

Insulin is Required for Prandial Ghrelin Suppression in Humans

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Accumulating evidence indicates that ghrelin plays a role in regulating food intake and energy homeostasis. In normal subjects, circulating ghrelin concentrations decrease after meal ingestion and increase progressively before meals. At present, it is not clear whether nutrients suppress the plasma ghrelin concentration directly or indirectly by stimulating insulin secretion. To test the hypothesis that insulin regulates postprandial plasma ghrelin concentrations in humans, we compared the effects of meal ingestion on plasma ghrelin levels in six C-peptide-negative subjects with type 1 diabetes and in six healthy subjects matched for age, sex, and BMI. Diabetic subjects were studied during absence of insulin (insulin withdrawal study), with intravenous infusion of basal insulin (basal insulin study) and subcutaneous administration of a prandial insulin dose (prandial insulin study). Meal intake suppressed plasma ghrelin concentrations (nadir at 105 min) by $32 \pm 4\%$ in normal control subjects, $57 \pm 3\%$ in diabetic patients during the prandial insulin study ($P < 0.002$ vs. control subjects), and $38 \pm 8\%$ during basal insulin study ($P = 0.0016$ vs. hyperinsulinemia; $P = NS$ vs. control subjects) but did not have any effect in the insulin withdrawal study ($P < 0.001$ vs. other studies). In conclusion, 1) insulin is essential for meal-induced plasma ghrelin suppression, 2) basal insulin availability is sufficient for postprandial ghrelin suppression in type 1 diabetic subjects, and 3) lack of meal-induced ghrelin suppression caused by severe insulin deficiency may explain hyperphagia of uncontrolled type 1 diabetic subjects. *Diabetes* 52:2923–2927, 2003

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (1,2), appears to play a key role in regulating food intake and energy homeostasis (3–5). Hormonal and nutritional factors might both affect ghrelin production. In lean subjects, plasma ghrelin levels rise progressively before meals and fall to a nadir within 1 h of eating, a pattern mirroring that of insulin (6). Ghrelin concentrations are decreased by oral or intravenous administration of glucose

(7) but not by filling the stomach with an equal volume of water (4,7). Potentially, one or more dietary nutrients could directly suppress ghrelin production or they could act indirectly by stimulating insulin secretion. The inverse temporal relationship between circulating concentrations of plasma ghrelin and insulin (6) suggests that postprandial hyperinsulinemia might inhibit ghrelin secretion during meal absorption.

At present, the effect of physiologic hyperinsulinemia on plasma ghrelin concentrations in healthy humans is controversial (8–14) and the contribution of postprandial hyperinsulinemia to plasma ghrelin suppression is unknown. In particular, it remains to be established whether a short-lived insulin peak or sustained hyperinsulinemia is required to induce plasma ghrelin decrease. Caixàs et al. (8) reported that, unlike food intake, a subcutaneous injection of a short-acting insulin analog (lispro) associated with a continuous glucose infusion did not affect plasma ghrelin concentration. Schaller et al. (9) observed an ~50% decrease in plasma ghrelin concentrations after an intravenous insulin infusion, resulting in supraphysiological hyperinsulinemia, but did not observe a significant change during an intravenous infusion of glucose stimulating endogenous insulin secretion. During a sustained hyperinsulinemic-euglycemic clamp, circulating insulin levels similar to or higher than those occurring after meal ingestion have been reported to suppress plasma ghrelin concentrations by 15–50% (10–14), suggesting that insulin might be involved in regulating postprandial ghrelin secretion.

The present study tested the hypothesis that insulin affects postprandial plasma ghrelin concentrations in humans. Theoretically, if insulin plays a key role in regulating the postprandial ghrelin decrease, food intake should not suppress plasma ghrelin concentration in conditions of severe insulin deficiency but should markedly suppress plasma ghrelin concentrations in conditions of hyperinsulinization. Therefore we compared the effects of meal ingestion on plasma ghrelin levels in six healthy subjects and in six C-peptide-negative type 1 diabetic patients who were studied under different experimental conditions. Postprandial plasma ghrelin concentrations in diabetic patients were measured during insulin deprivation (insulin withdrawal study), intravenous infusion of basal insulin (basal insulin study), and subcutaneous administration of a prandial insulin dose plus basal intravenous insulin infusion (prandial insulin study). During the prandial insulin study, to avoid the confounding variable of lower plasma glucose concentrations, plasma glucose was

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NEFA, nonesterified fatty acid.

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clamped at the values obtained during the basal insulin study. For ethical reasons, absolute insulin deficiency was carried out only for 90 min and was followed by an intravenous insulin bolus.

RESEARCH DESIGN AND METHODS

Study protocol. After the study had been approved by the Ethics Committee of Perugia University, informed written consent was obtained from all subjects. Six adult type 1 diabetic patients (male/female ratio: 3/3), aged 39 ± 4 (mean \pm SE; range 29–48 years), with a BMI of 22.9 ± 0.9 kg/m² (range 20–26), and HbA_{1c} levels of $6.5 \pm 0.5\%$ were included in the study. All subjects were affected by type 1 diabetes, which had been diagnosed 14 ± 2 years earlier. All had undetectable serum C-peptide concentrations, no history of severe or frequent hypoglycemic episodes, and no severe diabetic complications or other diseases, particularly chronic autoimmune gastritis, as shown by the negativity for serum gastric antiparietal cell antibodies. Hashimoto's thyroiditis had been diagnosed 4 years earlier in one female diabetic patient who was being treated with L-thyroxine (75 μ g daily). Insulin treatment consisted of multiple preprandial injections of regular or short-acting insulin (lispro) combined with a bedtime injection of intermediate insulin. Six healthy adult subjects matched with type 1 diabetic subjects for sex (male/female ratio: 3/3), age (37 ± 4 years, range 24–47), and BMI (22.7 ± 0.8 kg/m², range 21–25) served as control subjects.

For 1 week before starting the study and for the entire study period, all subjects followed a diet of 35 kcal \cdot kg⁻¹ \cdot day⁻¹ containing 55, 30, and 15% carbohydrate, fat, and protein, respectively. Normal control subjects were studied on one occasion; type 1 diabetic subjects were studied on three different occasions at 4- to 8-day intervals. Intermediate insulin administration was stopped 24 h before admission. On the day before each study, diabetic subjects were admitted to the clinical research center of our department at \sim 9:30 P.M., after dinner. At \sim 10:00 P.M., a 22-gauge plastic catheter needle was placed in an antecubital vein for overnight infusions of saline (0.2 ml/min; Vial Médical Pump, Grenoble, France) and regular human insulin (Humulin R; Eli Lilly, Indianapolis, IN) by two Harvard syringe pumps (Harvard Apparatus, South Natick, MA). A contralateral hand vein was cannulated in a retrograde fashion with a 21-gauge butterfly needle, and the hand was maintained at 65°C in a thermoregulated Plexiglas box for intermittent sampling of arterialized-venous blood (15). Intravenous infusion of regular human insulin was continued overnight to maintain plasma glucose levels within 6–8 mmol/l on the basis of frequent plasma glucose measurements. In diabetic subjects, the basal insulin study was followed by the prandial insulin and insulin deficiency studies. The sequence of the three studies was not randomized so as to avoid the confounding variable of different plasma glucose concentrations between the hyper- and the hypoinsulinemic studies. For this reason, during the prandial insulin study, plasma glucose was clamped at the values obtained during the previous basal insulin study.

Basal insulin study (meal and basal insulin). At \sim 8:00 A.M. (0 min), after fasting overnight, the diabetic patients ate a 350-kcal standard breakfast (60% carbohydrate, 25% fat, and 15% protein) in 15 min. Their usual subcutaneous insulin administration was omitted. The average insulin infusion rate required to maintain euglycemia over the preceding 2 h was continued throughout the study. In all studies, blood samples were collected before breakfast at -5 and 0 min and after breakfast at every 30 min until study completion (240 min) to measure the circulating concentrations of nonesterified fatty acids (NEFAs), glucagon, and growth hormone, every 15 min to measure ghrelin and insulin levels, and every 5 min to measure arterialized plasma glucose concentrations.

Prandial insulin study (meal and prandial insulin). At \sim 8:00 A.M., after the overnight fast session, patients ate the standard breakfast described above. The average insulin infusion rate required to maintain euglycemia over the preceding 2 h was continued throughout the study. In addition, 30 min before breakfast a subcutaneous injection of human regular insulin (0.1–0.15 mU/kg; Humulin R; Eli Lilly, Indianapolis, IN) was administered. To mimic the meal-induced rises in plasma glucose observed during the basal insulin study, a variable 20% glucose solution was infused by one Harvard syringe pump (Harvard Apparatus, South Natick, MA).

Insulin withdrawal study (meal and lack of insulin). At \sim 8:00 A.M., after the overnight fast, patients ate the standard breakfast. When patients started breakfast (0 min), the insulin infusion was stopped and the subjects remained without insulin for 90 min. At 90 min, they received an intravenous bolus of regular insulin to correct severe insulin deficiency, and the study was stopped at 150 min. Arterialized blood samples for pH and plasma bicarbonate assay were obtained at 0 and 90 min.

Control study. Healthy control subjects were admitted, after fasting overnight, at \sim 7:30 A.M. on the study day. At \sim 8:00 A.M. (0 min), the healthy

volunteers ate the same standard breakfast as the diabetic subjects in \sim 15 min. Blood samples were collected as described for the diabetic subjects.

Hormone assays. Plasma immunoreactive ghrelin levels were measured in duplicate using a commercial radioimmunoassay that uses ¹²⁵I-labeled bioactive ghrelin as a tracer and a rabbit polyclonal antibody raised against full-length octanoylated human ghrelin (Phoenix Pharmaceuticals, Belmont, CA) that recognizes both acylated and des-acylated ghrelin. The intra-assay coefficient of variation was $<10\%$ (11). Plasma concentrations of glucose were immediately determined using a Beckman glucose analyzer (Beckman Instruments, Palo Alto, CA). Plasma NEFAs were measured using a colorimetric assay (Kardia, Milan, Italy). Serum insulin (Technogenetics, Milan, Italy), growth hormone (Biodata, Norwell, MA), and plasma glucagon concentrations (DRG International, Marburg, Germany) were measured using commercial immunoradiometric assays.

Statistical analyses. All data were subjected to repeated measured ANOVA with Huynh-Feldt adjustment for nonsphericity. Post hoc comparisons (Tukey test) were carried out to pinpoint specific differences on significant means of interaction. Stepwise regression analyses were done to identify independent variables that contributed best to predict plasma ghrelin response. As a set of independent variables, we obtained plasma free fatty acids, growth hormone, and insulin concentrations, which we included in the multiple regression analysis. Ghrelin was the dependent variable. All time points in each curve were used as individual data points to determine the correlation between insulin and ghrelin over time and in the multiple regression analysis. Data are given as means \pm SE, and $P < 0.05$ was considered significant. Statistical analysis was carried out using NCSS 2001 software.

RESULTS

Glucose and insulin infusion rates. At baseline, no differences emerged in plasma glucose concentrations and the insulin infusion rates required to maintain normoglycemia in the three studies in diabetic subjects ($P = \text{NS}$). During the 240 min of the basal insulin study, subjects received a total of 97 ± 17 mU/kg of regular insulin. During the prandial insulin study, the intravenous infusion contained 97 ± 12 mU/kg and the subcutaneous injection 128 ± 19 mU/kg of regular insulin, for a total of 225 ± 27 mU/kg ($P < 0.001$ vs. basal insulin study).

Plasma glucose concentrations. After meal intake, plasma glucose concentrations in the insulin deficiency study (15–90 min) were higher ($P < 0.001$) than in the basal and prandial insulin studies (Fig. 1). In the prandial insulin study, a glucose infusion rate of 0.18 ± 0.07 mmol \cdot kg⁻¹ \cdot min⁻¹ was required to clamp plasma glucose at values that were not statistically different from those of the basal insulin study at all time points. Plasma glucose values of the healthy control subjects were lower ($P < 0.001$) than in the diabetic subjects in all three studies (Fig. 1).

Serum insulin concentrations. Serum insulin concentrations were significantly different ($P < 0.001$) in the four studies (Fig. 1). In the prandial insulin study, serum insulin gradually rose to peak at 75 min in diabetic subjects (253 ± 24 nmol/l) and was significantly higher than in normal control subjects from the 165th min onward ($P < 0.001$). In the basal insulin study, serum insulin concentrations were significantly lower from 30 to 240 min than in the prandial insulin study ($P < 0.001$) and from 15 to 105 min than in control subjects ($P < 0.001$). In the insulin withdrawal study, serum insulin was not detectable 30 min after insulin withdrawal; it reached a peak of 641 ± 62 nmol/l after the insulin bolus (105 min, $P < 0.001$ vs. other studies). After meal intake, insulin peaked earlier (at 30 min) in control subjects and declined more rapidly than it did in diabetic subjects in the prandial insulin study ($P < 0.001$ from 165 to 240 min).

Plasma NEFA concentrations. Plasma NEFA concentrations were suppressed in a similar manner by meal intake

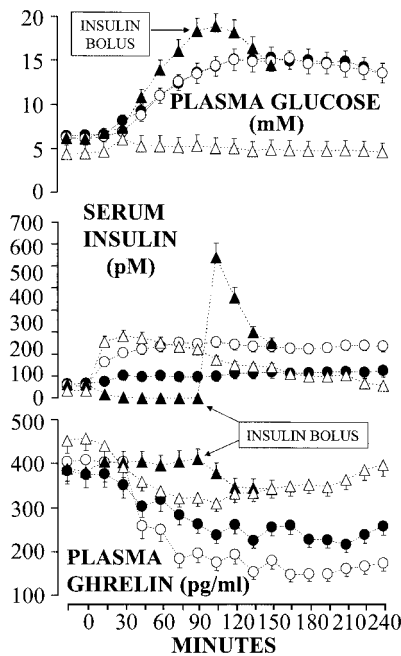


FIG. 1. Effects of meal intake on circulating concentrations of glucose, insulin, and ghrelin in six normal subjects and in six type 1 diabetic subjects during the prandial insulin, basal insulin, and insulin withdrawal studies. Δ , Normal subjects; \blacktriangle , diabetic subjects with no insulin; \circ , diabetic subjects with prandial insulin; \bullet , diabetic subjects with basal insulin.

during the prandial insulin, basal insulin, and control studies ($P = \text{NS}$, Fig. 2). In the insulin withdrawal study, plasma NEFA concentrations were higher ($P < 0.001$) than in all other studies from 30 to 90 min (Fig. 2). The intravenous insulin bolus (90 min) promptly decreased NEFAs from 852 ± 88 to 97 ± 16 mmol/l (120 min).

Serum growth hormone concentrations. Meal intake suppressed serum growth hormone concentrations in a similar way in all four studies ($P = \text{NS}$). In the insulin withdrawal study, the intravenous insulin bolus (90 min) was followed by a rapid increase in serum growth hormone from 1.4 ± 0.4 to 15.4 ± 5.8 $\mu\text{IU/ml}$ (150 min) (Fig. 2).

Plasma glucagon concentrations. No significant differences emerged in plasma glucagon concentrations in the prandial insulin, basal insulin, and control studies. In the insulin withdrawal study, plasma glucagon concentrations were higher ($P < 0.001$) than in the other studies from 30 to 150 min (Fig. 2).

Plasma ghrelin concentrations. Basal plasma ghrelin concentrations were not different in the four studies (Fig. 1). Meal intake reduced plasma ghrelin concentrations by $32 \pm 4\%$ in normal control subjects, $57 \pm 3\%$ in diabetic patients during the prandial insulin study ($P < 0.002$ vs. control subjects), and $38 \pm 8\%$ during the basal insulin study ($P < 0.002$ vs. hyperinsulinemia; $P = \text{NS}$ vs. control subjects). In the prandial insulin study, plasma ghrelin concentrations remained suppressed from the 135th min onward compared with that of control subjects ($P < 0.002$). During insulin withdrawal, meal intake did not change plasma ghrelin values ($P < 0.001$ vs. other studies), which, unlike the other studies, showed a trend to increase (0 min: 388 ± 52 , 90 min: 410 ± 58 pg/ml). The intravenous insulin bolus, given at 90 min, significantly reduced plasma ghrelin concentrations (150 min: 343 ± 54 pg/ml, $P < 0.001$).

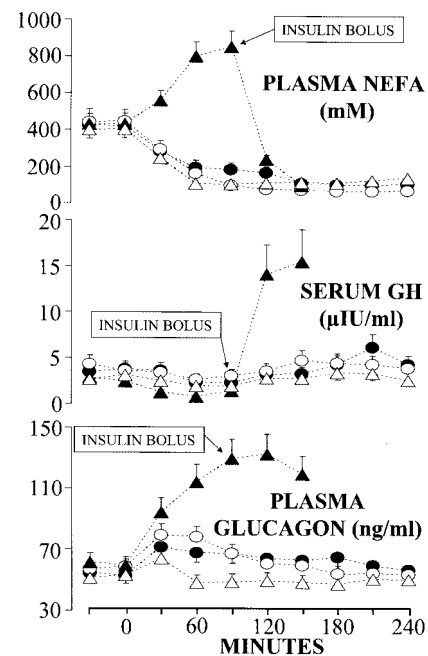


FIG. 2. Effects of meal intake on circulating concentrations of NEFA, growth hormone, and glucagon in six normal subjects and in six diabetic subjects during the prandial insulin, basal insulin, and insulin withdrawal studies. GH, growth hormone. Δ , Normal subjects; \blacktriangle , diabetic subjects with no insulin; \circ , diabetic subjects with prandial insulin; \bullet , diabetic subjects with basal insulin.

Correlations. In all subjects studied (type 1 diabetic and normal subjects), there was an inverse correlation ($r = -0.76$, $P < 0.001$) between serum insulin and plasma ghrelin concentrations. Multiple regression analysis showed plasma NEFA concentrations were best able to predict plasma ghrelin concentration by accounting for 89% of ghrelin variation ($P < 0.001$).

DISCUSSION

The present study, which measures plasma ghrelin concentrations for the first time in type 1 diabetic subjects, shows insulin is a decisive signal to ensure postprandial plasma ghrelin suppression in humans. Changes in serum insulin levels within the physiological range are associated with reciprocal changes in plasma ghrelin concentrations. Prandial insulinization (basal intravenous insulin plus subcutaneous prandial insulin dose) suppresses plasma ghrelin concentration more than in normal subjects (57 vs. 31%, respectively, $P < 0.002$), whereas partial insulinization (basal intravenous insulin) is sufficient for plasma ghrelin suppression (37%, $P = \text{NS}$ vs. normal control subjects and $P = 0.0016$ vs. hyperinsulinemia). In contrast, absolute insulin deficiency prevented prandial plasma ghrelin suppression until the insulin deficiency was corrected with an intravenous insulin bolus at 90 min.

Inverse patterns of plasma ghrelin and insulin concentrations have been described in a 24-h observation period in normal subjects (6), and fasting insulin and plasma ghrelin concentrations are correlated negatively in lean and obese individuals (16). Furthermore, hyperinsulinemia during either euglycemia (10–14) or hyperglycemia (9) is reported to suppress circulating ghrelin concentrations by 15–50%. Concurring with the above studies (6,9–16), our results demonstrate that physiologic increases in insulin

levels play a key role in regulating postprandial plasma ghrelin concentrations.

We also found an inverse correlation ($r = -0.76$, $P < 0.001$) between serum insulin and plasma ghrelin concentrations in both our diabetic and normal control subjects. Increases and decreases in serum insulin were closely associated with reciprocal changes in plasma ghrelin concentrations. In normal subjects, the rapid postprandial peak of serum insulin resulted in a more rapid decrease in plasma ghrelin concentrations than in the hyper- and hypoinsulinemic studies in type 1 diabetic subjects, and in the last part of the study, the prolonged hyperinsulinemia in diabetic subjects was associated with a more sustained suppression of plasma ghrelin concentrations.

How insulin affects plasma ghrelin concentrations remains to be established. Potentially, insulin might inhibit ghrelin synthesis or secretion by the X/A-like cells of the gastric mucosa either directly or indirectly. Studies in rats showed insulin-induced hypoglycemia increases (not decreases) ghrelin mRNA levels in the gastric fundus (17), thereby providing no evidence in support of a direct inhibitory action by insulin. However, in healthy humans, plasma ghrelin concentrations are decreased during insulin-induced hypoglycemia (11), suggesting species-specific differences between rodents and humans. As X/A-like ghrelin-producing cells are closely associated with the capillary network of the lamina propria of gastric mucosa, their function might be under endocrine control (18). However, at present we have not found any evidence for insulin receptors on ghrelin-producing cells in literature.

Indirect pathways for insulin inhibition of ghrelin synthesis or secretion include activation of hypothalamic insulin receptors (19) and modulation of the cellular flux of glucose and/or NEFAs. The latter hypothesis is supported by the very close relationship between insulin sensitivity to glucose and ghrelin suppression in healthy humans (11). The results of our basal and prandial insulin studies, demonstrating that plasma ghrelin was affected by different insulin concentrations in the presence of similar plasma glucose concentrations, suggest a potential additional role of nutrients and insulin in reducing circulating plasma ghrelin levels. In fact, it must be considered that peripheral glucose uptake was higher during the prandial insulin study, as shown by the glucose infusion required to match plasma glucose values with the basal insulin study. Thus, greater insulin-induced glucose uptake by X/A-like cells might have inhibited ghrelin synthesis and/or secretion. Whether NEFA flux is a signal of insulin action on ghrelin synthesis or secretion is still unclear. In our study, multiple regression analysis showed that the plasma NEFA concentration was the strongest independent variable and was able to predict 89% of variations in plasma ghrelin levels. However, it is difficult to determine whether this is simply because lipolysis and ghrelin-producing cells express a similar sensitivity to insulin or whether NEFAs have a direct role in regulating ghrelin secretion. The exquisite sensitivity to insulin of circulating NEFAs and ghrelin is demonstrated by the fact that basal insulinization (basal insulin study) was sufficient to suppress both parameters to the values of normal subjects. Möhlig et al. (13) examined the effects of increases in serum NEFA

concentrations (lipid emulsion infused into four healthy subjects) during a euglycemic-hyperinsulinemic clamp on plasma ghrelin concentrations, but as the infusion did not enhance the inhibitory effect of hyperinsulinemia on plasma ghrelin concentration, they concluded that NEFAs do not appear to play a direct role in modulating plasma ghrelin concentrations. In conclusion, we suggest that a minimal amount of insulin is essential for meal-related plasma ghrelin reduction. As ghrelin suppression in the basal insulin study was like that of normal control subjects and was greater in the prandial insulin study, one can speculate that ghrelin synthesis/secretion is very sensitive to insulin action. However, our data do not rule out a role for nutrients in the regulation of prandial ghrelin secretion. Postprandial hyperglycemia in type 1 diabetes may have enhanced the suppressive effect of insulin on plasma ghrelin concentrations during both the basal and prandial insulin studies. Thus whether, in the presence of the permissive effect of basal insulin concentrations, increased uptake of glucose reduces ghrelin synthesis and/or secretion by X/A-like cells remains to be established, bearing in mind that when insulin is absent, oral or intravenous nutrients do not affect plasma ghrelin concentrations.

During meal intake, the same pattern of reduction in serum growth hormone concentrations was observed in normal subjects and in the three studies in type 1 diabetic subjects. The results of the insulin withdrawal study originally show that meal-induced suppression of serum growth hormone is not mediated by ghrelin suppression because ghrelin concentrations remained at basal levels. Our data corroborate assertions that NEFAs are potent inhibitors of growth hormone release (19,20). In fact, when insulin (at 90 min) was given as a bolus, there was a rapid drop in NEFAs and a concomitant rise in serum growth hormone to well above basal levels (Fig. 2). Finally, lipolysis is also confirmed as highly sensitive to insulin-induced nutrient disposal because prandial plasma NEFA suppression was not different among the diabetic (basal and prandial insulin studies) and normal subjects.

The role played by insulin in postprandial regulation of plasma ghrelin concentrations may have a physiologic and clinical impact in modulating the feeling of hunger and consequent food intake. Ghrelin has a potent orexigenic effect that seems to be mediated, at least in part, through activation of NPY/Agouti gene-related protein neurons in the hypothalamic arcuate nucleus (3–5,21,22). Indeed, the fall in plasma ghrelin concentration could well limit the intake of additional food (23). If this is true, impaired suppression of plasma ghrelin during meal intake in obese humans (24) might contribute and possibly account for increased food intake in obesity. The data in the present study demonstrate that insulin has a key role regulating postprandial ghrelin suppression and suggest a link between insulin resistance and increased food intake in obesity that requires additional study. On the other hand, since lack of insulin prevents postprandial ghrelin suppression, we hypothesize that hyperphagia, which is common in severe insulin-deficient diabetic subjects, might be the result of loss of the physiologic regulation of ghrelin secretion. This hypothesis is supported by recent data in rat studies (25,26).

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