Impaired Leptin Responsiveness in Aged Rats

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We previously reported that adiposity and serum leptin levels increase with age in male F-344×BN rats and that when physiological levels of serum leptin are manipulated by fasting, there is a corresponding reciprocal change in hypothalamic neuropeptide Y (NPY) mRNA in young rats, but there are no changes in older rats. These findings suggest that the regulation of hypothalamic NPY mRNA by leptin may be impaired with age. To test this hypothesis, we infused saline or leptin for 7 days into ad libitum-fed rats and compared these with saline-infused rats that were pair-fed the amount of food consumed by the leptin-treated rats. We examined daily food consumption, body weight, whole-body oxygen consumption, serum leptin, and NPY mRNA in the hypothalamus. Food consumption decreased by 50% in the leptin-infused compared with the saline-infused young rats but only decreased by 20% in the aged rats. In the leptin-treated young rats, there was a 24% increase in oxygen consumption compared with the pair-fed rats, but there were no changes in oxygen consumption in the aged rats. Leptin infusion diminished hypothalamic NPY levels by nearly 50% compared with pair-fed young rats, whereas there were no changes in the hypothalamic NPY mRNA levels in senescent rats. In summary, aged rats demonstrate a reduced responsiveness to leptin, including a diminished decrease in food intake and no increase in energy expenditure. These diminished responses to leptin were associated with and may be the result of an impaired suppression of hypothalamic NPY mRNA levels. This leptin resistance may be due to either the elevated obesity and serum leptin with age or due to age itself, or both. Diabetes 49:431-435, 2000

Leptin, synthesized by white adipose tissue (WAT), is an afferent signal molecule that interacts with the appetite and satiety centers in the brain to regulate body weight, and this hormone contributes to the regulation of both food intake and energy expenditure (1-4). Leptin modulates a number of neuropeptides in the hypothalamus, including neuropeptide Y (NPY) (5). NPY stimulates feeding (6) and suppresses thermogenesis in brown adipose tissue (BAT) (7). One mechanism by which leptin increases energy expenditure is through increasing thermogenesis in BAT (4,8). Thus, leptin, by inhibiting NPY synthesis, reduces food intake and increases energy expenditure, and this may be one mechanism by which leptin promotes weight loss.

In the absence of functional leptin (such as the ob/ob mouse) or in the absence of a functional leptin receptor (such as the db/db mouse), obesity results. However, obesity in humans is not usually associated with leptin deficiency (9). Serum leptin is markedly elevated in most obese humans, and obesity persists in spite of the elevated leptin, which should promote weight loss (9,10). It has been suggested that human obesity as well as many rodent models of obesity are associated with leptin resistance, which becomes more pronounced with progressive degrees of obesity (10-13).

A common form of obesity, late-onset obesity, is characterized by a steady weight gain as adults age until early senescence, after which body weight declines (14). The F-344×BN rat strain is a useful model for late-onset human obesity because it demonstrates a steady increase in body fat into early senescence, followed by a decline (15). In our previous study (15), we found that adiposity in male F-344×BN rats increased with age, which could not be attributed to either increased food intake, impaired leptin gene expression, or impaired peripheral leptin production. In fact, serum leptin levels and leptin mRNA levels in WAT were actually increased with age in these rats (15). Thus, the aged rats become obese in spite of the elevated leptin, suggesting the relationship between leptin, adiposity, and food intake is altered with age.

Furthermore, in our recent study, when physiological levels of serum leptin were manipulated by fasting, there was a corresponding reciprocal change in hypothalamic NPY mRNA in young rats, but there were no changes in older rats (16). These findings suggest that the regulation of hypothalamic NPY mRNA by physiological levels of leptin may be impaired with age. It is unknown whether hypothalamic NPY mRNA in aged rats will respond to regulation by a pharmacological dose of leptin.

To test whether aged rats will respond to a pharmacologic dose of leptin, we infused saline or leptin for 7 days into ad libitum-fed young and senescent rats and compared these with rats saline-infused and pair-fed the amount of food consumed by the leptin-treated rats. We examined parameters associated with both food intake and energy expenditure, including daily food consumption, body weight, whole-body oxygen consumption, serum leptin, and NPY mRNA in the hypothalamus.

RESEARCH DESIGN AND METHODS

Animals. There were 6- and 30-month-old male F-344×BN rats obtained from Harlan Sprague-Dawley (Indianapolis, IN). On arrival, rats were examined and
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Data are means ± SE of eight rats per group. P = 0.0001 for difference with age or pair-feeding by two-way ANOVA. *P = 0.0001 for difference between young and old (ad libitum–fed) and young and old (pair-fed) rats; †P = 0.0036 (young) and P = 0.0034 (old) for difference between ad libitum–fed and pair-fed rats. There are no significant differences between serum leptin levels in the leptin-infused young and old rats.

remained in quarantine for 1 week. Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals. Rats were housed individually with a 12:12 h light:dark cycle (0700–1900). Ambient temperature was 26°C—thermoneutral for these rats (17).

Experimental design and leptin administration. Rats (8 per group) were administered either saline (control and pair-fed groups) or mouse leptin by a minipump (1 mg leptin/day) for 7 days. Osmotic minipumps (model 2ML1; Alzet, Palo Alto, CA) were implanted subcutaneously along the backs of the rats. Control and leptin-treated rats were allowed access to food ad libitum, whereas pair-fed rats were pair-fed the amount of food consumed by the leptin-treated rats. The pair-fed rats began the experiment 1 day later than the leptin-treated rats. The amount of food consumed by the leptin-treated group was then provided to the pair-fed group once a day in the evening. Food consumption was measured on a daily basis. Rats were killed on the seventh day after minipump implantation.

Chemicals. Mouse leptin was a gift from Amgen (Thousand Oaks, CA). All other chemicals were obtained from Sigma (St. Louis, MO). O2 consumption. O2 consumption was assessed in up to four rats simultaneously with an Oxyscan analyzer (OX-54; Omnitech Electronics, Columbus, OH) as described previously (18). Flow rates were 2 l/min with a 30-s sampling time at 5-min intervals. The rats were placed into the chamber for 90 min with the O2 consumption values for the last 30 min used in the calculations. The temperature was maintained at 26°C. Food was not available. Results were expressed on a mass-independent basis (ml · min−1 · kg−0.75).

Tissue harvesting. Rats were killed by cervical dislocation under 85 mg/kg pentobarbital sodium anesthesia. Blood samples were collected by heart puncture, and serum was harvested by a 30-min centrifugation in serum separator tubes. The circulatory system was perfused with 20 ml of cold saline and epididymal (EWAT), perirenal (PWAT), and retroperitoneal (RTWAT) white adipose tissues, and BAT and hypothalamus were excised. The hypothalamus was removed by making an incision medial to the piriform lobes, caudal to the optic chiasm, and anterior to the cerebral crus to a depth of 2–3 mm.

Leptin radioimmunoassay. Serum leptin levels were measured with a rat leptin radioimmunoassay kit (Linco Research, St. Charles, MO).

mRNA levels. Total cellular RNA was extracted using a modification of the method of Chomczynski and Sacchi (19). The integrity of the isolated RNA was verified using agarose gels (1%) stained with ethidium bromide. The RNA was quantified by spectrophotometric absorption at 260 nm using multiple dilutions of each sample. The rat pre-pro NPY cDNA was provided by Janet Allen (University of Pennsylvania, Philadelphia, PA) and labeled using a random primer kit (Prime-a-Gene, Promega, Madison, WI). For dot-blot analysis, multiple concentrations of the RNA were immobilized on nylon membranes using a dot-blot apparatus (Bio-Rad, Richmond, CA). The membranes were baked at 80°C for 2 h. The baked membranes were prehybridized in 10 ml Quikhyb (Stratagene, La Jolla, CA) for 20 min followed by hybridization in the presence of a labeled probe and 100 µg denatured salmon sperm DNA. After hybridization for 2 h at 65°C, the membranes were washed and exposed to a phosphor imaging screen for 48 h. The latent image was scanned using a Phosphor Imager (Molecular Dynamic, Sunnyvale, CA) and analyzed by ImageQuant Software (Molecular Dynamics). Intensities per microgram total cellular RNA were calculated by comparison to internal laboratory standards of hypothalamic mRNA present on each nylon membrane. In some cases, nylon membranes probed for specific mRNA were stripped and rehybridized with g-actin.

Statistical analysis. Data were analyzed by one-way or two-way analysis of variance (ANOVA). When the main effect was significant, a post-hoc test (either Tukey-Kramer or Scheffé’s S test) was applied to determine individual differences between means. A value of P < 0.05 was considered significant.

RESULTS

Leptin infusion and food consumption. Endogenous levels of serum leptin were 2.7-fold greater in aged rats compared with young rats (Table 1). This increase in serum leptin with age may reflect both the twofold increase in fat depot size with age (Table 2) and the increase in leptin mRNA levels per unit of WAT with age (15). Leptin (1 mg/rat) was administered to both young and old rats based on the observation that lean body mass is unchanged between these two age-groups (20). Leptin administration for 7 days raised serum leptin levels to >100 ng/ml in both young and old rats (Table 1). The final serum concentration of leptin was not significantly different between young and old rats (Table 1). Leptin treatment decreased food intake in both young and old rats but to a greater extent in young rats (Fig. 1). After 1 day of leptin, there was a 44% decrease in food intake in the young rats and a 13% reduction in the aged rats (P = 0.001) compared with corresponding pretreatment food consumption. By the seventh day, food consumption was diminished by 65% in the young rats.
rats compared with 35% in the old rats (P = 0.0001, Fig. 1). Food consumption was unchanged in the ad libitum-fed rats, except for the first day after the minipump implantation, in which case food intake was diminished in both young and old rats (data not shown). Over the course of 1 week, food consumption decreased by nearly 50% in the leptin-infused compared with the saline-infused young rats (102 ± 3 vs. 54 ± 4 g), but only 20% in the aged rats (99 ± 5 vs. 81 ± 3). A third group of young and old rats were pair-fed the daily amount of food consumed by the leptin-treated rats. Pair-feeding decreased endogenous serum leptin 50% in the young rats and nearly 40% in the aged rats; however, serum leptin levels remained 3.3-fold higher in the old rats compared with young rats (Table 1).

**Body weight and adiposity.** In our previous study, a 7-day leptin infusion in young rats did not reduce body weight beyond that due to the leptin-induced reduction in food intake (4). Similarly, in the present study, in young rats, both pair-feeding and leptin administration resulted in a significant loss of body weight compared with pretreatment weight levels (P = 0.0001), but there was no greater loss of body weight in the leptin-treated rats (−29.3 ± 5.2 g) compared with the pair-fed rats (−30.4 ± 2.1 g). With pair-feeding in the aged rats, the weight loss was 50% less (−13.3 ± 2.5 g) compared with the pair-fed young rats (P = 0.02), but again there was no greater loss of body weight in the leptin-treated aged rats (Table 2). The adiposity level, as assessed by the sum of three WAT depots, EWAT, PWAT, and RTWAT, was two-fold greater in the senescent rats compared with the young rats (Table 2). Similarly, the adiposity index (the sum of the weight of EWAT, PWAT, and RTWAT divided by body weight × 100 (21)) was greater in the senescent rats compared with the young rats (2.71 ± 0.07 vs. 1.93 ± 0.05, P < 0.0001). After leptin infusion in young rats, there was a 28% decrease in the combined fat depot size compared with the ad libitum-fed rats (Table 2). In contrast to the changes in body weight, the decrease in adiposity could not be accounted for by pair-feeding; there was a 20% decrease in fat depot weights in the leptin-infused compared with the pair-fed young rats (Table 2). In the senescent rats, despite the small loss of body weight, there was no change in the combined fat depot size with either pair-feeding or leptin infusion (Table 2).

**Oxygen consumption.** Whole-body oxygen consumption was determined on the sixth day after minipump implantation. In the young rats, pair-feeding resulted in a 16% decrease in oxygen consumption compared with the ad libitum-fed rats (Fig. 2). With leptin administration in young rats, there was a highly significant 24% increase in oxygen consumption compared with the pair-fed rats (Fig. 2). By observation, all rats were inactive during oxygen consumption measurements, and there were no observed differences in activity levels between groups. In contrast to young rats, there were no changes in oxygen consumption with either pair-feeding or leptin administration in the aged rats (Fig. 2).

**Hypothalamic NPY gene expression.** As expected in the young rats, both pair-feeding and leptin infusion modulated hypothalamic NPY mRNA levels. There was a 30% increase in hypothalamic NPY mRNA levels in the pair-fed rats compared with the ad libitum-fed young rats (Fig. 3). After leptin infusion, NPY levels diminished by nearly 50% compared with the pair-fed rats and 35% compared with the ad libitum-fed rats (Fig. 3). Among the ad libitum-fed rats, basal NPY levels were 18% less with age in the senescent compared with the young rats (Fig. 3). Qualitatively, with pair-feeding and leptin administration, the same pattern was observed in old rats as in the young rats: an increase with pair-feeding and a decrease with leptin; however, in contrast to changes in the young rats, these changes were not significantly different in the senescent rats (P = 0.081, Fig. 3).
DISCUSSION
The male F-344×BN rat demonstrates a gradual increase in adiposity, serum leptin, and body weight between 3 and 24 months of age followed by a slight decline between 24 and 30 months of age (15). Thus, the male of this rat strain is not weight stable at any age. In addition, our previous data indicate that food intake is unchanged at five different ages ranging from 3 to 30 months (15,16), and the present data indicate basal oxygen consumption is unchanged with age. Thus, there is a steady increase in adiposity and serum leptin with age despite unchanged food intake and basal oxygen consumption. Because leptin contributes to both the negative regulation of food intake and the positive regulation of energy expenditure (2–4), these data suggest that the relationship between leptin and both oxygen consumption and food intake is altered with age. Furthermore, when challenged with a 48-h fast, both young and senescent rats responded with a decrease in serum leptin (16). However, there was a corresponding increase in hypothalamic NPY mRNA only in the young rats, with no increase in the aged rats (16). In addition, serum leptin and hypothalamic NPY mRNA were strongly correlated in young rats but demonstrated no correlation in either the 24- or 31-month-old rats (16). These data indicate that young male rats can respond to small physiological changes in serum leptin (from 2 to 7 ng/ml) with a corresponding reciprocal change in hypothalamic NPY mRNA, whereas older male rats are unresponsive to changes in serum leptin between 10 and 18 ng/ml. These data alone suggest that the regulation of hypothalamic NPY mRNA by serum leptin is blunted with age. However, because basal levels of serum leptin are higher in older rats, small increments in serum leptin represent smaller percentage changes in serum leptin in the older rats. Possibly, the hypothalamus responds to threshold percentage increases rather than absolute increases in leptin. In addition, the higher basal serum leptin in older rats may already have maximally suppressed hypothalamic NPY mRNA levels. In the present study, we administered a pharmacological dose of leptin, which results in serum leptin levels >100 ng/ml in both young and old rats. At this dose, the young rats responded by decreasing food intake and increasing oxygen consumption, whereas these responses were blunted or absent in the older rats. A number of different neuropeptides in the hypothalamus are believed to mediate the responses of leptin (5). One of these, NPY, both stimulates food consumption and suppresses thermogenesis in BAT (6,7). Leptin, by inhibiting NPY synthesis, reduces the NPY increase in food intake and relieves the NPY suppression of energy expenditure, thus promoting weight loss. In the present report, 7 days of leptin infusion suppressed NPY mRNA levels in the hypothalamus of young rats but not in the hypothalamus of senescent rats. These data suggest that the leptin regulation of hypothalamic NPY mRNA is greatly diminished in senescent rats and that the impaired ability of leptin to decrease food intake and increase oxygen consumption in the senescent rats may, at least partially, be due to an inability to suppress NPY mRNA levels in the hypothalamus. Moreover, in the pair-fed young and old rats, there was a 40–50% decrease in serum leptin with a corresponding increase in hypothalamic NPY mRNA levels only in the young rats. These data suggest that the upregulation of hypothalamic NPY mRNA is also impaired in aged rats and challenge the idea that the elevated serum leptin levels with age have already maximally suppressed hypothalamic NPY mRNA levels.

In the present study, the final concentration of serum leptin after leptin infusion was not significantly different between young and old rats. However, because the initial concentration of serum leptin was lower in the young rats, the relative incremental increase in leptin after infusion was greater in the young rats. It is possible that the older rats may have fully responded to a higher dose of leptin that produced the same relative incremental increase in leptin observed in the young rats. However, the difference in initial basal levels of leptin between young and old rats was <10 ng/ml compared with the serum leptin levels of >100 ng/ml after the pharmacological dose of leptin. More importantly, hypothalamic NPY mRNA levels demonstrated a reciprocal regulation with both physiological changes in leptin (16) and pharmacological changes in leptin (present study) in the young rats, whereas neither physiological nor pharmacological changes in leptin altered hypothalamic NPY mRNA in the aged rats.

It has been proposed that human obesity may lead to leptin resistance, which becomes more pronounced with progressive degrees of obesity (10). In addition, diet-induced obese mice develop resistance to leptin (11–13). Thus, aged rats, like obese humans and diet-induced obese rodents, may be leptin resistant because they are fat rather than because they are old. However, the degree of obesity in aged F 344×BN rats is not comparable to the degree of obesity in typical mice or rats with genetic or diet-induced obesity. Both of the latter demonstrate twofold body weight gains over 2- to 3-month periods, whereas the male F 344×BN rat demonstrates a 40% increase over 24 months. Thus, aged F 344×BN rats are best characterized as senescent rather than obese and more represent a model for the gradual increase in body weight with age observed in most humans (14) rather than a model for obesity. Nevertheless, the present report cannot distinguish whether the leptin resistance observed in these aged rats is a consequence of either senescence or mild obesity and most likely is due to both.

The mechanism of the leptin resistance is unknown but may be multifactorial. Potential possibilities include impaired transport of leptin across the blood-brain barrier, reduced leptin signal transduction, antagonism of leptin action, or impaired end organ responsiveness. Diet-induced obese A K R mice and New Zealand obese mice are resistant to peripheral but not central administration of leptin (12,13), suggesting the leptin is not reaching the target sites in the brain. However, diet-induced obese Wistar rats (11) and obese A y mice (13) are resistant to central-administered leptin, suggesting an impairment at or downstream of leptin target sites in the brain. One possibility is a desensitization to leptin. For example, in F 344×BN rats, serum leptin levels increase with age (15) and may contribute to the leptin resistance by desensitization of the receptor/leptin signal transduction pathway. In addition, a recent report suggested that leptin resistance may be a result of increased levels of SOCS-3 (suppressor of the cytokine-signaling factor) (22). This factor, which suppresses the leptin activation of the Janus kinase signal transducer and activation of transcription (JAK-STAT) signal transduction pathway, is induced by leptin and is elevated in leptin-resistant obese A y/a mice. The latter or any of the above possibilities could be contributing to the failure of leptin and pair-feeding to modulate hypothalamic NPY levels in aged rats.

In the present study, leptin infusion did not induce any body weight loss other than the weight loss due to the leptin-induced decrease in food intake, that is, the reduction in body
weight was the same in pair-fed rats compared with leptin-treated rats. These findings are similar to our two previous reports (4,8) and suggest that the body weight loss was secondary to the leptin-induced food reduction. Similarly, in the older rats, the reduction in body weight was the same in pair-fed rats compared with leptin-treated rats. However, because the decrease in food intake in the leptin-treated young rats was greater than the decrease in food intake in the senescent rats, the corresponding weight loss in the older rats was less than in the young rats. In contrast to the changes in body weight, the leptin-induced decrease in WAT depot weight in young rats was, at least partially, independent of the reduction in food intake. Leptin treatment significantly decreased WAT depot size, whereas with pair-feeding, WAT depot size was between that of the ad libitum–fed and leptin-treated groups and not significantly different from either. These data suggest that pair-feeding induced a small nonsignificant decrease in WAT depot weight, whereas leptin treatment induced a larger highly significant decrease, indicating a metabolic effect of leptin independent of the reduction in food intake.

The present data indicate that in aged rats, the leptin-induced decrease in food intake is blunted, whereas the leptin-induced increase in oxygen consumption is absent. The leptin-induced increase in oxygen consumption was apparently not due to an increase in activity levels. By observation, all rats remained inactive during periods of measurement, and there were no observed differences in activity levels between groups. However, it is possible that one component of the leptin-induced increase in oxygen consumption is due to an increase in activity. In our previous report, we found that the increase in oxygen consumption in young rats did not occur until the fourth day after leptin administration, even though the decrease in food intake was evident after the first day (4). With age, physiological responses may be delayed and reduced in magnitude. We previously demonstrated that thermogenesis in brown adipose tissue in response to a bacterial infection was delayed in aged rats (23). The thermogenic response after a bacterial infection is mediated by cytokines (24). Leptin, whose receptor is from the cytokine receptor family (25), increases thermogenesis in BAT that is associated with an increase in oxygen consumption (4,8). Thus, it seems probable that in the older rats, any leptin-induced increase in oxygen consumption would be both reduced in magnitude and delayed, such that by 6 days of infusion, there is no evidence of an increase in oxygen consumption. We would predict that longer infusions of leptin would result in an increase in oxygen consumption but to a lesser extent than in the younger rats.

In summary, aged male rats demonstrate a reduced responsiveness to leptin, including a diminished decrease in food intake and no increase in energy expenditure. These diminished responses to leptin were associated with and may be the result of an impaired suppression of hypothalamic NPY mRNA levels. This leptin resistance may be due to either the elevated obesity and serum leptin with age or due to age itself, or both.

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