b	6.35 $\pm$.40 ^b	0.3 $\pm$.2	1.3 $\pm$.3 ^{ab}

* Values are mean $\pm$ SEM. Means within an age and gender with different superscripts differ significantly ($p < 0.05$).

† Weight and adipose analyses adapted from Oberbauer et al. (6).

Figure 1. Circulating leptin concentrations (nanograms per milliliter) in male (hatched bars) and female (open bars) mice pooled across genotypes at varying ages. Values are expressed as least squares means $\pm$ SEM and asterisks denote significant ($p < 0.05$) differences between males and females.

leptin levels appear to be uncoupled from their physiological effects in inactivated TG mice.

The development of obesity in the face of elevated leptin in mice in which the GH transgene was inactivated was unexpected. The GH transgene is not expressed at the same time that visible obesity develops in these mice. Therefore, the actual accrual of adipose tissue must involve down-

stream effects of the transgene GH expression responsible for the capacity to become obese. Oberbauer et al. (8) have previously published data on the body composition of the mice used in this study (up to 105 days of age). They found that the obese phenotype that occurs in these TG mice does not appear to depend on insulin resistance, development of diabetes, or decreased brown fat thermogenic capacity. It

Table 2. Correlation coefficients (and significance levels*) for circulating leptin with TBF, gonadal fat pad, mesenteric fat pad, and inguinal fat pad weights for male and female mice pooled across genotypes at varying ages

	TBF	GFP	MFP	IFP
Males				
42 days	-0.68 ($p < 0.005$)	NS*	NS	NS
63 days	NS	NS	NS	NS
84 days	0.68 ($p < 0.001$)	0.68 ($p < 0.001$)	0.70 ($p < 0.001$)	0.60 ($p < 0.001$)
105 days	0.63 ($p < 0.001$)	0.60 ($p < 0.001$)	0.65 ($p < 0.001$)	0.62 ($p < 0.001$)
Females				
42 days	NS	NS	-0.57 ($p < 0.02$)	-0.51 ($p < 0.04$)
63 days	NS	NS	NS	NS
84 days	0.86 ($p < 0.001$)	0.88 ($p < 0.001$)	0.84 ($p < 0.001$)	0.84 ($p < 0.001$)
105 days	0.84 ($p < 0.001$)	0.78 ($p < 0.001$)	0.79 ($p < 0.001$)	0.76 ($p < 0.001$)

GFP, gonadal fat pad; MFP, mesenteric fat pad; IFP, inguinal fat pad.

* NS, nonsignificant ($p > 0.1$).

Figure 2. Circulating leptin expressed per gram of TBF for WT (hatched bars), TG (gray bars), and inactivated TG (striped bars) pooled male and female mice at varying ages. Values are expressed as least squares means $\pm$ SEM and asterisks denote significant ($p < 0.05$) differences from the WT genotype.

would seem plausible that chronically high GH levels do result in leptin resistance mediated through the hypothalamus by decreased receptor sensitivity or leptin transport. Our activated TG mice have chronically high GH levels. Physiologically, these mice may decrease hypothalamic sensitivity to leptin as an attempt to decrease perceived continued pituitary release of GH. It is not clear why this resistance would persist past 63 days when the high-level expression of the transgene is terminated in inactivated TG mice. However, in GH-deficient human patients, higher than expected plasma concentrations of leptin are seen (21), although this may reflect the enlarged adipose mass that accompanies GH deficiency. It is unclear in many patients whether this is the result of GH deficiency or decreased sensitivity to plasma leptin (22). In these human patients and in the mice with the inactivated transgene in this study, leptin resistance may impair the release of adequate GH from the pituitary (23,24).

Hormonal control of adipocyte function may be an important determinant of leptin expression; however, long-term basal leptin expression may actually be more closely correlated with indices of fat cell size. In some experiments, plasma leptin has been tightly correlated with fat cell size (25). Oberbauer et al. (8) demonstrated that WAT increases up to 270% by 105 days of age after transgene inactivation at maturity (63 days of age). The adipose tissue in these animals is composed of cells that are both larger and more numerous. It is expected that with increasing adipocyte cell number and/or size, leptin levels would increase correspondingly. In the oMT1a-oGH model, inactivated TG mice had more numerous small fat cells and the male inactivated TG mice had a greater number of large adipocytes, whereas the female inactivated TG mice had significantly larger large adipocytes. Because the effect of high GH levels is to negate the difference in growth rate between males and

females in this model, female TG mice put on proportionally more lean body mass over control females than male TG mice do over control males. This dimorphic response may represent a greater reserve growth potential or it may represent a differential sensitivity to the effects of elevated GH. In the latter case, females may be stretched closer to their genetic capacity under the influence of TG GH expression. Males may thus have more immediate resources available to produce more preadipocytes than do females who are putting more energy into developing lean body mass over the period of transgene expression from 21 to 63 days in this experiment. A large number of large-sized cells may yield relatively low leptin values (males), whereas a low number of very large cells yields high plasma leptin concentrations (females). This is supported by the data of leptin expressed on a per gram of TBF basis. Male inactivated TG mice had much lower adjusted leptin levels when compared with the data from the female inactivated TG mice.

Alternatively, leptin levels may not literally represent the amount of body fat but rather may be related to adipocyte cell membrane stretching or adipocyte glucose metabolism (26). It is interesting to note that Murray et al. (27) reported that expression of the oMT1a-oGH transgene results in altered cell membrane composition. Some membrane-associated compounds may be related to the signaling process involved in leptin expression or to the physiological response of adipocytes to inhibitory signals resulting from leptin activity. The cell membrane components may be altered in the oMT1a-oGH model.

Jorgensen et al. (28) found that leptin was an important determinant of resting basal metabolic rate (BMR). Long-term GH therapy in human subjects induces a new energy balance state characterized by the return of depressed plasma leptin concentrations to basal levels and by increased BMR. Body fat in these subjects remains reduced and indicates that a new energy balance is achieved (29). Unfortunately, Karlsson et al. (29) did not examine leptin and BMR in a post-therapy state in which the opposite could be predicted to be true (i.e., elevated leptin and decreased BMR). The TG mice in this study also appear to have established a new postinactivation steady state of energy balance that has been triggered by TG GH expression. The nature of this interaction needs to be investigated.

In this study, we examined the plasma concentrations of leptin in both sexes of mice that demonstrate an obese phenotype on cessation of high levels of transgene GH expression. We have shown that plasma leptin concentrations in the obese inactivated TG mice follow predicted patterns with regard to gender, genotype, and age. However, examining the amount of leptin produced per gram of body fat indicates a significant reduction in the amount of leptin secreted per gram of lipid in animals subjected to elevated concentrations of GH (activated TG) and in animals in which the elevated GH has been removed (inactivated TG).

It is possible that the elevated GH alters glucose utilization in the adipose cell by promoting insulin resistance. The activated TG mice have elevated circulating insulin concentrations without alteration of circulating glucose levels, suggesting that these animals are experiencing insulin resistance (8). Decreased glucose utilization is known to depress leptin secretion by the adipocyte (6,7). Although we have not directly measured glucose metabolism in adipocytes from animals exposed to elevated GH, it is well known that excess GH can induce insulin resistance. It is possible that the lowered leptin production on a per gram of fat basis is due to a decrease in insulin-mediated glucose metabolism within adipose tissue. If adipose tissue is using less glucose, the production of leptin would be diminished (6,7). If this were the case for the activated TG animal, the elevated GH would serve to prevent excess fat deposition; however, on withdrawal of the elevated GH, as seen in the inactivated TG, the lower leptin on a per gram fat basis would no longer be counterbalanced by the elevated GH and the obese phenotype could develop as the adipocytes hypertrophy.

The data suggest that normal physiological responses to elevated plasma leptin are impaired in response to prolonged elevation of GH. Although the most likely site of impairment is in the hypothalamic axis (leptin resistance), GH effects at the adipocyte cellular level are also possible particularly because a number of adipocyte characteristics are altered by GH transgene expression (e.g., cell size, number, and membrane composition) (8,27). One of the most important factors in the production of the obese phenotype must be the recruitment and differentiation of preadipocytes. We have shown that GH causes an obese phenotype as well as the development of an abnormal physiological response to increasing plasma leptin concentrations. The results of the present study suggest that alterations of leptin production and action induced by overexpression of GH contribute to increased adiposity in this model.

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