Reduced anorexic effects of insulin in obesity-prone rats fed a moderate-fat diet

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The Pancreatic Hormone Insulin regulates food intake and energy expenditure through its action in the brain (27, 33, 42). Plasma insulin concentrations are positively correlated with body fat (3, 32) and circulating insulin enters the brain and cerebrospinal fluid by a transport-mediated process (4, 12). Obese humans have elevated cerebrospinal fluid, as well as plasma insulin levels, compared with normal weight controls, and these levels decrease in both groups after fasting (29). Chronic and acute administration of insulin into the 3rd-cerebral ventricle (iv3t) reduces food intake and body weight of baboons and rats (27, 42). Conversely, reduction of insulin signaling in the brain by selective knockout of insulin receptors (6) or by local administration of antibodies (25, 36) or antisense oligonucleotides to the insulin receptor (28) results in hyperphagia and weight gain. All of these findings indicate that insulin, once it gains access to the brain, provides a signal that is proportional to body fat and functions as one of the negative feedback signals that reduce intake and inhibit the development of obesity.

Whereas administration of insulin into the brain reduces food intake and body weight of normal rats, it is less effective in rats maintained on high-fat diets (2, 8). However, those studies did not differentiate between the effects of consuming a diet with high fat content and those due to the metabolic concomitants of the consequent obesity (including hyperinsulinemia), which develops on such diets. One way to address this critical issue is through the use of the diet-induced obesity (DIO) rat model. This is a polygenic model of obesity in which DIO rats gain no more carcass adiposity than diet-resistant (DR) rats when fed a low-fat chow diet but rapidly become obese, hyperinsulinemic, and hyperleptinemic when fed a 31% fat, high-energy (HE) diet (16–19). Both outbred (OutB) DIO Sprague-Dawley rats and rats selectively bred (SB) to develop DIO share a number of neural abnormalities involved in the regulation of energy homeostasis before the onset of obesity (15, 18, 19, 22). Among these is a reduced sensitivity to the anorectic effects of leptin (16, 18) and a reduced sensitivity to the modulatory effects of glucose on specialized glucosensing neurons (14, 21, 24, 35). When fed the HE diet, DR rats reduce their caloric intake and resist the development of obesity, whereas DIO rats become hyperphagic and obese, despite early increases in their plasma leptin and insulin levels (19). Given their defects in central responses to leptin and glucose, we postulated that DIO rats would also have a reduced responsivity to the anorectic effects of insulin. Further, in light of prior studies demonstrating reduced central insulin sensitivity in rats fed a high-fat diet, we also postulated that DR rats would become centrally insulin-resistant on the HE diet, even though they do not become obese or hyperinsulinemic on this diet (17, 19, 21). The current studies were carried out to test these hypotheses.

**DESIGN AND METHODS**

*Animals and procedures.* OutB male Sprague-Dawley rats (Charles River Labs, Kingston, NY) and Sprague-Dawley rats selectively bred (SB) to express DIO and DR (Crl:CD(SD)DIOBR; Charles River Labs, Kingston NY) were housed in individual tub cages and maintained on a 12:12 light:dark cycle with ad libitum access to water and Purina lab chow. After 5 weeks, after a 1-week acclimation period, six groups of rats were fed the HE diet: OutB DR and DIO rats, and SB DR and DIO rats. The current studies were carried out to test these hypotheses.
tained in a temperature-controlled room at 19–23°C, which was illuminated from 0100 to 1300. All animals were fed a pelleted chow diet (Harlan Teklad Rat Chow; 6% fat) for the initial experiments, and subsequently, they were given a nutritionally complete, defined high-energy (HE) diet (Research Diets no. C11024F, New Brunswick, NJ), which contains 4.47 kcal/g with 21% of the metabolizable energy as protein, 31% as fat, and 48% as carbohydrate, 50% of which is sucrose (21). Water was available ad libitum. All procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee and were in compliance with the guidelines of the American Physiological Society (1).

Third ventricular (iv3t) cannula placements were carried out in rats anesthetized with ketamine-xylazine (70 mg/kg). Coordinates were on the midline, 2.2 mm posterior to bregma, and 7.5 mm ventral to dura (7). The guide cannulas were cemented to anchor screws attached to the skull and plunged with obturators. After a 10-day recovery period, during which body weight returned to baseline, placement of each iv3t cannula was confirmed by infusion of 10 ng ANG II in 1 μl saline in water-replete rats. Only animals drinking in excess of 5 ml over 1 h after ANG-II infusion were included in the study. Twenty-seven of 30 rats met this criterion in experiment 1 and 57 of 60 animals met it in experiment 2 (see below).

Experiment 1: Relationship between insulin-induced anorexia and the development of DIO in OutB rats. One week after the ANG II tests, the 350- to 400-g Sprague-Dawley rats were adapted to a regimen on which chow was removed 4 h before the onset of the dark each day. Rats were individually handled for 5 min on four successive days to equilibrate their arousal levels before the experiment. On separate days spaced 7–10 days apart, one group of control rats (n = 11) then received two injections of 2 μl iv3t saline and another group (n = 16) received 4 mU or 8 mU insulin (Iletin II regular pork insulin, Eli Lilly, Indianapolis, IN) dissolved in saline. Food intake was assessed at 4 and 24 h after the injections. After the 16 rats had received both insulin injections and the 11 rats had received two saline injections, they were all changed to the HE diet for 4 wk. At the end of 4 wk on the HE diet, the lowest (≤191 g; n = 7) and highest (≥206 g; n = 9) weight gainers among the insulin-injected rats were retrospectively characterized as DR and DIO, respectively (Fig. 1) (21). Of the 16 insulin-injected rats carried through the 4 wk on the HE diet, four DR and four DIO rats maintained functional iv3t cannulas. These rats were retested for 4 h and 24-h food intake after 8 μl iv3t insulin injections. Of the 11 saline-injected animals, 8 could be retested with iv3t saline after 4 wk on HE diet, and these were similarly retested for 4-h and 24-h intake. Twenty-four hours after the final injections, rats were anesthetized with CO2 and decapitated. Trunk blood was taken for assessment of plasma insulin and leptin levels.

Experiment 2: Insulin-induced anorexia in SB DR and DIO rats. One week after ANG II testing, SB DIO (340–375 g) and DR (260–300 g) rats were administered either iv3t saline or 8 μl insulin on separate days spaced 7 to 10 d apart. Food intake was assessed at 4 and 24 h after the injections. After each animal received both saline and insulin injections, 15 DIO and 15 DR were changed to the HE diet, and 14 DIO and 13 DR were maintained on chow for 4 wk. At that time, 7 DIO rats on chow and 8 on HE diet and 5 DR rats on chow and 7 on HE diet maintained patent iv3t cannulas. These rats were all reweighed with both saline and 8 μl insulin again.

Experiment 3: Expression of arcuate nucleus insulin receptor mRNA in SB DIO and DR rats. Male SB DIO and DR (n = 16 per group) rats were fed rat chow ad libitum from weaning. At 6 wk of age, half of each genotype was kept on chow, and the other half was fed HE diet for 4 wk. They were then decapitated in the nonfasting state during the 2 h after lights on. Brains were quickly removed, frozen on dry ice, and stored at –70°C until assay. A 300-μm section was cut from frozen brains on a cryostat at –12°C such that the compact portion of the dorsomedial nucleus (DMN) was in the center of the slice. Cut sections were placed in RNA Later. One week after at least 1 h, sections were placed under a dissecting microscope and the arcuate nucleus was micropunched bilaterally by minor modifications (13) of the method of Palkovits et al. (30). The punched samples were kept in RNA Later at 4°C for ≥2 wk before being reverse-transcribed to cDNA.

Micropunch samples of arcuate nucleus were subsequently assayed by real-time (quantitative) PCR as previously described (18). After homogenization in a solution containing guanidinium thiocyanate and nucleotides, mRNA was purified using silica columns (Ambion RNAqueous kit). After removal of genomic DNA with DNase, mRNA was reverse-transcribed with random hexamer priming with Superscript 3 (Invitrogen). Samples were then treated with RNaseH (Ambion), and the resulting purified cDNA was aliquoted and frozen. Primer sets for cyclophilin and insulin receptor mRNA were designed by reference to published sequences, and their specificity was verified using GenBank and by comparing the sequenced PCR product for both cyclophilin and insulin receptor to these references. For each mRNA species, a pair of conventional primers was used in combination with a sequence-specific 6-carboxyfluorescin (FAM)-labeled probe to allow real-time PCR quantitation using an Applied Biosystems 7700 Sequence detector set for 40 PCR cycles. The primers for cyclophilin [constitutive gene (39)] were GenBank (NM_017101): forward beginning at 253 bp, CCAATAGCTATTCCACAAACAAG; reverse beginning at 330 bp, AAGTGTGTGAATCATCAGTGTATAC; FAM-labeled probe TCTACGGAGAAATT. The primers for the insulin receptor were Genebank (NM_017071): Forward beginning at 1250 bp: AACTGTTAAGTACTGAGTGGTCA- GAGT; Reverse beginning at 1321 bp: CCTGGTTCTCCAGTGAT- TCAA; and FAM-labeled probe TGCTATGCACGAGTGGC. Reference standards were created for cyclophilin and insulin receptor from pooled aliquots of the arcuate nucleus samples, and these were used to generate standard curves from which quantitative data were read. Data were then expressed as the ratio of insulin receptor to cyclophilin mRNA.

Plasma leptin and insulin levels. Plasma leptin and insulin were determined by radioimmunoassays (Linco, St. Charles, MO).

**Statistical analysis.** In experiment 1, chow intake at 4 and 24 h after 8 μl iv3t insulin (there was no effect of 4 mU) was compared with that animal’s subsequent body weight gain when switched to the HE diet over 4 wk by Pearson’s correlation coefficient test. Comparisons of 4- and 24-h food intake among saline-injected controls and retrospectively identified, insulin-injected DIO and DR rats were made by one-way ANOVA with post hoc Tukey’s HSD tests. Comparisons of body weights and plasma leptin and insulin levels were made by unpaired t-test between DIO and DR rats fed the HE diet for 4 wk. In experiment 2, food intake of each SB DR or DIO rat after iv3t insulin was compared with that animal’s food intake after saline using
Table 1. Body weights and plasma leptin and insulin levels for outbred and selectively bred diet-resistant as diet-induced obesity rats

<table>
<thead>
<tr>
<th></th>
<th>OutB DR n = 6</th>
<th>OutB DIO n = 10</th>
<th>SB DR Chow n = 15</th>
<th>SB DR HE n = 15</th>
<th>SB DIO Chow n = 15</th>
<th>SB DIO HE n = 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, initial, g</td>
<td>377±7</td>
<td>385±4</td>
<td>258±6*</td>
<td>275±6*</td>
<td>361±16b</td>
<td>357±6b</td>
</tr>
<tr>
<td>Body weight gain, g</td>
<td>537±10</td>
<td>623±16*</td>
<td>368±12*</td>
<td>377±9*</td>
<td>476±9b</td>
<td>514±11c</td>
</tr>
<tr>
<td>Body weight gain, g</td>
<td>160±3</td>
<td>237±14*</td>
<td>110±14*</td>
<td>102±6*</td>
<td>115±14*</td>
<td>157±7b</td>
</tr>
<tr>
<td>Plasma leptin, ng/ml</td>
<td>18.6±1.4</td>
<td>26.3±2.5*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>716±127</td>
<td>1074±82*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are given as means ± SE. Body weights and plasma leptin and insulin levels are provided of outbred (OutB) rats retrospectively identified as diet-resistant (DR) and diet-induced obese (DIO) when maintained on chow (body weight, initial) and after 4 wk on the high-energy (HE) diet (body weight, final and body weight gain). Also, comparable data are shown for selectively bred (SB) DR or DIO rat strains on chow and after 4 wk on either chow or the HE diet. *P < 0.05 when data were compared between OutB DIO and DR rats. For SB DIO and DR rats, data with differing superscripts differ by P < 0.05 or less by post hoc analysis after intergroup differences were found by two way ANOVA. ND, not done.

RESULTS

Experiment 1: Relationship between insulin-induced anorexia and the development of DIO in OutB rats. There were no significant effects of 4 mU of iv3t insulin on food intake in any group. Following 8 mU of iv3t insulin, there was a highly variable anorectic effect (Fig. 1). At least 50% of this variability was correlated with the weight gain phenotype when rats were subsequently fed the HE diet for 4 wk. Thus there was a significant inverse correlation between the initial levels of Chow intake after 8 mU iv3t insulin and the subsequent 4 wk body weight gain on the HE diet (Fig. 1: r = 0.72; P = 0.002; n = 16). There was no significant correlation between 24-h intake after iv3t insulin and subsequent body weight gain on HE diet. When rats were retrospectively divided into the DIO and DR groups according to their 4 wk weight gain on the HE diet, those defined as DIO gained 48% more body weight and had 41% higher plasma leptin and 50% higher plasma insulin levels than those defined as DR (Table 1). When grouped in this manner, 8 mU iv3t insulin produced a 53% reduction in 4-h food intake in Chow-fed DR rats compared with the saline-injected controls, whereas DIO rats had only a small (17%) insulin-induced reduction in 4-h intake compared with saline-injected rats (Fig. 2). Thus insulin had an anorectic effect in DR rats that was threefold the magnitude of the change in DIO rats. Also, the 8 mU insulin dose significantly reduced 24-h intake by 69% in DR rats, but only by 27% in DIO rats (Fig. 2). This represented a 2.5-fold greater effect of insulin in DR than DIO rats. In contrast to the anorectic effects of iv3t insulin observed when the rats were consuming chow, 4 wk on HE diet reduced both the 4- and 24-h anorectic response to iv3t insulin by 45% in DR rats and completely abolished the effect in DIO rats compared with saline-injected controls (Figs. 2).

Experiment 2: Insulin-induced anorexia in SB DR and DIO rats. While maintained on chow, SB DR rats had a 21% decrease in 4-h (P = 0.014) and a 22% decrease in 24-h intake (P = 0.003) after 8 mU iv3t insulin compared with their own saline-injected intake by repeated-measures ANOVA. In contrast, Chow-fed SB DIO rats had no significant change in food intake at either time after iv3t insulin (Fig. 3). After 4 wk on their respective diets, SB DR rats fed HE diet gained the same amount of weight as DR rats fed Chow over the same period (Table 1). On the other hand, SB DIO rats fed HE diet gained 37% more body weight than those fed Chow over 4 wk (Table 1). When SB DR rats were retested after 4 wk on HE diet, there was a small but nonsignificant reduction in food intake after 8 mU insulin injections in the DR rats and no effect in the DIO rats (Fig. 3).

Experiment 3: Assessment of arcuate nucleus insulin receptor mRNA levels. There were no significant differences in the expression of arcuate nucleus insulin receptor mRNA (relative
to the constitutively expressed control) between SB DIO and DR rats fed either chow or HE diet for 4 wk (Table 2).

DISCUSSION

We previously reported that both OutB (16) and SB DIO rats (18) have a reduced anorectic response to leptin before the development of obesity on the HE diet. Here, we show that both OutB and SB DIO rats have a similar preexisting reduction in their central anorectic response to insulin compared with DR rats before exposure to HE diet. In addition, DR rats developed a greatly diminished anorectic response to iv3t insulin after 4 wk on an HE diet, even though they did not become obese or hyperinsulinemic on this diet (17, 23). It is possible that the results from the OutB insulin-injected rats might have been biased because rats were retrospectively identified as DR and DIO and because they were tested against an independent group of saline-treated rats. For this reason, we undertook similar studies in SB DIO and DR rats and tested each individual rat with both insulin and saline. The results from these genetically distinct strains, which were derived from the original OutB rats used here (17), are in agreement with those from the OutB rats. Our results demonstrate both genetically determined and diet-induced defects in central insulin signaling. Importantly, these studies confirm that it is the content of the HE diet that produces central insulin resistance in DR rats and not the metabolic consequences that accompany the development of obesity on this diet. Such a discrimination cannot be made in other studies where high-fat diets reduced central insulin sensitivity but also produced obesity (2, 8). In light of the development of insulin resistance in DR rats on HE diet, it is puzzling that deficits in central insulin signaling alone can produce obesity (6, 25, 28, 36). However, although DR rats do not become obese after 4 wk on the HE diet (16, 19) despite the development of central insulin resistance, they do eventually become obese on the HE diet, but this takes 5 mo (20). These findings suggest that it is the combination of reduced insulin and leptin sensitivity that predisposes DIO rats to develop early hyperphagia and obesity on HE diet but that processes other than central insulin responsiveness must regulate energy homeostasis in DR rats during the early phases of chronic exposure to high-fat diets.

In DR rats, leptin and insulin might provide the critical early negative feedback signals, which allow them to adapt rapidly to the increased caloric density of the HE diet by reducing their intake (19). The selective inhibitory effect of central insulin on fat intake might be an important factor in this reduced intake in DR rats (8). On the other hand, DIO rats fail to compensate for the increased caloric density of the HE diet. DIO rats continue to eat the same weight of HE diet as they did of chow 4 wk after initial exposure to the HE diet and thus remain hyperphagic despite early increases in their plasma leptin and insulin levels (19). As in obese (fa/fa) Zucker rats (9, 41), the combined defects in central leptin and insulin signaling may underlie the failure of DIO rats to reduce their caloric intake and regulate their body weight (18, 19). Although these defects are not as pronounced in DIO as they are in fa/fa rats, reduced DIO central hormonal signaling would certainly promote the development of obesity when dietary fat and caloric content were increased. The site of defective leptin and insulin signaling is unclear. DIO rats do have reduced expression of arcuate Lepr-b mRNA, as well as reduced leptin-induced phosphorylation of STAT3 before the onset of obesity (18, 19). However, even though insulin exerts its anorectic effect within the arcuate nucleus (40), SB DIO rats had no reduction in arcuate insulin receptor mRNA expression comparable to the decrease seen in Lepr-b expression (18, 19). This makes it likely that the inherent reduction in insulin signaling of DIO rats occurs downstream of the transcriptional regulation of the receptor. Since both leptin- and insulin-induced anorexia are dependent on the activation of phosphoinositide-3 kinase (27), defects in the activation of this common regulatory step might underlie the reduced hormonal signaling in DIO rats.

While DIO rats had reduced central insulin signaling before the onset of obesity, DR rats lost or had greatly attenuated responsiveness to the anorectic effects of insulin after 4 wk on HE diet. This occurred even though they did not become obese or hyperinsulinemic (16, 19). The fact that DR rats do not develop obesity, despite losing their anorectic response to iv3t insulin suggests that defective central insulin signaling alone is not sufficient to produce obesity. The reduction in central insulin signaling in DR rats after 4 wk on HE diet is in keeping with other studies showing deleterious effects of dietary fat on hormonal signaling. This reduced signaling may well be due to alterations in neuronal membrane fatty acid composition and/or fluidity leading to reduced binding of ligands to their receptors (22, 37). For example, increasing cholesterol in the medium of

Table 2. Real-time PCR assessment of arcuate nucleus insulin receptor mRNA in SB DIO and DR rats fed chow or HE diet for 4 wk beginning at 6 wk of age

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>4 wk HE Diet</th>
</tr>
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<tbody>
<tr>
<td>DR</td>
<td>0.92±0.09</td>
<td>0.75±0.09</td>
</tr>
<tr>
<td>DIO</td>
<td>0.90±0.10</td>
<td>0.86±0.07</td>
</tr>
</tbody>
</table>

Data are means ± SE ratios of insulin receptor to cyclophilin mRNA.

Fig. 3. Effects of saline vs. 8 mU iv3t insulin on 4-h and 24-h intake in selectively bred (SB) rats on chow and after 4 wk on the HE diet. Data are means ± SE. Data with different superscript letters differ from each other by P < 0.05 or less by post hoc comparison after intergroup differences were found by ANOVA.
cultured neurons reduces insulin binding and insulin-induced receptor tyrosine phosphorylation and downstream signaling (37). Also, intake of HE diet is accompanied by altered synaptosomal fatty acid composition and reduced binding to brain α2-adrenoceptor (22) and sulfonylurea receptors (15). Intake of a high-fat diet also reduces the anorectic effects of melanocortins (10), a pathway which is critical for the catalytic effects of centrally administered insulin (5). In addition to pathways involved in energy homeostasis, intake of high-fat diets also adversely affects other neural functions such as synaptic plasticity (26), memory (11), stress responsivity (31, 38), and reproductive function (34).

In conclusion, we have found that rats predisposed to develop DIO on an HE diet have a preexisting reduction in insulin’s central anorectic effect. Because this occurs in both OutB and SB DIO rats, it is likely to be an inherited trait (22) that, along with defects in leptin signaling, may be a critical predisposing factor to the development of DIO when dietary fat and energy content are elevated. However, intake of HE diet reduces central insulin sensitivity, even in rats that do not become obese or hyperinsulinemic on such diets. This failure of DR rats to become obese within 4 wk on HE diet in the face of central insulin resistance suggests that reduced central insulin sensitivity alone is insufficient to alter the regulation of energy homeostasis in these rats. It is likely that the combination of central insulin, as well as leptin ‘resistance,’ together with other possible defects in central metabolic sensing may promote the development of obesity in genetically susceptible animals when the caloric and fat density of the diet is increased.

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