Abnormalities of leptin and ghrelin regulation in obesity-prone juvenile rats

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Diet-induced obesity (DIO) in the rat shares several features with human obesity. Approximately one-half of outbred rats fed a diet of moderately high fat and caloric density [high-energy (HE) diet] increase their caloric intake and become obese with consequent hyperinsulinemia and insulin resistance (8, 33). The remaining rats are HE diet resistant (DR), gaining no more weight than low-fat chow-fed controls do (33). Both the DIO and DR traits appear to be polygenic, but selective breeding for these phenotypes from the outbred Sprague-Dawley strain (30). Whereas outbred rats become obese only when provided a HE diet, selectively bred DIO rats begin to increase their weight, even on chow, between 5 and 7 wk of age. This observation suggests that, at that age, DIO rats experience an unidentified error in the control of energy homeostasis.

The systems controlling energy homeostasis respond to alterations of energy stores by compensatory changes in appetite and energy expenditure that resist these changes. Implicit in this system are “adiposity signals” that communicate the status of body energy stores to the brain (25). Peripheral hormones serving this function should satisfy several key criteria. They should circulate in relation to adipose mass, modulate the activity of neurons known to be involved in energy balance, and alter body weight in opposite directions when either administered or blocked chronically. Leptin is the prototypic adiposity signal that unequivocally satisfies these criteria (41). This adipocyte-derived hormone circulates in proportion to body-fat stores (18) and exerts long-term catabolic effects by regulating numerous classical body-weight control centers in the brain through the long (signaling) splice variant (Ob-Rb) of its receptor (41, 45). Chronic leptin administration promotes weight loss, and ablation of leptin signaling leads to massive obesity (6, 19, 22).

Ghrelin is a recently discovered, 28-amino acid peptide secreted primarily from the stomach and small intestine (3, 5, 12, 26). Somewhat surprisingly for a gut hormone, ghrelin displays all of the qualities articulated above for an adiposity signal that participates in long-term body-weight regulation. Circulating ghrelin levels are inversely proportionate to body mass index and are modulated bidirectionally by changes in body weight (11, 39, 47). Peripheral and central ghrelin administration increases food intake in humans and other species as potently as any known compound (50, 51). This effect occurs, at least in part, through activation of neurons in the hypothalamic arcuate nucleus that coexpress neuropeptide Y and agouti-related protein, both prototypic anabolic neuropeptides that promote weight gain (4, 10, 16, 23, 24, 26, 38, 42). These same neurons are inhibited by leptin (41, 48). Chronic

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ghrelin administration increases body weight, not only by stimulating food intake but also by inhibiting energy expenditure and fat catabolism (46). Conversely, blockade of ghrelin signaling reduces food intake, fat mass, and body weight (37, 38, 43). These findings suggest that ghrelin may be an anabolic counterpart to leptin in energy homeostasis.

We hypothesized that abnormal adiposity signaling triggers the spontaneous hyperphagia that occurs at 5–7 wk in DIO rats. This was based, in part, on the finding that obesity-prone outbred DIO rats have reduced sensitivity to the anorectic effects of leptin, which might lead them to become obese on a HE diet (29). Thus selectively bred DIO rats might also have decreased hypothalamic Ob-R expression or decreased leptin levels. Also, given the potent anabolic effects of ghrelin, we postulated that selectively bred DIO rats might have increased plasma ghrelin levels and/or increased hypothalamic ghrelin receptor expression. To test these hypotheses, we examined hormone levels and central receptor expression for leptin and ghrelin in selectively bred DIO rats from 5 to 7 wk of age, when these animals begin their spontaneous increase in food intake and body weight.

METHODS

Animals and diet. Animal usage was in compliance with the Animal Care Committee of the East Orange VA Medical Center and the Guidelines of the American Physiological Society (1). These experiments were designed to establish the relationships among plasma leptin, ghrelin, and insulin levels and the food intake and body weight gain of DIO and DR rats on chow and HE diet at a time when the DIO rats begin to gain weight spontaneously. Male 5-wk-old Sprague-Dawley rats selectively bred for the DIO (n = 18) or DR (n = 18) traits were obtained from our resident breeding colonies (30). At weaning (~3 wk of age), they were placed on a reversed 12:12-h dark-light schedule with lights off at 1000 (dark onset) and on at 2200 (light onset). During weaning, rats were fed ad libitum with Purina rat chow (no. 5001), which contains 3.3 kcal/g with 23.4% as protein, 4.5% as fat, and 72.1% as carbohydrate that is primarily in the form of complex polysaccharide (31). From 5 to 6 wk of age, baseline body weights and caloric intakes of chow were assessed daily. A separate group of 6-wk-old DIO and DR rats (n = 12 per genotype) also had their food intake monitored over 24 h with measures made specifically during the 4 h before dark onset (0600–1000), the first 6 h after dark onset (1000–1600), and the entire 24-h period. Additional groups of 18 DIO and 18 DR 6-wk-old chow-fed rats had 0.5 ml of tail blood sampled at both dark onset (1000) and 6 h into the dark phase (1600) for baseline measures of plasma ghrelin, leptin, and insulin. Then nine rats of each genotype were placed on HE diet ad libitum, and the remaining nine rats per genotype were continued on chow. The HE diet is composed of 8% corn oil, 44% sweetened condensed milk, and 48% Purina rat chow (Research Diets no. C11024F, New Brunswick, NJ). It contains 4.5 kcal/g, with 21% of the metabolizable energy content as protein, 31% as fat, and 48% as carbohydrate, 50% of which is sucrose (31). Food intake and body weight were assessed daily, and tail blood was taken at both 1000 and 1600 on days 3, 7, and 10. The animals were killed by decapitation on day 11 between 1000 and 1200. Brains were removed and frozen quickly on powdered dry ice, and epididymal, retroperitoneal, perirenal, and mesenteric fat pads were removed and weighed.

Plasma ghrelin, insulin, and leptin levels. Total immunoreactive ghrelin levels were measured with a radioimmunoassay that uses a polyclonal antibody raised against acylated rat ghrelin and 131I-labeled ghrelin as the tracer (Phoenix Pharmaceuticals, Belmont, CA). This assay detects both acylated and des-acyl ghrelin. Although only acylated ghrelin is bioactive (26), levels of total ghrelin are a good surrogate for those of acylated ghrelin, because the ratio of the two remains constant under a wide variety of conditions (2, 37). Plasma insulin and leptin levels were measured using radioimmunoassay kits (Linco, St. Charles, MO), with rat insulin and leptin as standards.

Leptin receptor, ghrelin, and ghrelin receptor mRNA assays by in situ hybridization. Serial 15-μm sections were taken through the rostrocaudal extent of the arcuate (ARC), dorsomedial (DMN), and ventromedial (VMN) hypothalamic nuclei. Sections were processed for in situ hybridization by minor modifications of previously described methods (28). Briefly, cRNA was synthesized and radiolabeled from a radioactive probe corresponding to the intracellular domain of the long form of the leptin receptor (Ob-Rb, amino acids 930–1063) kindly supplied by J. K. Elmquist, Harvard Medical School; an 853-bp probe for the ghrelin receptor (GHS-R) kindly supplied by N. Vrang, Rheoscience; and a 501-bp probe for ghrelin kindly supplied by M. Tschöp, German Institute of Human Nutrition. Sense probes were generated for each of the antisense probes and run to control for nonspecific hybridization. Frozen sections of brain (15 μm) were freeze-thawed onto gel-coated slides and fixed in 4% paraformaldehyde and 10% neutral buffered formalin. The probes were then subjected to our standard method for in situ hybridization (28). On completion of hybridization, slides were exposed to SB-5 X-ray film (Kodak, Rochester, NY) for 3–28 days.

The resulting autoradiograms were read by an experimentally “blinded” observer using computer-assisted densitometry (Drexel University, Philadelphia, PA) (28). Areal (mm2) or optical density (OD) measures were made through the entire rostrocaudal extent of the hypothalamus in the ARC, VMN, and DMN. The outlines of the autoradiographic images of GHS-R expression were well defined. These images were quantitated using our standard method by which a line is drawn around the exposed image of each nucleus, and the area within the boundaries of the outlined anatomic structure is measured (28). The Ob-Rb images were less well defined than those for GHS-R, such that no distinct borders for a given hypothalamic nucleus could be clearly discerned. For these images, the brain section used to generate the individual autoradiographic image was stained with cresyl violet, and the digitized image of that histological section was superimposed upon the digitized autoradiographic image. A line was drawn around the anatomic boundaries of the ARC, VMN, and DMN on the histological image, and the OD was read from the superimposed autoradiographic image.

Statistics. Body weight gain, daily food intake, and plasma ghrelin, leptin, and insulin measures were compared among various groups by two-way analysis of variance (ANOVA) for repeated measures with post hoc Scheffé comparisons when significant differences were found. Total body weight gain, cumulative food intake and fat pad weights, and GHS-R areal and Ob-Rb OD measures were compared by two-way ANOVA with post hoc Scheffé comparisons where appropriate. Feed efficiency (amount of weight gained relative to caloric intake) was calculated as the gain in body weight (g) per food intake (kcal) over the period of observation.
RESULTS

Food intake, body weights, and fat pad weights. At 5 wk of age, despite comparable 24-h caloric intake of chow, selectively bred chow-fed DIO rats were already 9% heavier than DR rats (Fig. 1; Table 1). The DIO rats gained 19% more weight and consumed 6% more calories than DR rats over the next 7 days on chow, although there was no difference in the amount of weight gained as a function of energy intake (feed efficiency; Table 1). This 7th day was the one on which the first baseline plasma samples were drawn for leptin, ghrelin, and insulin levels at dark onset (1000) and 6 h into the dark phase (1600). Because the amount and pattern of food intake can affect these plasma levels, we used a different group of rats to assess intake separately during the 4 h before and the 6 h after dark onset and for the entire 24 h on that day. There were no differences in the intake patterns between genotypes during the periods before and after dark onset. DIO caloric intake (7.6 ± 0.9 kcal) over the 4 h before dark onset was comparable to DR intake (9.3 ± 1.3 kcal). Similarly, DIO intake (30.9 ± 1.0 kcal) over the first 6 h after dark onset was comparable to that of DR rats (27.2 ± 2.0 kcal). Neither did the 24-h caloric intake differ significantly between DIO (62 ± 3 kcal) and DR rats (57 ± 3 kcal).

Over the next 10 days, rats were either switched to HE diet or continued on chow. Blood samples were drawn just before the switch (baseline) and at 3, 7, and 10 days after the switch. Over this 10-day period, DIO rats gained 33% more body weight [Table 1; F(1,32) = 15.57; P = 0.0001], and their final body weights were 12% greater than those of DR rats [F(1,32) = 37.55; P = 0.0001]. Overall, there was no significant effect of diet on weight gain. During the first few days of HE diet intake, both DIO and DR rats increased their caloric intake markedly (Fig. 2). In fact, they continued to eat approximately the same weight of food as they had on chow, but because the caloric density of the HE diet is 35% higher than that of chow, their total caloric intake was increased. DR rats on HE diet made a relatively rapid adjustment to this change in caloric density by reducing their caloric intake of HE diet to that of chow-fed controls after 4 days. However, whereas DIO rats did reduce their intake somewhat, they never fully compensated for the increased caloric density of the diet and maintained a significantly higher intake of HE diet than did all other groups over the entire 10-day period [phenotype F(1,8) = 15.13, P = 0.003; diet F(1,8) = 11.72, P = 0.009; phenotype × diet F(1,18) = 4.56, P = 0.05]. Cumulatively, DIO rats ate more than DR rats during the 10 days on either chow or HE diet [Table 1; F(1,32) = 37.42; P = 0.001], and rats on HE diet ate more than those on chow (P = 18.55; P = 0.001). However, these differences were

Table 1. Food intake, feed efficiency body weight and body weight gain of selectively bred DR and DIO rats on chow and HE diet

<table>
<thead>
<tr>
<th></th>
<th>DR Chow</th>
<th>DR HE</th>
<th>DIO Chow</th>
<th>DIO HE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial 24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>118 ± 5a</td>
<td>129 ± 3b</td>
<td>165 ± 6a</td>
<td>156 ± 4b</td>
</tr>
<tr>
<td>24-h Food intake, kcal</td>
<td>60.1 ± 3.0a</td>
<td>62.3 ± 2.2a</td>
<td>47 ± 3a</td>
<td>56 ± 4b</td>
</tr>
<tr>
<td>Chow 7 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final body weight, g</td>
<td>498 ± 16a</td>
<td>530 ± 12b</td>
<td>218 ± 7a</td>
<td>212 ± 6a</td>
</tr>
<tr>
<td>Body weight gain, g</td>
<td>106 ± 5a</td>
<td>106 ± 5a</td>
<td>507 ± 12a</td>
<td>597 ± 17a</td>
</tr>
<tr>
<td>Food intake, kcal</td>
<td>107 ± 4a</td>
<td>90.7 ± 3.1b</td>
<td>218 ± 7a</td>
<td>212 ± 6a</td>
</tr>
<tr>
<td>Feed efficiency, kcal × 10³</td>
<td>870 ± 23b</td>
<td>78.6 ± 2.5c</td>
<td>870 ± 23b</td>
<td>78.6 ± 2.5c</td>
</tr>
</tbody>
</table>

DR, diet resistant; DIO, diet-induced obesity; HE, high energy. Selectively bred 5-wk-old DIO (n = 18) and DR (n = 18) rats were observed for 7 days on chow, and then one-half of each group was fed either HE diet or chow for an additional 10 days. Data are means ± SE. Initial body weight and 24-h food intake were measured on chow at 5 wk of age. Other measures were made after 7 days on chow and 10 days on either chow or HE diet. Feed efficiency is body weight gain (g)/food intake (kcal) × 10³ over the 7-day and 10-day periods. Data with differing superscripts differ from each other at P ≤ 0.05 by post hoc t-test after 1-way ANOVA showed significant intergroup differences.
had 42–72% heavier epididymal, 88–192% heavier retroperitoneal, 21–60% heavier perirenal, and 84–122% heavier mesenteric fat depots than did all other groups (Table 2).

Plasma hormones. Baseline leptin levels were not significantly higher in DIO than in DR rats at 6 wk of age (Table 3) at either dark onset or 6 h into the dark phase. Leptin levels at 6 h into the dark phase were 65% higher than those at dark onset for both DIO and DR rats \( F(1,20) = 3.70; P = 0.010 \). Plasma leptin levels were consistently higher in DIO rats on HE diet than in all other groups by repeated-measures ANOVA [Fig. 3; \( F(1,8) = 11.86; P = 0.009 \)], and both DIO and DR rats on HE diet had higher levels than those on chow \( F(1,8) = 10.98; P = 0.011 \). These differences were due to the fact that leptin levels were much higher in DIO rats on HE diet than in all other groups. Most importantly, plasma leptin levels were already elevated in DIO rats on HE diet above all other groups only 3 days after start of the HE diet. At this time, their levels were 66–235% greater at dark onset and 83–137% greater at 6 h into the dark phase than those in all other groups (Fig. 3). Throughout the rest of the 10 days, plasma leptin levels in DIO rats on HE diet remained 50–343% greater than in all other groups. Also, their leptin levels remained consistently higher at 6 h into the dark phase than at dark onset on each of the days tested.

At 6 wk of age, chow-fed DIO rats had basal ghrelin levels at dark onset that were 29% lower than basal levels in chow-fed DR rats (Table 3; \( P = 0.200 \)). Furthermore, although basal ghrelin levels tended to fall from dark onset to 6 h into the dark phase in DR rats, they actually rose by 30% from dark onset (464 ± 33 pg/ml) to 6 h after dark onset (604 ± 45 pg/ml; \( P = 0.01 \)) in DIO rats. Although this diurnal pattern did not persist on HE diet, DIO rats had consistently lower plasma ghrelin levels than did DR rats, regardless of diet, throughout the entire 10-day period of observation on chow or HE diet [Fig. 4; \( F(1,30) = 16.24; P = 0.001 \)]. This was primarily due to the lower ghrelin levels in DIO rats at dark onset (0.81 ± 0.18 pg/ml; \( P = 0.002 \)), because values at 6 h into the dark cycle did not differ significantly between the phenotypes across the 10 days.

Plasma insulin levels were comparable at 6 wk of age in chow-fed DIO and DR rats (Table 3). Insulin levels were assessed only at dark onset after introduction of

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**Table 2. Fat depot weights at 7.5 wk of age in rats described in Table 1**

<table>
<thead>
<tr>
<th>Fat Depot</th>
<th>DR Chow</th>
<th>DR HE</th>
<th>DIO Chow</th>
<th>DIO HE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epididymal, g</td>
<td>1.50 ± 0.14a</td>
<td>1.68 ± 0.11a</td>
<td>1.84 ± 0.04a</td>
<td>2.62 ± 0.14b</td>
</tr>
<tr>
<td>Retroperitoneal, g</td>
<td>0.62 ± 0.16a</td>
<td>0.86 ± 0.10a</td>
<td>0.96 ± 0.08a</td>
<td>1.81 ± 0.18b</td>
</tr>
<tr>
<td>Perirenal, g</td>
<td>0.21 ± 0.04a</td>
<td>0.28 ± 0.03a</td>
<td>0.20 ± 0.02a</td>
<td>0.34 ± 0.04b</td>
</tr>
<tr>
<td>Mesenteric, g</td>
<td>1.38 ± 0.17a</td>
<td>1.58 ± 0.10a</td>
<td>1.67 ± 0.14a</td>
<td>3.07 ± 0.20b</td>
</tr>
<tr>
<td>Total, g</td>
<td>3.71 ± 0.48a</td>
<td>4.39 ± 0.28a</td>
<td>4.67 ± 0.24a</td>
<td>7.84 ± 0.48b</td>
</tr>
<tr>
<td>Percent body wt</td>
<td>1.70 ± 0.13a</td>
<td>2.07 ± 0.19a</td>
<td>1.88 ± 0.18a</td>
<td>3.15 ± 0.21b</td>
</tr>
</tbody>
</table>

Values are means ± SE. Total, total weight of all four depots. Percent body wt, total weight of 4 adipose pads/ final body wt (see Table 1) × 100. Data with differing superscripts differ from each other at \( P \leq 0.05 \) by post hoc t-test after 1-way ANOVA showed significant intergroup differences.
Leptin and ghrelin levels were determined from tail blood at 6 wk of age at dark onset (1000) and at 6 h into the dark phase (1600) while animals were on chow. Insulin levels were determined at dark onset. Data are means ± SE. Data with differing superscripts differ from each other at P ≤ 0.05 by post hoc t-test after 1-way ANOVA showed significant intergroup differences.

Table 3. Plasma leptin, ghrelin, and insulin levels from DIO and DR rats

<table>
<thead>
<tr>
<th></th>
<th>DR Dark Onset</th>
<th>DR 6 h Into Dark</th>
<th>DIO Dark Onset</th>
<th>DIO 6 h Into Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin, ng/ml</td>
<td>0.66 ± 0.18a</td>
<td>1.21 ± 0.32a</td>
<td>0.91 ± 0.15a</td>
<td>1.39 ± 0.32a</td>
</tr>
<tr>
<td>Ghrelin, pg/ml</td>
<td>665 ± 58a</td>
<td>606 ± 29a</td>
<td>463 ± 43b</td>
<td>604 ± 30a</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.81 ± 0.20a</td>
<td>1.19 ± 0.23a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Leptin and ghrelin levels were determined from tail blood at 6 wk of age at dark onset (1000) and at 6 h into the dark phase (1600) while animals were on chow. Insulin levels were determined at dark onset. Data are means ± SE. Data with differing superscripts differ from each other at P ≤ 0.05 by post hoc t-test after 1-way ANOVA showed significant intergroup differences.

the HE diet. Across all time points, repeated-measures ANOVA showed significantly higher insulin levels in DIO than in DR rats [Fig. 5; F(1,20) = 7.10; P = 0.010], irrespective of diet. However, post hoc analysis showed that insulin levels became significantly greater by 7 days in DIO rats on HE diet than in DR rats on HE diet and were significantly greater than in all other groups after 10 days. Finally, there was no correlation between plasma ghrelin and either plasma leptin or insulin levels over the course of these studies.

Ob-Rb, ghrelin, and GHS-R mRNA. Expression of Ob-Rb mRNA by in situ hybridization showed that DIO rats had reduced expression of Ob-Rb mRNA in the ARC, VMN, and DMN [Fig. 6; F(1,99) = 5.78; P = 0.010]. When individual nuclei were assessed, DIO rats had 10% lower Ob-Rb expression than DR rats in the ARC, independent of dietary exposure [Fig. 6; F(1,130) = 8.37; P = 0.007]. In the VMN, chow-fed DR rats had the highest Ob-Rb expression of all groups [Fig. 6; F(1,130) = 7.01; P = 0.013]. Exposure to HE diet for 10 days reduced Ob-Rb expression in DR rats by 10% compared with DR rats on chow and reduced expression in DIO rats by 7% compared with DIO rats fed chow (Fig. 6). In the DMN, chow-fed DR rats had 10% higher Ob-Rb expression than DIO rats fed either chow or HE diet [Fig. 7; F(1,30) = 5.78; P = 0.018].

In situ hybridization assays run on sections taken through the rostrocaudal extent of the hypothalamus showed no demonstrable mRNA for ghrelin above background or compared with films generated with the sense-strand ghrelin probe. In contrast, GHS-R mRNA was readily detected (Fig. 7). In the ARC, the largest area of GHS-R mRNA expression was in the most rostral sections. There was a relatively uniform rostrocaudal distribution of GHS-R mRNA in the DMN. In the ARC, the average area of GHS-R expression in DIO rats (0.295 ± 0.010 mm$^2$) was 13% less than in DR rats (0.338 ± 0.010 mm$^2$; F(1,131) = 9.17; P = 0.003). There was no effect of diet on ARC GHS-R mRNA expression. In the DMN, the average area of GHS-R expression in DIO rats (0.191 ± 0.010 mm$^2$) was 15% less than in DR rats (0.224 ± 0.013 mm$^2$; F(1,74) = 4.30; P = 0.042). In addition, HE diet was associated with 16% higher DMN GHS-R expression compared with chow [F(1,74) = 4.02; P = 0.050]. Neither genotype nor diet altered in GHS-R expression in the VMN.

Fig. 3. Plasma leptin levels in DIO and DR rats (n = 9/group) were drawn at 6 wk of age and again at 3, 7, and 10 days after one-half of each genotype was switched to HE diet or continued on chow. Blood samples were taken at dark onset (1000) and again at 6 h after dark onset (1600). Data are means ± SE. Arrows, dark onset. *P ≤ 0.05 when leptin levels were compared by paired t-test at dark onset vs. 6 h after dark onset in DIO rats on HE diet.

Fig. 4. Plasma ghrelin levels in DIO and DR rats described in Fig. 3. Data are means ± SE. *P ≤ 0.05 by repeated-measures ANOVA when values in all DIO rats were compared with DR rat values.
DISCUSSION

This study was undertaken to test the hypothesis that 5- to 7-wk-old selectively bred DIO rats spontaneously increase their food intake and body weight gain because they have abnormal regulation of, or responses to, plasma leptin or ghrelin. Selectively bred DIO rats were derived originally from among the outbred Sprague-Dawley rats that were high-weight and high-fat gainers on HE diet (30). Although the outbred DIO rats gain no more weight than outbred DR rats when fed chow from weaning (32, 33), the current studies show that selectively bred DIO rats begin to eat more and gain more weight starting at 5–6 wk of age than comparable DR rats. By 6 wk of age, juvenile DIO rats were heavier and ate more chow than DR rats over a 7-day period. Six-week-old DIO and DR rats had comparable plasma levels of leptin [an index of total body adiposity (41)] and insulin [an index of visceral adiposity (9, 14)]. Even at 7.5 wk of age, the increased body weight of the chow-fed DIO rats was not matched by significantly heavier fat pads (particularly compared with their slightly higher body weight) or by higher leptin or insulin levels than those seen in chow-fed DR rats. In confirmation of the assumption that 6- to 7-wk-old chow-fed DIO rats are not fatter than DR rats, we have recently shown (40) that neither total carcass adiposity nor relative adiposity differs between chow-fed selectively bred DIO and DR rats at either 6 or 7 wk of age. However, it should be pointed out that the period from 4 to 8 wk of age is one of very rapid lean body-mass growth, as well as a phase of changing hormonal and metabolic status. These factors might have an important impact on the findings reported here.

These data show that chow-fed juvenile DIO rats are larger but not fatter than comparable DR rats. However, when placed on HE diet, DIO rats rapidly increased their caloric intake and, unlike DR rats, failed to compensate appropriately for the increased caloric density of the HE diet. Their persistent hyperphagia produced no increase in weight gain but did lead to increased fat storage associated with significantly higher leptin and insulin levels than in all other groups. Thus, as in outbred DIO rats (32, 33) and adult selectively bred DIO rats (30), exposure to increased dietary calorie and fat density is required for juvenile selectively bred DIO rats to develop true DIO. Even as they increased their body adiposity, selectively bred DIO rats had reduced feed efficiency compared with DR rats. This suggests that their hyperphagia did evoke some thermogenic response, presumably mediated by sympathetic activation (15). If so, any increase in caloric expenditure was insufficient to prevent excess fat deposition. Similarly, juvenile outbred Sprague-Dawley rats show considerable sympathetic activation but still become obese when fed HE diet from weaning (34). For selectively bred juvenile DIO rats, their hyperphagia appears to be the most important factor leading to DIO on HE diet during this period of rapid growth.

We originally predicted that decreased plasma leptin levels, decreased central Ob-Rb expression, increased plasma ghrelin levels, and/or increased GHS-R expression might underlie the spontaneous hyperphagia that develops in chow-fed juvenile DIO rats. While chow-fed DIO and DR rats had comparable leptin levels, DIO rats had a small but significant reduction in hypothalamic Ob-Rb mRNA expression in all three assessed
hypothalamic nuclei compared with DR rats. Although it does not necessarily follow that reduced mRNA expression equates to reduced receptor function, these data are compatible with the idea that selectively bred DIO rats have reduced leptin sensitivity similar to that seen in adult outbred DIO rats (29). If leptin signaling were reduced, then DIO rats might overeat to compensate. Consuming a very low-fat (4.5%) chow diet could lead to increased growth but little excess adipose deposition, especially in rapidly growing juvenile rats. However, with the increased caloric density and fat content of the HE diet, DIO rats became overtly hyperphagic. They continued to consume approximately the same number of grams of food as they had on chow and failed to compensate for their increased caloric intake, despite early increases in plasma leptin and insulin levels that might ordinarily be expected to inhibit intake. This lends support to the contention that the reduced hypothalamic Ob-Rb expression might underlie a diminished capacity of DIO rats to monitor feedback signals associated with increased adipose stores sufficiently to prevent the development of obesity. Of course, this contention must be directly tested before it can be accepted as an important mechanism underlying the development of DIO.

A reduced ability to monitor and regulate leptin levels might be one consequence of reduced leptin signaling. It is possible that the early increase in leptin levels after only 3 days on HE diet simply mirrored a comparable early increase in adiposity. Although carcass adiposity was not independently evaluated at that time, it is unlikely that adipose mass had increased sufficiently to explain a ~75% rise in leptin levels after only 3 days on HE diet. In fact, a similar increase in plasma leptin levels has been described in unselected (by weight-gain phenotype) adult Sprague-Dawley rats after only 3-day intake of a highly palatable, 33% fat diet (49). Similarly, both obesity-prone Osborne-Mendell and obesity-resistant SB5/Pl rats fed a 56% fat diet (35) show a comparable early rise in leptin levels. On the other hand, some obesity-prone mice (44) and rats (20) have a reduced plasma leptin response to high-fat diets. Thus the early responses to diets of increased caloric and fat content appear to vary as a function of strain and species. Here we show that increased plasma leptin levels are a very specific feature of the selectively bred DIO rat, which is not found in DR rats bred from the same parent strain. This early increase may reflect their decreased ability to detect and inhibit their elevated leptin levels. Although this early increase in leptin levels might be a function of the young age of the animals studied here, the fact that some adult rodents also show such an early leptin rise on high-fat diets (35, 49) supports the contention that this is an important intrinsic characteristic of the DIO rat that contributes to its obesity-prone phenotype.

Although DIO rats did appear to have reduced leptin sensitivity, our prediction of elevated plasma ghrelin levels or increased GHS-R expression proved incorrect. On the contrary, DIO rats had both reduced plasma ghrelin levels and reduced hypothalamic expression of GHS-R mRNA. This suggests that they would be less, rather than more, responsive to the central orexigenic effects of ghrelin. Even though one study suggested that the orexigenic actions of circulating ghrelin are vagally mediated (13), it is clear that centrally administered ghrelin can markedly increase food intake (4, 24, 38, 42), apparently by activating anabolic ARC neuropeptide Y neurons (10, 24, 27, 42). Although we were unable to document the presence of ghrelin mRNA in the hypothalamus by in situ hybridization, others have identified both ghrelin mRNA and immunoreactive ghrelin protein in the hypothalamus (10, 26, 36). Thus brain ghrelin might provide an endogenous ligand for GHS-R and the orexigenic effects of centrally administered ghrelin. If so, it could be responsible for

Fig. 7. Ghrelin receptor mRNA expression by in situ hybridization through the rostrocaudal extent (A–F) of the VMN hypothalamus (n = 6/group). The left-hand portion of each photo is the cresyl violet-stained section used to generate the autoradiogram in the matching right-hand side of the photo. A: DM, dorsomedial; C and VL, central and ventrolateral subdivisions, respectively. B and E: DMND or D, dorsal; C, compact; V, ventral.

AJP-Endocrinol Metab • VOL 285 • NOVEMBER 2003 • www.ajpendo.org
the spontaneous hyperphagia in chow-fed juvenile DIO rats. However, our results suggest that, if this occurs, any release of endogenous ghrelin would interact with a reduced complement of ghrelin receptors, especially in the ARC where ghrelin has been shown to activate anabolic neuropeptide Y neurons (10, 24, 27, 42).

Thus the finding of low plasma ghrelin levels and reduced hypothalamic GHS-R mRNA in DIO compared with DR rats was unexpected, since we had originally postulated that increased ghrelin levels or responsivity might be an underlying cause of the spontaneous hyperphagia of DIO rats on chow. Ghrelin levels are generally inversely related to adiposity (7, 11, 17, 47). Obese humans have reduced plasma ghrelin levels (7, 11, 17, 47), possibly due to insulin resistance (21). Genetically obese fa/fa rats and ob/ob and db/db mice also have reduced ghrelin levels (2). However, the humans and animals in such studies were already obese and insulin resistant at the time of testing. The current studies demonstrate that both reduced plasma ghrelin levels and hypothalamic GHS-R mRNA expression are intrinsic properties of selectively bred DIO rats that antedate their increases in carcass adiposity or insulin resistance. At present, there is little reason to believe that either of these findings contributes to their spontaneous hyperphagia on chow or their susceptibility to DIO on HE diet.

Finally, it is important to point out that these studies were carried out in juvenile rats that were in the peripubertal period. A large number of hormonal changes are likely to be occurring during this time that might not be reflected in the adult condition. Thus the rapid growth and altered neurohumoral milieu associated with this developmental period might easily contribute to the genotypic differences described here. This in no way diminishes the importance of these differences between DIO and DR rats and their potential role in the propensity to develop obesity on HE diet.

In conclusion, juvenile Sprague-Dawley rats bred for their genetic predisposition to develop DIO have reduced hypothalamic Ob-Rb expression and an accentuated, early plasma leptin response when the caloric and fat density of their diet is increased. They also have inherently low plasma ghrelin levels and reduced hypothalamic GHS-R mRNA expression. All of these factors appear to be intrinsic properties of the DIO rat, since they antedate the onset of obesity. Reduced sensitivity to the catabolic effects of leptin might explain the increased plasma leptin levels and rapid onset of DIO when caloric density is increased. However, our data showing reduced plasma ghrelin levels and hypothalamic GHS-R expression in DIO rats before the onset of obesity suggest that ghrelin does not contribute directly to the hyperphagia of those animals.

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DISCLOSURES

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