Obesity-prone rats have preexisting defects in their counterregulatory response to insulin-induced hypoglycemia

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Tkacs, Nancy C., and Barry E. Levin. Obesity-prone rats have preexisting defects in their counterregulatory response to insulin-induced hypoglycemia. Am J Physiol Regul Integr Comp Physiol 287: R1110–R1115, 2004; doi:10.1152/ajpregu.00312.2004.—Rats that develop diet-induced obesity (DIO) on a 31% fat [high-energy (HE)] diet have defective sensing and responding to altered glucose levels compared with diet-resistant (DR) rats. Thus we postulated that they would also have defective counterregulatory responses (CRR) to insulin-induced hypoglycemia (IIH). Chow-fed selectively bred DIO and DR rats underwent three sequential 60-min bouts of IIH separated by 48 h. Glucose levels fell comparably, but DIO rats had 22–29% lower plasma epinephrine (Epi) levels during the first two bouts than DR rats. By the third trial, despite comparable Epi levels, DIO rats had lower 30-min glucose levels and rebounded less than DR rats 85 min after intravenous glucose. Although DIO rats gained more carcass and fat weight after 4 wk on an HE diet than DR rats, they were unaffected by prior IIH. Compared with controls, DR rats with prior IIH and HE diet had higher arcuate nucleus neuropeptide Y (50%) and proopiomelanocortin (POMC; 37%) mRNA and an inverse correlation (r = 0.85; P = 0.004) between POMC expression and body weight gain on the HE diet. These data suggest that DIO rats have a preexisting defect in their CRR to IIH but that IIH does not affect the expression of their hypothalamic neuropeptides or weight gain as it does in DR rats.

diet-induced obesity; epinephrine; norepinephrine; neuropeptide Y; proopiomelanocortin; hypoglycemia-associated autonomic failure

THE ADVENT OF TIGHT CONTROL of blood glucose levels in diabetic patients has led to a dramatic reduction in the complications associated with chronic hyperglycemia (10). However, this has also resulted in more frequent bouts of insulin-induced hypoglycemia (IIH; see Ref. 11). Such recurrent bouts are associated with reduced awareness of hypoglycemia and a down-regulation of the counterregulatory responses (CRR) that restore glucose homeostasis (2, 8, 9, 13). We have shown that a single bout of hypoglycemia can reduce the plasma epinephrine (Epi) and corticosterone response to a second bout of hypoglycemia 48 h later in outbred rats (37). This was associated with a reduction in the hypothalamic arcuate nucleus (ARC) mRNA expression of the anabolic neuropeptide Y (NPY) and proopiomelanocortin (POMC), a precursor of the catabolic neuropeptide, α-MSH (38). In addition, a single bout of hypoglycemia led to an upregulation of ARC glucokinase (GK), the putative gatekeeper of neuronal firing in brain glucosensing neurons (12, 15, 16, 28, 30, 34, 35, 40). This suggested that, despite the downregulation of neuropeptides involved in energy homeostasis, the compensatory upregulation of GK might lead to increased sensitivity to lower glucose levels, resulting in a reduced CRR to subsequent bouts of hypoglycemia.

The involvement of ARC NPY and POMC neurons in IIH also suggested that repeated bouts might lead to an alteration in weight gain and adiposity. We previously showed that rats selectively bred to develop diet-induced obesity (DIO) also have increased hypothalamic GK expression (12) and a number of other defects in glucosensing at both the cellular and whole animal levels compared with diet-resistant (DR) rats (17, 19, 24, 26, 36). This suggested that their upregulation of hypothalamic GK expression might be a compensatory response to these multiple glucosensing deficits. Thus we postulated that the preexisting elevation of hypothalamic GK expression in DIO rats would be associated with a blunted CRR to IIH as it does in unselected rats after a single bout of IIH. We also postulated that the downregulation of ARC NPY and POMC expression that follows recurrent bouts of IIH would alter body weight gain and carcass adiposity when rats were subsequently fed a diet with increased caloric and fat content [high-energy (HE) diet]. The present studies show that DIO rats do, indeed, have a reduced CRR to IIH but that prior IIH has no effect on DIO weight gain, adiposity, or hypothalamic neuropeptide expression. However, prior IIH does increase the expression of NPY and POMC and alter the weight gain pattern of DR rats after 4 wk of HE diet.

METHODS

Animals. Male selectively bred DR (n = 16) and DIO (n = 17) rats from our in house colonies were used at ~3 mo of age (DR = 336 ± 15 g; DIO = 379 ± 20 g; P = 0.05), in compliance with the Animal Care Committee of the East Orange Veterans Medical Center and the American Physiological Society guidelines (1). Rats were singly housed and fed Purina rat chow (no. 5001) and water ad libitum from weaning and were kept on a 12:12-h light-dark schedule (lights out at 1800). Purina rat chow contains 3.3 kcal/g, with 23.4% of total energy as protein, 4.5% as fat, and 72.1% as carbohydrate. Approximately one-half of each genotype was injected three times with saline (n = 7 DR; n = 9 DIO), and one-half was subjected to three bouts of IIH separated by 48 h (n = 9 DR; n = 8 DIO). After the last injections, rats were fed a HE diet (no. C11024F; Research Diets, New Brunswick, NJ), which contains 4.47 kcal/g, with 21% of the metabolizable energy content as protein, 31% as fat, and 48% as carbohydrate, 50% of which is sucrose (25). They were maintained on this diet for 4 wk and killed by decapitation within 2–4 h of light onset.

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Surgery and experimental protocol. Rats were anesthetized with ketamine (45 mg/kg)-xylazine (9 mg/kg)-buprenorphine (0.2 mg/kg) intraperitoneally. Surgery was performed under aseptic conditions, as previously described (37). Briefly, a Silastic catheter was implanted via the jugular vein in the junction of the right atrium and superior vena cava, sutured in place, and tunneled subcutaneously to exit between the scapulae. Catheters were filled with a suspension of 20% polyvinylpyrrolidone in heparinized saline (100 U/ml) and plugged. Catheters were flushed daily. The rats were allowed to recover for at least 5 days before experiments were performed. At that time, all rats were eating and drinking normally and had surpassed their preoperative weight.

For the experimental treatment (recurrent IHH vs. recurrent saline), rats were fasted every other night, before each of three episodes. On the experiment days, rats had blood samples (1.0 ml) drawn for baseline glucose, lactate, and catecholamine levels. The blood was centrifuged, plasma was removed, and red blood cells were suspended in saline and reinfused in the animal (37). At that time, rats were also injected intravenously with either regular insulin (5 U/kg in 1 ml/kg) or saline (1 ml/kg; controls). Additional blood samples were drawn 30 and 60 min after treatment. When red blood cells from the 60-min sample were reinfused, rats were treated with 1 g/kg D-glucose intravenously, and food was returned to the rat for the next 29 h. A final sample for glucose (20 μl) was taken at 85 min. This protocol

Fig. 1. Plasma glucose, lactate, and catecholamine values. Diet-induced obesity (DIO) and diet-resistant (DR) rats were subjected to three trials of insulin-induced hypoglycemia separated by 48 h. Plasma from an indwelling jugular catheter was analyzed at baseline and at 30 and 60 min after insulin (5 U/kg iv). Data are means ± SE.
was followed two more times, each time after an overnight fast. In some of the rats, catheters failed, so those rats were used as controls; they were fasted at the same time as the other rats, and they were used for the weight gain part of the experiment. A total of seven DIO and six DR rats was carried through all three injections of insulin. Food intake and weight gain were monitored for an additional 4 wk. After six DR rats was carried through all three injections of insulin. Food intake and weight gain were monitored for an additional 4 wk. After

In situ hybridization for ARC NPY and POMC mRNA. Brains were processed for in situ hybridization by minor modifications of previously described methods (20, 21, 39). Serial 15-μm sections were taken through the rostrocaudal extent of the ARC and dorsomedial (DMN) and ventromedial (VMN) hypothalamic nuclei. Briefly, cRNA was synthesized from the 511-bp probe [derived from the original probe of Higuchi et al. (14) for NPY, the 923-bp probe for POMC (kindly provided by D. Richard), and subcloned in a pBluescript SK(+) vector at an EcoRI I site. Sense probes were generated for each of the antisense probes and run to control for nonspecific hybridization. Frozen sections of brain were freeze-thawed on 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. On completion of hybridization, slides were opposed to SB-5 X-ray film (Kodak, Rochester, NY) for 3–4 days.

The resulting autoradiograms were read by an experimentally “blinded” observer using computer-assisted densitometry (Drexel University, Philadelphia, PA; see Ref. 21). Areal measures (mm²) or optical density measures were made through the entire rostrocaudal extent of the hypothalamus in the ARC, VMN, and DMN. The outlines of the autoradiographic images of NPY and POMC 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 anatomical structure was measured (21).

Assay of plasma constituents. Plasma catecholamines were assayed by HPLC with electrochemical detection, as previously described (37). Glucose and lactate were assayed by an automated analyzer (Yellow Springs Instruments).

Statistics. Data were analyzed by two-way ANOVA for single parameters and by ANOVA with repeated-measures design for plasma values. When ANOVA results showed significant intergroup differences, post hoc comparisons were compared by Bonferroni correction for individual sets of data.

**RESULTS**

Effects of recurrent hypoglycemia on CRR. Three bouts of IIH had similar effects on plasma glucose levels in DIO and DR rats over the first 60 min of all but the third episode. In all bouts, glucose levels reached a nadir at 30 min and then increased by at least 100% in both DIO and DR rats from 60 to 85 min after intravenous glucose administration (Fig. 1). However, during the third bout, glucose fell to 31% lower levels at 30 min and rebounded to 35% lower levels after glucose administration in DIO than DR rats (Fig. 2). In 89% of DR and 95% of DR rats the peak Epi levels were attained at 60 min in the three trials (Fig. 1). Summated 30- and 60-min Epi levels differed between DIO and DR rats across the three trials by repeated measures [F(1,11) = 4.58; P = 0.05]. Analysis at each trial showed that DIO rats had 22% lower summated Epi responses during the first bout (P = 0.05) and 29% lower response during the second bout than DR rats (P = 0.05; Fig. 3). There was no significant difference in Epi levels between DIO and DR rats after the third bout because of the fact that DR, but not DIO, rats showed a trend (P = 0.08) toward reduced summated Epi levels between the second and third bout (Fig. 3). However, DIO rats exhibited no such trend toward downregulation of their Epi responses to repeated bouts of hypoglycemia. Lactate levels rose progressively from baseline to 60 min into the hypoglycemic bouts but then fell back to baseline levels after glucose administration from 60 to 85 min (Fig. 1). There were no differences between DIO and DR plasma lactate responses to hypoglycemia, nor was there a change in the lactate response across the three bouts in either DIO or DR rats. Also, there were no genotype nor interbout differences in plasma NE levels (Fig. 1).

Effects of recurrent hypoglycemia on body and adipose gain. Three bouts of hypoglycemia affected neither body weight gain nor adipose pad weights in either DIO or DR rats fed HE diet for 4 wk. As expected, DIO rats gained 100% more body weight and had 150% heavier total adipose pad weights than DR rats after 4 wk on the HE diet (Table 1). However, there was an interaction effect on body weight gain between genotype and treatment [F(1,29) = 3.62; P = 0.05] whereby DR

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**Fig. 2.** Plasma glucose levels in DR vs. DIO rats during the third trial of insulin-induced hypoglycemia. At 60 min after iv insulin injections, rats were injected with glucose (1 g/kg iv), and blood was drawn at 85 min. Data are means ± SE for the plasma glucose levels during the third trial of IIH. *P = 0.05 or less when values in DR rats were compared with those in DIO rats by post hoc t-test after repeated-measures ANOVA showed a significant intergroup difference.

**Fig. 3.** Summated plasma epinephrine (Epi) levels (Σ pg/ml) during 3 trials of insulin-induced hypoglycemia. Plasma Epi levels at 30 and 60 min after insulin injection were summed for each trial × genotype. Data are means ± SE. *P = 0.05 or less when values in DR rats were compared with those in DIO rats for each trial.
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POMC expression and body weight gain over the preceding 4 wk had higher expression of both NPY (1,24) and POMC (1,24) F(1,24) than saline-treated controls, with no main effect of genotype. These hypoglycemia-induced increases reached statistical significance only in DR rats compared with their saline controls. Also, there was a negative correlation between ARC POMC expression and body weight gain over the preceding 4 wk on the HE diet for DR rats subjected to three bouts of IIH ($r = -0.85; P = 0.004$). This relationship did not occur for any of the other groups or for NPY expression and body weight gain.

DISCUSSION

Before they become obese, DIO rats have a number of defects in their ability to sense and respond to glucose (36). Here we show that rats selectively bred to express the DIO phenotype also have a reduced Epi response compared with the response in DR rats to two bouts IIH spaced 48 h apart. After a third bout, DIO and DR rats had similar Epi responses because of a tendency to lower Epi responses across trials in DR but not DIO rats. Furthermore, DIO rats exhibited a greater reduction in glucose levels and rebounded less than DR rats after an intravenous glucose “rescue” bolus during their third bout of IIH. There are many potential sites at which such defects in responding to IIH might occur. DIO rats have reduced numbers of glucosensing neurons in their ventromedial hypothalamus (36), a site that is critical to the CRR (3–6, 35). Neurons in this area have reduced sensitivity of their ATP-sensitive K+ channels to ATP and sulfonylureas (unpublished observation, V. H. Routh) and an associated reduction in low-affinity sulfonylurea binding (22). This ATP-sensitive K+ channel is essential for glucosensing in hypothalamic glucose-excited neurons (24). DIO rats also have defective glucose-regulated α2-adrenoceptor binding in hypothalamic areas containing glucosensing neurons (26). When infused with glucose through the carotid arteries, they fail to activate hypothalamic neurons comparably to DR rats (24), and they activate their sympathetic nervous system aberrantly after both intravenous (27) and intracarotid (17) glucose infusions.

Any of these defects in glucosensing alone might be sufficient cause for the reduced Epi response to IIH in DIO rats, since their responsiveness to low glucose levels would be impaired. Another possibility is that DIO rats have aberrant regulation of glycolysis in specialized glucosensing neurons. This regulatory step is controlled by GK and is a key component of glucosensing in both pancreatic β-cells (31) and glucosensing neurons (12, 18, 28, 29, 32, 40). We have shown that both glucose-excited and glucose-inhibited hypothalamic neurons express GK mRNA (16) and drugs that inhibit GK activity alter firing rates in both types of these glucosensing neurons (12, 16, 40). A single bout of hypoglycemia that induces hypoglycemia-associated autonomic failure is associated with an upregulation of hypothalamic GK mRNA expression in selectively bred DIO and DR rats (12). Also, the CRR to 2-deoxyglucose-induced glucoprivation is reduced after third ventricular injections of alloxan, which increase GK expression (35). In fact, selectively bred DIO rats also have elevated hypothalamic GK mRNA expression before they become obese (12). If this upregulation were translated into increased GK enzyme activity, it could enable the neurons that express GK to maintain adequate ATP production during times of lowered glucose levels. Because glucosensing neurons alter their firing rates in direct relationship to intracellular ATP levels, elevated GK activity in such neurons of DIO rats would provide more ATP than in DR rats at comparably low glucose levels.

Table 1. Body and organ weights of selectively bred DR and DIO rats subjected to 3 serial bouts of IIH separated by 48 h

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<th></th>
<th>Saline (7)</th>
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<td><strong>DR</strong></td>
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<tr>
<td>Initial body weight, g</td>
<td>329±16*</td>
<td>343±14*</td>
<td>387±15†</td>
<td>370±24†</td>
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<tr>
<td>Final body weight, g</td>
<td>398±15*</td>
<td>418±13*</td>
<td>546±20†</td>
<td>514±25†</td>
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<tr>
<td>Body weight gain, g</td>
<td>69.4±4.6*</td>
<td>75.2±5.0*</td>
<td>159±8†</td>
<td>143±10†</td>
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<td>Fat pad weights, g</td>
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<td>Inguinal</td>
<td>5.57±0.45*</td>
<td>5.06±0.52*</td>
<td>13.3±1.4†</td>
<td>10.8±1.5†</td>
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<td>Mesenteric</td>
<td>4.03±0.66*</td>
<td>3.44±0.24*</td>
<td>13.3±1.7†</td>
<td>10.9±2.0†</td>
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<td>Retroperitoneal</td>
<td>4.53±0.76*</td>
<td>5.37±0.83*</td>
<td>14.8±1.9†</td>
<td>11.8±1.9†</td>
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<td>Perirenal</td>
<td>0.81±0.22*</td>
<td>0.68±0.07*</td>
<td>1.46±0.14†</td>
<td>1.41±0.26†</td>
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<tr>
<td>Total</td>
<td>14.9±1.9*</td>
<td>15.5±1.6*</td>
<td>42.9±5.0†</td>
<td>34.9±5.8†</td>
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<tr>
<td>Body weight, %</td>
<td>3.7±0.3*</td>
<td>3.7±0.3*</td>
<td>7.7±0.7†</td>
<td>6.5±0.8†</td>
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<tr>
<td>Liver weight, g</td>
<td>13.9±0.6*</td>
<td>14.6±0.5*</td>
<td>22.8±0.9†</td>
<td>21.2±1.4†</td>
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<th>Saline (7)</th>
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<td><strong>DIO</strong></td>
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Data are means ± SE; no. of rats in parentheses. DR, diet resistant; DIO, diet-induced obese; IIH, insulin-induced hypoglycemia (5 U/kg). Control rats were injected with saline. All rats were then placed on a high-energy (31% fat) diet for 4 wk. Data with dissimilar superscripts differ from each other by $P < 0.05$ by post hoc t-test after intergroup differences were found by 2-way ANOVA.

Fig. 4. Arcuate neuropeptide Y (NPY) and proopiomelanocortin (POMC) mRNA expression after 3 trials of insulin-induced hypoglycemia (IIH) and 1 mo on a high-energy diet. Data are means ± SE. Bars with differing superscripts differ from each other by $P < 0.05$ by post hoc t-test after 1-way ANOVA showed significant intergroup differences.
levels. This would be interpreted by glucosensing neurons as having sufficient glucose and should evoke a reduced CRR in DIO rats. Thus elevation of hypothalamic G K expression, as well as other potential defects in neuronal glucosensing, might well underlie the reduced Epi response of DIO rats to their first two bouts of IHH compared with DR rats.

Despite their impaired Epi response during the first two IHH bouts, DIO rats had comparable decreases in glucose levels to DR rats. This suggests that other responses, particularly glucagon, might compensate for their reduced sympathoadrenal responses. However, by the third bout, glucose levels fell to lower levels and DIO rats failed to rebound comparably to DR rats after glucose administration. This too might be because of impaired glucagon responses but could also be caused by differences in insulin degradation by the liver in DIO rats.

Recurrent hypoglycemia had no significant overall effect on weight gain or adiposity when dietary fat and caloric density were increased for a 4-wk period in either DIO or DR rats. However, there was a tendency for DR rats to gain more and DIO rats to gain less weight after prior IHH. We previously showed that both ARC NPY and POMC expression were downregulated after three bouts of IHH in association with reduced Epi and corticosterone responses in outbred Sprague-Dawley rats (37). Although we did not assess the short-term effects of IHH on NPY and POMC expression here in selectively bred DIO and DR rats, it is reasonable to assume that the previously reported acute reductions in outbred Sprague-Dawley rats would occur here as well, since the DIO and DR rats were selectively bred from that strain (23). If so, hypoglycemia-induced acute reductions were apparently reversed after 4 wk on the HE diet in DIO rats, whereas levels were actually increased in DR rats. Even if recurrent IHH did not affect ARC NPY and POMC expression in selectively bred DIO and DR in a similar manner to outbred rats, it is clear that the end result of recurrent hypoglycemia followed by 4 wk of HE diet intake did have a selective effect on the expression of these peptides in DR rats. Only recurrently hypoglycemic DR rats had increased NPY and POMC expression and an inverse relationship between POMC expression and body weight gain on HE diet. As opposed to the other groups, only recurrently hypoglycemic DR rats, which gained the most weight, were those that had the lowest expression of POMC, the precursor of the catabolic neuropeptide α-MSH (38). This peptide interacts with melanocortin-3 and -4 receptors, chronic activation of which reduces food intake, energy expenditure, and body weight gain (7, 33). Because no such relationship was seen between body weight gain and NPY expression, this suggests that the effect of recurrent hypoglycemia and HE diet exposure on DR POMC expression might have been an important contributor to body weight regulation in these rats. However, since this was only a correlation and because no effects on α-MSH were examined, no causal relationship can be confirmed. Most important, our data show that repeated bouts of IHH can have long-lasting, genotype-specific sequelae with respect to brain pathways involved in energy homeostasis.

In conclusion, DIO rats have several preexisting defects in central glucosensing that alter their pattern of glucose-induced autonomic activation. Here we show that their adrenal medullary response to IHH is attenuated and does not show the trend toward reduced Epi secretion seen with repeated bouts in DR rats. Furthermore, DR rats showed a selective increase in both ARC NPY and POMC expression after three bouts of hypoglycemia followed by 4 wk of HE diet intake and a selective inverse relationship between POMC expression and body weight gain on HE diet. Although prior IHH had no significant overall effect on body weight gain or adiposity, there was a tendency for DR rats to gain more weight in inverse proportion to the ARC expression of the catabolic peptide POMC. These results support our prior studies showing that DIO rats have defective central glucosensing that might contribute to their genetic predisposition to develop DIO. They also suggest that IHH can have a genotype-selective, long-term effect on body weight and the expression of hypothalamic neuropeptides involved in energy homeostasis.

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