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Leptin acts in the brain to influence hypoglycemic counterregulation: disparate effects of acute and recurrent hypoglycemia on glucagon release

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Leptin CM, Ding Y, Sherwin R. Leptin acts in the brain to influence hypoglycemic counterregulation: disparate effects of acute and recurrent hypoglycemia on glucagon release. Am J Physiol Endocrinol Metab 309: E960–E967, 2015. First published October 27, 2015; doi:10.1152/ajpendo.00361.2015.—Leptin has been shown to influence hypoglycemic counterregulation, hyperinsulinemic hypoglycemic (∼45 mg/dl) clamps were performed on rats exposed to or not exposed to recurrent hypoglycemia (3 days, ∼40 mg/dl). Intracerebroventricular artificial cerebral spinal fluid or leptin was infused during the clamp. During acute hypoglycemia, leptin decreased glucagon responses by 51% but increased epinephrine and norepinephrine by 24 and 48%, respectively. After recurrent hypoglycemia, basal plasma leptin levels were undetectable. Subsequent brain leptin infusion during hypoglycemia paradoxically increased glucagon by 45% as well as epinephrine by 19%. In conclusion, leptin acts within the brain to diminish glucagon secretion during acute hypoglycemia but increases epinephrine, potentially limiting its detrimental effects during hypoglycemia. Exposure to recurrent hypoglycemia markedly suppresses plasma leptin, whereas exogenous brain leptin delivery enhances both glucagon and epinephrine release to subsequent hypoglycemia. These data suggest that recurrent hypoglycemia may diminish counterregulatory responses in part by reducing brain leptin action.

hypoglycemia; recurrent hypoglycemia; leptin; glucagon

Rodent data suggest these adaptive hormonal changes produced by antecedent hypoglycemia may at least in part be linked to increased GABA and diminished glutamate VMH neurotransmission (2, 27, 29). Whether recurrent hypoglycemia (RH) alters circulating leptin levels, and whether such changes in leptin might contribute to defective glucose counterregulation after recurrent bouts of insulin-induced hypoglycemia, has not been explored.

Leptin, a hormone secreted by adipocytes, has been studied extensively for its effects on food intake and body weight. However, leptin’s role in glucose metabolism has only recently become appreciated. Chronic systemic or brain infusion of leptin in insulin-deficient diabetic rodents has been shown to reduce hyperglycemia independently of insulin (4, 15, 16, 28). It has been suggested that the effects of chronic intracerebroventricular (icv) leptin delivery may be mediated by alterations in the function of GABAergic and proopiomelanocortin neurons (14). Leptin receptors are highly expressed in the arcuate nucleus and dorsomedial, ventromedial, and lateral hypothalami (21). In addition to the hypothalamus, the parabrachial nucleus (PBN) of the hindbrain is also an important site of leptin action that feeds forward to the VMH to elicit its actions (12). Ablation of leptin receptors on the PBN neurons was shown to enhance the counterregulatory response to hypoglycemia, suggesting that leptin action in the hindbrain inhibits hypoglycemic counterregulation (12). Thus, it remains uncertain as to which specific brain region is predominantly responsible for leptin’s capacity to regulate counterregulatory hormone secretion.

Multiple mechanisms have been proposed to account for leptin’s antidiabetic effects, including reductions in glucagon secretion and hepatic glucose production as well as increased glucose uptake into peripheral tissues (4, 15, 16, 28). However, the data regarding the suppression of glucagon levels have been inconsistent (15, 17) and generally derived from studies employing chronic leptin administration. On the other hand, chronic brain delivery of leptin has been reported to increase sympathetic nerve activity (8) and epinephrine secretion (25). It is noteworthy that no studies have examined leptin’s effect on glucagon release during insulin-induced acute hypoglycemia or following recurrent hypoglycemia, an effect that could be detrimental. Additionally, the effects of leptin on epinephrine secretion during hypoglycemia or following recurrent hypoglycemia have not been explored and could potentially offset a leptin-induced glucagon-lowering effect. Since leptin has been proposed as a potential glucose-lowering agent in the treatment of insulin-deficient diabetes (5), a better understand-
ing of how leptin acts under hypoglycemic conditions is needed before such an approach can be applied therapeutically.

MATERIALS AND METHODS

Animals. Adult male, nondiabetic Sprague-Dawley rats (Harlan Laboratories, South Easton, MA) were used at ~300 g body wt and were individually housed in the Yale Animal Resources Center in temperature- and humidity-controlled rooms with a 12-h light-dark cycle. The rats had free access to normal rodent chow (Harlan-Teklad, Indianapolis, IN) and water. Principles of laboratory animal care were followed, and experimental protocols were approved by the Institutional Animal Care and Use Committee at Yale University.

Surgery. Rats underwent stereotaxic and vessel cannulation surgery prior to the studies, as described previously (3). Briefly, vascular catheters were implanted into the left carotid artery for blood sampling and the right jugular vein for infusion. For stereotaxic surgery, the rats were immediately placed in a stereotaxic frame and single stainless-steel guide cannulae (internal: 31 g; guide: 24 g, 9.5 mm below pedestal; dummy: 31 g; Plastics One, Roanoke, VA) inserted icv into the third ventricle (from bregma: posterior -2.6 mm, medial-lateral 0 mm, dorsal-ventral 8.2 mm).

Hyperinsulinemic hypoglycemic clamp. Overnight-fasted rats were connected to the infusion pumps and allowed to rest for 2 h. A primed, continuous infusion of artificial extracellular cerebrospinal fluid (aECF) or leptin [1 μg·kg⁻¹·h⁻¹ (19), mouse leptin; Sigma, St. Louis, MO] was infused icv 30 min prior to and throughout the duration of the clamp procedure. A hyperinsulinemic (20 mU·kg⁻¹·min⁻¹, Humulin R; Eli Lilly, Indianapolis, IN) hypoglycemic (~45 mg/dl; 20% dextrose) clamp was performed for 2 h on awake, unrestrained rats. Blood samples were taken every 10 min for plasma glucose (Analox Instruments USA, Lunenburg, MA) and every 30 min for hormone measurements. Following each sample collection, the red blood cells were resuspended in an equivalent volume of artificial plasma and reinfused back into the animal to prevent volume depletion and anemia. At the end of the study, rats were euthanized with an overdose of pentobarbital sodium and the brains removed and immediately frozen on dry ice. Probe placement was checked through histoch-
**Assays.** Plasma leptin was measured by ELISA. Plasma catecholamines were measured by high-performance liquid chromatography using electrochemical detection (Thermo Fisher Scientific). Plasma glucagon (Linco Research, St. Charles, MO) and insulin (Linco Research) were measured by radioimmunoassay.

**Western blot.** To test the efficacy of leptin delivery to the brain, Western blots were performed on whole hypothalamus for phosphorylated (Tyr705) and total STAT3 (Cell Signaling Technology, Beverly, MA).

**Statistics.** All statistics were performed using GraphPad Prism 6. Student’s t-test was performed on plasma glucose, epinephrine, norepinephrine, glucagon, insulin, and leptin levels and glucose infusion rates for comparison of the means, peaks, and area under the curve. One-way ANOVA was used to compare basal vs. clamp data between the groups and the results of glucagon from both studies. A repeated-measures ANOVA was used to compare glucose infusion rates. All data are expressed as means ± SE.

**RESULTS**

**Acute hypoglycemia-icv leptin infusion.** Glucose levels during the hypoglycemic clamp for aECF and leptin-infused rats were 46 ± 1 and 47 ± 2 mg/dl, respectively (n = 7/group; Fig. 1A). However, the glucose infusion rate required to maintain this glycemic level was 2.2-fold higher in the leptin- (2.8 ± 0.7) compared with aECF (1.3 ± 0.4 mg·kg⁻¹·min⁻¹) brain-infused rats between 20 and 120 min (P < 0.04; Fig. 1B). Insulin levels were unaffected by brain leptin infusion (Fig. 1C). As shown in Fig. 2A, glucagon release in response to hypoglycemia was reduced by 51% in the leptin-infused rats. Glucagon levels during the first 20–40 min of the clamp for the aECF and leptin groups were 1,003 ± 247 and 496 ± 76 pg/ml, respectively (P < 0.03; Fig. 2A). In contrast, epinephrine levels were significantly higher during the first 40 min in the leptin-infused rats (11,195 ± 493 vs. 9,055 ± 964 pg/ml in controls, P < 0.05; Fig. 2B). Norepinephrine levels were also significantly increased in leptin-infused rats (1,661 ± 207 vs. aECF: 1,124 ± 117 pg/ml, P < 0.05; Fig. 2C). To ensure that brain leptin delivery did not significantly spill over into the blood, plasma leptin levels were measured and were not significantly different between the groups (Fig. 2D).

**Acute hypoglycemia-VMH leptin microinjection.** To assess whether the effects of leptin on glucagon release were mediated via changes in VMH function, a separate experiment was conducted in which leptin (or aECF) was targeted specifically to the VMH just before the onset of acute hypoglycemia. Glucose levels during hypoglycemia were evenly matched between the two groups (VMH-aECF: 48 ± 3, n = 8; VMH-leptin: 45 ± 2 mg/dl, n = 6; Fig. 3A). The glucose infusion rate required to maintain this glycemic state was increased 2.8-fold...
in the VMH-leptin-injected rats (1.5 ± 0.23 mg·kg⁻¹·min⁻¹) compared with the VMH-aECF control rats (0.53 ± 0.28 mg·kg⁻¹·min⁻¹, P < 0.02; Fig. 3B). VMH leptin injection reduced plasma glucagon by 24%, but this decrease did not quite reach statistical significance (VMH-aECF 1,657 ± 135 pg/ml; open bars), compared with controls (VMH-aECF 1,267 ± 141 pg/ml, P < 0.06; black bars), but this just missed significance. In VMH-leptin rats (1.53 ± 0.35 µM) compared with control rats (2.32 ± 0.71 µM). Data are represented as means ± SE; n = 6–8/group. *P < 0.05.

**RH-icv leptin infusion.** As shown in Fig. 4A, mean glucose levels during RH on days 1, 2, and 3 were similar for the control rats (aECF-RH; 39 ± 2, 40 ± 3, and 39 ± 2 mg/dl) and the leptin-infused rats (leptin-RH; 44 ± 2, 40 ± 2, and 37 ± 2 mg/dl). However, following an overnight fast on day 4 before the hypoglycemic clamp study, basal plasma leptin levels were consistently below the threshold for measurement in the assay (<25 pg/ml), whereas control rats that had not been exposed to recurrent hypoglycemia had basal plasma leptin levels of 105 ± 50 pg/ml (Fig. 4B). As shown in Fig. 5, A and B, during the hypoglycemic clamp study, plasma glucose levels (47 ± 1 in the aECF-RH and 45 ± 2 mg/dl in the leptin-RH groups, respectively) and plasma insulin levels were similar. However, the mean glucose infusion rate required to maintain this level of glucose was reduced by 48% in the leptin-RH group (0.89 ± 0.2, P < 0.04) compared with aECF-RH rats (2.17 ± 0.6 mg·kg⁻¹·min⁻¹; Fig. 5C). This change in the leptin-treated RH rats was associated with an increase in both glucagon and epinephrine secretion. There was a 45% increase in the glucagon response in the leptin-treated rats in the first 40 min of the hypoglycemic clamp (970 ± 147 vs. 531 ± 139 pg/ml in the aECF-RH controls, P < 0.05; Fig. 6A). Epinephrine levels were also initially increased by 24% in leptin-RH rats in the first 40 min of the study (3,675 ± 306 vs. 2,975 ± 317 pg/ml in the aECF-RH controls, P < 0.05; Fig. 6B). Norepinephrine levels were not significantly different between the groups (leptin-RH: 2,674 ± 339 vs. aECF-RH: 2,451 ± 233 pg/ml; Fig. 6C). As shown in Fig. 6D, hypothalamic phosphorylated STAT3 protein expression was increased twofold in the leptin-RH group compared with controls, verifying the efficacy of leptin VMH delivery.

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**Fig. 3. Ventromedial hypothalamus (VMH) leptin microinjection with acute hypoglycemia.** A: glucose levels were evenly matched during the acute hypoglycemic clamp in control (VMH-aECF, 48 ± 3 mg/dl; ○) and leptin-injected rats (VMH-leptin, 45 ± 2 mg/dl; ■). B: the GIR was significantly increased in the VMH-leptin-injected rats (mean: 1.5 ± 0.23 mg·kg⁻¹·min⁻¹, P < 0.02) compared with controls (0.53 ± 0.28 mg·kg⁻¹·min⁻¹). C: glucagon was decreased in VMH-leptin rats (1,267 ± 141 pg/ml, P < 0.06; black bars) compared with controls (VMH-aECF: 1,657 ± 135 pg/ml, open bars), but this just missed significance. D: mean VMH glutamate levels trended to be lower in VMH-leptin rats (1.53 ± 0.35 µM) compared with control rats (2.32 ± 0.71 µM). Data are represented as means ± SE; n = 6–8/group. *P < 0.05.

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The mechanisms by which leptin acts to lower blood glucose have been reported to be mainly through reductions in glucagon secretion and hepatic glucose production (4, 15, 16, 28). However, previous studies in which leptin was given to reduce elevations in blood glucose in diabetes did not test whether leptin exerts similar glucose-lowering effects during hypoglycemia, which could be detrimental. The current study demonstrates that leptin acts in the brain to blunt the glucagon response during acute hypoglycemia in nondiabetic rats. However, its capacity to increase both epinephrine and norepinephrine release during acute hypoglycemia diminishes this potential adverse effect. In contrast, rats exposed to recurrent hypoglycemia display a paradoxical increase in glucagon release in conjunction with an increase in epinephrine release as well. Of particular interest, we observed, unexpectedly, that basal circulating leptin levels were markedly suppressed in response to antecedent recurrent hypoglycemia. These data imply that reduced leptin action in the brain may play an important role in the maladaptation that arises from antecedent hypoglycemia and that leptin treatment might have clinical implications to help combat impaired counterregulation in type 1 diabetic individuals.

Although leptin receptors are expressed throughout the brain (21), and the current studies have focused on third ventricle delivery of leptin, our data suggest that the VMH is likely to be a key site of leptin’s action in the brain to modulate glucose counterregulation (1, 18). Selective VMH microinjection of leptin just before the onset of acute insulin-induced hypoglycemia increased the glucose infusion rate during the hypoglycemic clamp study and tended to diminish glucagon responses. Nevertheless, further studies are required to better define the role of VMH leptin action in hypoglycemic counterregulation. Additionally, it has been reported recently that leptin availability modulates the activation of neurons in the PBN region of the brain that project to the dorsomedial division of the VMH (12). Leptin-induced inhibition of PBN neurons was shown to diminish hypoglycemic counterregulation (12), suggesting that it may be another potential site of leptin’s effect to alter the response to hypoglycemia. This will need to be addressed in future studies. It is possible that the changes in glucose infusion rates induced by brain leptin infusion were not simply caused by alterations in glucagon and epinephrine secretion and that other factors modulating liver or peripheral glucose metabolism might be involved (4, 15, 16, 28). However, since leptin levels in the peripheral circulation were not altered during brain leptin delivery, it is highly unlikely that the metabolic effects observed could be attributed to leptin leakage into the periphery.

Although the mechanisms by which leptin increases the epinephrine response to hypoglycemia are not fully understood, previous studies have demonstrated that full activation of the counterregulatory response to hypoglycemia requires VMH GABA neurotransmission to decrease (2, 29) and, in turn, VMH glutamate neurotransmission to increase. In keeping with the latter possibility, previous studies have shown that suppression of VMH glutamate receptors (VGLUT2) using SF1-Cre VGLUT2-knockout mice impairs glucagon and epinephrine secretion during hypoglycemia, indicating that a rise in glutamate neurotransmission within the VMH is important to combat hypoglycemia (27). Previous studies have reported that leptin increases glucagon uptake into astrocytes (13), and the current data, in keeping with these findings, show that VMH leptin microinjection diminishes glutamate levels in VMH interstitial fluid during hypoglycemia, implying that leptin-induced alterations in glutamate neurotransmission contribute to the blunted glucagon response. In striking contrast, following antecedent recurrent hypoglycemia, brain leptin infusion paradoxically stimulated the release of plasma glucagon during subsequent acute hypoglycemia compared with controls. The mechanisms leading to these differences in the capacity of brain leptin to affect glucagon secretion are uncertain but might be attributed to effects on the alterations in VMH neurotransmission that develop following exposure to recurrent hypoglycemia, such as absent changes in VMH GABA and glutamate levels, inactive/closed kATP channels, suppressed AMP kinase activity, or alterations in the function of neurons in other brain regions that act to modulate the activity of VMH glucose-sensing neurons (2, 6, 9, 20, 23, 26).

Unexpectedly, basal plasma leptin levels were undetectable in rats that were exposed to recurrent hypoglycemia, which was in striking contrast to rats studied after an overnight fast. The suppression of leptin under these conditions may represent an attempt to stimulate carbohydrate consumption to protect...
against hypoglycemia (24). Importantly, restoring leptin levels in the brain by local delivery of leptin into the third ventricle not only enhanced the epinephrine response but also caused stimulation of glucagon secretion in rats exposed to recurrent hypoglycemia. Interestingly, clinical trials of obesity, type 1 and type 2 diabetes, and lipodystrophy have also shown that only individuals who are hypoleptinemic are able to effectively respond to leptin treatment (10, 11, 22). These data suggest that the markedly diminished levels of leptin within the brain after recurrent antecedent hypoglycemia are a key contributor to the impaired counterregulatory response observed under these conditions. This conclusion is underscored by the fact that there is high expression of leptin receptors in the hypothalamus and the fact that the hypothalamus is a well-established regulator of hypoglycemic counterregulation. We recognize that the current data were designed specifically to restore brain leptin availability, without changing peripheral levels, so as to isolate the effects of leptin within the brain without changing peripheral leptin levels. Whether or not whole body restoration of leptin levels after recurrent hypoglycemia will increase the glucagon and epinephrine responses to subsequent hypoglycemia remains to be determined.

The current rodent data demonstrate that recurrent insulin-induced hypoglycemia unexpectedly produces a striking reduction in circulating leptin and suggest that these changes diminish brain delivery of leptin, which in turn may play an important unrecognized role in the development of defective glucose counterregulation that arises from antecedent hypoglycemia. These observations also have potential clinical implications for the management of patients with brittle type 1 diabetes receiving intensive insulin treatment in whom plasma leptin levels are suppressed. In such patients, glucagon release during hypoglycemia fails to occur, and glucose recovery is impaired by an inadequate ability to release epinephrine during hypoglycemia. Such insulin-requiring diabetic patients might benefit from the addition of leptin treatment not only to reduce hyperglycemia but also to more effectively increase their capacity to defend against hypoglycemia.

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DISCLOSURES

No potential conflicts of interest, financial or otherwise, are reported. R. Sherwin is the guarantor of this research and takes full responsibility of the data presented.

AUTHOR CONTRIBUTIONS

C.M.R. and R.S. conception and design of research; C.M.R. and Y.D. performed experiments; C.M.R. analyzed data; C.M.R. and R.S. interpreted...
results of experiments; C.M.R. prepared figures; C.M.R. drafted manuscript; C.M.R. and R.S. edited and revised manuscript; C.M.R. and R.S. approved final version of manuscript.

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