Reduced Anorexic Effects of Insulin in Obesity-Prone Rats Fed a Moderate Fat Diet.

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Key words: insulin receptor, diet-induced obesity, arcuate nucleus, metabolic sensing

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ABSTRACT

Rats prone to develop diet-induced obesity (DIO) have reduced central sensitivity to many metabolic and hormonal signals involved in energy homeostasis. High fat diets produce similar defects in diet-resistant (DR) rats. To test the hypothesis that genotype and diet exposure would similarly affect central insulin signaling, we assessed the anorectic effects of 8mU third ventricular (iv3t) insulin before and after 4wk intake of a 31% fat, high energy (HE) diet intake in outbred (OutB) rats. Rats were retrospectively designated as DR or DIO by their low or high weight gains on HE diet. Prior to HE diet, iv3t insulin reduced 4h and 24h chow intake by 53% and 69% in DR rats but by only 17% and 27% in DIO rats, respectively. Also, the anorectic response to iv3t insulin in OutB rats was inversely correlated (r=0.72, P=0.002) with subsequent 4wk weight gain on HE diet. Similarly, in selectively bred (SB) chow-fed DR rats, 8mU iv3t insulin reduced 4h and 24h intake by 21% and 22%, but had no significant effect in SB DIO rats. Four week HE diet intake reduced 4h and 24h insulin-induced anorexia by 45% in OutB DR rats and completely abolished it in SB DR rats. Reduced insulin responsiveness was unassociated with differences in arcuate nucleus insulin receptor mRNA expression between DIO and DR rats or between rats fed chow or HE diet. These data suggest that DIO rats have a pre-existing reduction in central insulin signaling which might contribute to their becoming obese on HE diet. However, since HE diet reduced central insulin sensitivity in DR rats but did not make them obese, it is likely that other brain areas are involved in insulin’s anorectic action or that other pathways contribute to the development and maintenance of obesity.
INTRODUCTION

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 to normal weight controls and these levels decrease in both groups following 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-
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 prior to 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 (13, 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. Outbred (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 in a temperature-controlled room at 19-23°C which was illuminated from 0100 to 1300 h. All animals were fed a pelleted chow diet (Harlan Teklad Rat Chow; 6% fat) for the initial experiments, and subsequently given a nutritionally complete, defined high-energy (HE) diet (Research Diets no. C11024F, New York, NY).
Brunswick, NY) which contains 4.47kcal/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 IACUC 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 (70mg/kg). Coordinates were on the midline, 2.2mm posterior to bregma, and 7.5mm ventral to dura (7). The guide cannulae were cemented to anchor screws attached to the skull and plugged with obturators. After a 10d recovery period, during which body weight returned to baseline, placement of each iv3t cannula was confirmed by infusion of 10ng angiotensin II in 1µl saline in water-replete rats. Only animals drinking in excess of 5ml over 1h after angiotensin-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 following the angiotensin-II tests, the 350-400g Sprague-Dawley rats were adapted to a regimen on which chow was removed 4h prior to the onset of the dark each day. Rats were individually handled for 5min on 4 successive days to equilibrate their arousal levels before the experiment. On separate days spaced 7 to 10 d apart, one group of control rats (n=11) then received two injections of 2µl iv3t saline and another group (n=16) received 4mU or 8mU insulin (Iletin II regular pork insulin, Eli Lilly, Indianapolis, IN) dissolved in saline. Food intake was assessed at 4h and 24h 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 4wk. At the end 4wk on HE diet, the lowest (<191g; n=7) and highest (>206g; n=9) weight gainers among the insulin-injected rats were retrospectively characterized as DR and DIO, respectively (Figure 1) (21). Of the 16 insulin-injected rats carried through the 4wk on the HE diet, 4 DR and 4 DIO rats maintained functional iv3t cannulae. These rats were re-tested for 4h and 24h food intake following 8mU iv3t insulin injections. Of the 11 saline-injected animals, 8 could be re-tested with iv3t saline after 4wk on HE diet and these were similarly retested for 4h and 24h 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 following angiotensin-II testing, SB DIO (340-375g) and DR (260-300g) rats were administered either iv3t saline or 8mU insulin on separate days spaced 7 to 10d apart. Food intake was assessed at 4h and 24h 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 4wk. 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 i3vt cannulae. These rats were all re-injected with both saline and 8mU 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 6wk of age, half of each genotype was kept on chow and the other half was fed HE diet for 4wk. They were then decapitated in the non-fasting state during the 2h after lights on. Brains were quickly removed, frozen on dry ice and stored at –70°C until assay. A 300µm section was cut from frozen brains on a cryostat at –12°C such that the compact portion of the DMN was in the center of the slice. Cut sections were placed in RNALater (Ambion). After at least 1h, sections were placed under a dissecting microscope and the arcuate nucleus was micropunched bilaterally by minor modifications (14) of the method of Palkovits et al (30). The punched samples were kept in RNALater at 4°C for less than 2wk before being reversed transcribed to cDNA.

Micropunch samples of arcuate nucleus were subsequently assayed by real-time (quantitative) polymerase chain reaction (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 Genebank 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-carboxyfluoroscein (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 Genebank (NM 017101):
Forward beginning at 253bp: CCAATACGTCATTCAACAACAAG; Reverse beginning at 330bp: AAGTTGCTGGAATTTCATGGTATAGC; FAM-labeled probe TCTACGGAGAGAAATT. The primers for the insulin receptor were Genebank (NM_017071): Forward beginning at 1250bp: AACCTGTGAGGATGAGTGTCAGAGT; Reverse beginning at 1321bp: CCTTGCTCTTCACTCATGTTCCA; FAM-labeled probe TGCATCCCGAGGTGC. 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 24h following 8mU iv3t insulin (there was no effect of 4mU) was compared with that animal’s subsequent body weight gain when switched to the HE diet over 4wk by Pearson’s correlation coefficient test. Comparisons of 4h and 24h food intake among saline-injected controls and retrospectively identified, insulin-injected DIO and DR rats were made by 1 way ANOVA with post hoc Tukey’s HSD tests. Comparisons of body weights, plasma leptin and insulin levels were made by unpaired t-test between DIO and DR rats fed the HE diet for 4wk. In Experiment 2, food intake of each SB DR or DIO rat following iv3t insulin was compared to that animal’s food intake following saline using ANOVA with repeated measures. They were also compared by paired t-test between saline and insulin injections.
for both DIO and DR rats. Comparisons of body weights and arcuate nucleus insulin receptor mRNA data were compared by 2 way ANOVA (genotype, diet) and then by post hoc Tukey’s HSD tests. In all cases significance was set at P < 0.05, two-tailed. All data are expressed as mean ± SEM.

RESULTS

Experiment 1: Relationship between insulin-induced anorexia and the development of DIO in OutB rats. There were no significant effects of 4mU of iv3t insulin on food intake in any group. Following 8mU of iv3t insulin, there was a highly variable anorectic effect (Figure 1). At least 50% of this variability was correlated with the weight gain phenotype when rats were subsequently fed the HE diet for 4wk. Thus, there was a significant inverse correlation between the initial levels of chow intake following 8mU iv3t insulin and the subsequent 4wk body weight gain on the HE diet (Figure 1; R = 0.72; p = 0.002; n=16). There was no significant correlation between 24h intake after iv3t insulin and subsequent body weight gain on HE diet. When rats were retrospectively divided into DIO and DR groups according to their 4wk 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, 8mU iv3t insulin produced a 53% reduction in 4h food intake in chow-fed DR rats as compared to the saline-injected controls, whereas DIO rats had only a small (17%) insulin-induced reduction in 4h intake when compared to saline-injected rats (Figure 2). Thus, insulin had an anorectic effect in DR rats that was three-fold the magnitude of the change in DIO rats. Also, the 8mU insulin dose significantly reduced 24h intake by 69%.
in DR rats, but by only by 27% in DIO rats (Figure 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, 4wk on HE diet reduced both the 4h and 24h anorectic response to iv3t insulin by 45% in DR rats and completely abolished the effect in DIO rats as compared to saline-injected controls (Figures 2).

**Experiment 2: Insulin-induced anorexia in SB DR and DIO rats.** While maintained on chow, SB DR rats had a 21% decrease in 4h (P=0.014) and a 22% decrease in 24h intake (P=0.003) following 8mU iv3t insulin compared to their own saline-injected intakes by repeated measures ANOVA. In contrast, chow-fed SB DIO rats had no significant change in food intake at either time after iv3t insulin (Figure 3). After 4wk 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 4wk (Table 1). When SB DR rats were retested after 4wk on HE diet, there was a small but non-significant reduction in food intake following 8 mU insulin injections in the DR rats and no effect in the DIO rats (Figure 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 4wk (Table 2).
DISCUSSION

We previously reported that both OutB (16) and SB DIO rats (18) have a reduced anorectic response to leptin prior to the development of obesity on HE diet. Here we show that both OutB and SB DIO rats have a similar pre-existing reduction in their central anorectic response to insulin in comparison to DR rats before exposure to HE diet. In addition, DR rats developed a greatly diminished anorectic response to iv3t insulin after 4wk on a 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 which 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 defects in central insulin signaling alone can produce obesity (6, 25, 28, 36). However, although DR rats do not become obese after 4wk on HE diet (16, 19) despite the development of central insulin resistance, they do eventually become obese on HE diet but this takes 5mo (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 weeks 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 calorie 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 prior to 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 phosphoinositol-3 kinase (27), defects in the activation of this common regulatory step might underlie the reduced hormonal signaling in DIO rats.

While DIO rats have reduced central insulin signaling prior to the onset of obesity, DR rats lost or had greatly attenuated responsiveness to the anorectic effects of insulin after 4wk 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 4wk 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 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 catabolic 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 HE diet
have a pre-existing reduction in insulin’s central anorectic effect. Since 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 4wk 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.
ACKNOWLEDGEMENTS

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<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>
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<td>275±6^a</td>
<td>361±16^b</td>
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<td>476±9^b</td>
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Table 1. Body weights and plasma leptin and insulin levels 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 HE diet (Body weight, final and Body weight gain) and comparable data for selectively bred (SB) DR or DIO rat strains on chow and after 4wk on either chow or HE diet. Data are mean ± SEM; *=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 2 way ANOVA. ND = not done.
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 6wk of age. Data are mean ± SEM ratios of insulin receptor to cyclophilin mRNA.

<table>
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<td>0.75±0.09</td>
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<td>DIO</td>
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FIGURES

Figure 1. Relationship of 4h food intake following 8mU of iv3t insulin in outbred (OutB) chow-fed rats to their subsequent body weight gain rats after 4wk on HE diet.

Figure 2. Effects of saline vs. 8mU iv3t insulin on 4h and 24h intake in OutB chow-fed rats and after 4wk on the HE diet. Chow-fed rats were tested for food intake after either iv3t injections of saline (n=11) or both 4 and 8mU insulin (n=16). They were then placed on HE diet for 4wk. Insulin-injected rats were retrospectively identified as diet-resistant (DR) and diet-induced obese (DIO) by their low and high body weight gains, respectively and were retested with 8mU insulin (n=4 DIO, n=4 DR). Saline-injected rats (n=8) were re-tested with iv3t saline. Data are mean ± SEM. 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.

Figure 3. Effects of saline vs. 8mU iv3t insulin on 4h and 24h intake in selectively bred (SB) rats on chow and after 4wk on the HE diet. Data are mean ± SEM. 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.
Figure 1

8mU Insulin
4h FI

R = 0.718
P = 0.002
Figure 2
Figure 3