Ghrelin Is Not Necessary for Adequate Hormonal Counterregulation of Insulin-Induced Hypoglycemia

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Ghrelin, a 28–amino acid hormone, was recently identified in the stomach as the endogenous ligand for the growth hormone (GH) secretagogue receptor (1). Ghrelin is a potent stimulator of GH secretion (2); promotes ACTH, cortisol (2,3), and epinephrine (3) release; increases food intake, possibly by augmenting hypothalamic mRNA levels of neuropeptide Y and agouti gene-related protein (4); and increases plasma glucose in normal subjects (5). Recently, it was shown in rats and agouti gene-related protein (4); and increases plasma glucose; and increases food intake by inducing the feeling of hunger. These characteristics make ghrelin a potential counterregulatory hormone. At present, it is not known whether ghrelin increases in response to insulin-induced hypoglycemia. To answer this question, we compared plasma ghrelin concentrations after a short-term insulin infusion that was allowed or not (euglycemic clamp) to cause hypoglycemia (2.7 ± 0.2 mmol/l at 30 min) in five healthy volunteers. In both studies, plasma ghrelin concentrations decreased (P < 0.01) after insulin infusion (hypoglycemia by 14%, euglycemia by 22%), reached a nadir at 30 min, and returned to baseline at 60 min, without differences between the hypoglycemia and the euglycemia studies. Glucagon, cortisol, and GH increased in response to hypoglycemia despite the decreased ghrelin. There was a strong correlation (R² = 0.91, P < 0.002) between the insulin sensitivity of the subjects and the percentage suppression of ghrelin from baseline. These data demonstrate that ghrelin is not required for the hormonal defenses against insulin-induced hypoglycemia and that insulin can suppress ghrelin levels in healthy humans. These results raise the possibility that postprandial hyperinsulinemia is responsible for the reduction of plasma ghrelin that occurs during meal intake. Diabetes 51:2911–2914, 2002

RESEARCH DESIGN AND METHODS

Study protocol. The study was conducted according to the principles of the Helsinki Declaration, and five of the authors (P.L., G.M., A.D.C., N.P., and P.D.F.), who, naturally, knew in detail the procedure of the study, volunteered to participate. All subjects (three women and two men) were in good health as determined by medical history, physical examination, and routine laboratory evaluation. They ranged in age from 23 to 46 years (31 ± 4, mean ± SE), in body weight from 53 to 85 kg (62 ± 6), and in BMI from 18 to 25 kg/m² (21 ± 1).

All subjects were studied at random on two different occasions (hypoglycemia or euglycemia) at 4- to 7-day intervals. A week before and until the completion of the entire study, the volunteers consumed a diet of 35 kcal · kg⁻¹·day⁻¹ containing 55% carbohydrate, 30% fat, and 15% protein. They were admitted to the Clinical Research Center of our Department at ~0730 h, after fasting overnight. At ~0800 h, a 22-gauge plastic cathether needle was placed in an antecubital vein for the infusions of saline (0.5 ml/min; Vial Medical pump, Grenoble, France), regular human insulin (Humulin R; Eli-Lilly, Indianapolis, IN) and, in the euglycemic study, a variable 20% dextrose solution with 20 mEq of KCl by two Harvard syringe pumps (Harvard Apparatus, Ealing, South Natick, MA). A contralateral hand vein was cannulated in a retrograde manner.

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GH, growth hormone.
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 (14). In both studies, at ~000 h (0 min), the infusion of regular human insulin (rate 160 mU · m⁻² · min⁻¹) was started and continued for 10 min. In the hypoglycemic study, plasma glucose concentration was allowed to fall spontaneously. In the euglycemic study, plasma glucose was kept at the basal concentration by a variable 20% dextrose infusion, adjusted on the basis of immediate measurements of plasma glucose, sampled every 5 min. Twelve milliliters of blood was collected at ~15, 0, 15, 30, 45, 60, 90, and 120 min to measure the circulating concentrations of insulin, glucagon, ghrelin, GH, and cortisol.

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%. The plasma concentrations of glucose were immediately determined using a Beckman glucose analyzer (Beckman Instruments, Palo Alto, CA). The serum concentrations of insulin (Technogenetics, Milan, Italy) and GH (Biodynamics, Ares Serono, Norwell, MA) and the plasma concentrations of glucagon (ICN Pharmaceuticals, Diagnostic Division, Orangeburg, NY) were measured using commercial immunoradiometric assays. The serum concentrations of cortisol were determined by enhanced chemiluminescence using kits of Ortho-Clinical Diagnostics (Johnson & Johnson, New Brunswick, NJ).

Statistical analyses. Data in text are given as mean ± SE and were considered to be significantly different at P < 0.05. All data were subjected to repeated measures ANOVA followed by Tukey’s test to pinpoint specific differences on interaction means. Regression analyses were conducted according to the least squares method.

RESULTS
Plasma glucose and glucose infusion rate. In the hypoglycemic study, plasma glucose concentration reached a nadir at 30 min (2.7 ± 0.2 mmol/l) and returned to the basal values at 50 min (4.5 ± 0.2 mmol/l). In the euglycemic study, the glucose infusion rate required to clamp plasma glucose reached a peak at 30 min (0.50 ± 0.07 mmol · kg body wt⁻¹ · min⁻¹). The exogenous glucose infusion clamped plasma glucose concentrations at values not statistically different from the baseline (mean between −15 and 0 min; Fig. 1).

Circulating hormone concentrations. There were no differences in serum insulin concentrations (peaks at 15 min) between the euglycemic and the hypoglycemic studies (P = NS; Figs. 1 and 2).

In the euglycemic study, after insulin infusion, plasma ghrelin concentrations (pmol/l) declined, reaching the nadir at 30 min (209 ± 41 vs. basal 268 ± 49; P < 0.01), and returned to values not statistically different from basal at 60 min (225 ± 41). Similarly, in the hypoglycemic study, plasma ghrelin concentrations declined, reaching the nadir at 30 min (198 ± 40 vs. basal 230 ± 40; P = 0.03), and returned to values not statistically different from basal at 60 min (220 ± 46). There were no differences (NS) between plasma ghrelin concentrations of the euglycemic and hypoglycemic studies before and after the insulin infusion. There was a strong linear correlation (r² = 0.91, P < 0.002) between the insulin sensitivity of the subjects (measured as the average amount of glucose required to maintain basal plasma glucose concentrations during the first 30 min of the euglycemic clamp) and the percentage suppression from baseline of ghrelin at nadir (30 min; Fig. 2).

In the hypoglycemic study, the circulating concentrations of the counterregulatory hormones, glucagon, GH, and cortisol, increased (P < 0.05) at 15 (glucagon) or 45 (GH, cortisol) min; glucagon reached a peak at 30 min, GH reached a peak at 45 min, and cortisol reached a peak at 60 min. Glucagon returned to values not different from basal at 90 min, and cortisol and GH returned to values not different from basal at 120 min.

DISCUSSION
Our results demonstrate for the first time that circulating ghrelin concentration is reduced by insulin-induced hypoglycemia in healthy humans and that even though ghrelin levels decreased, glucagon, cortisol, and GH increased appropriately in response to hypoglycemia (15). The de-
crease of plasma ghrelin is induced by hyperinsulinemia and not by the plasma glucose decrease, because plasma ghrelin was similarly suppressed during the euglycemic study. The inhibition of ghrelin levels was strictly related to the insulin sensitivity of the volunteers.

The results of this study reject the hypothesis that ghrelin is necessary to activate the mechanisms of defense against insulin-induced hypoglycemia. To our knowledge, this is the first time that circulating ghrelin concentrations have been measured during insulin-induced hypoglycemia. Our results are apparently in contrast with those reported by Toshinai et al. (13). These authors demonstrated that the expression of ghrelin mRNA is upregulated in gastric mucosa cells of rats in response to insulin-induced hypoglycemia (13). The different results might be species related, or, alternatively, hyperinsulinemia might inhibit the secretion but not the synthesis of ghrelin by oxyntic mucosa cells. Additional studies are required to answer this question.

That plasma ghrelin level is decreased and not increased in response to insulin-induced hypoglycemia rules out that during insulin-induced hypoglycemia ghrelin contributes to the stimulation of hepatic glucose production (5,12), the suppression of endogenous insulin secretion (5), or the initiation of the feeling of hunger (8,16) through its orexigenic activity (8,9,17,18). Furthermore, our results indicate that the responses of glucagon, GH, and cortisol to insulin-induced hypoglycemia are not mediated by ghrelin. Cortisol and GH do not play a role in defense against acute hypoglycemia (15,19) but are important counterregulatory hormones in prolonged hypoglycemia because they increase hepatic glucose production and decrease peripheral glucose utilization (10,11,15). It has been clearly demonstrated that ghrelin strongly stimulates GH secretion in animals and in humans (1,2,8,16) and that the hormone also increases ACTH and, consequently, cortisol secretion (2). However, the suppression of circulating ghrelin during insulin-induced hypoglycemia clearly indicates that the responses of GH, ACTH, and cortisol are triggered by mechanisms independent of ghrelin. This conclusion can be extended also to physical exercise, another classic stimulus of GH secretion. In fact, Kallio et al. (20) recently reported that the response of GH to physical exercise occurred in the absence of changes of plasma ghrelin concentrations. Our results demonstrate that three (glucagon, GH, and cortisol) of the four classical counterregulatory hormones increased appropriately in response to insulin-induced hypoglycemia, even though ghrelin levels decreased. Because epinephrine was not measured in our study, it is not possible to rule out that ghrelin might play a role in the epinephrine response to hypoglycemia.

According to the preliminary results of the literature, the key physiologic role of ghrelin should be to signal the need to conserve energy (9). Ghrelin concentrations increase before meals and are reduced by meal intake (6,7), with changes that occur in the opposite direction and are inversely related to those of insulin (6,7). On the basis of our data demonstrating that hyperinsulinemia reduces ghrelin levels, we hypothesize that insulin modulates the meal-related changes in plasma ghrelin concentrations. Thus, during meal absorption, postprandial hyperinsulinemia would reduce ghrelin secretion, whereas during fasting, the progressive decline in circulating insulin would favor ghrelin production. However, our hypothesis must be confirmed by specific studies because in our study the amount of insulin given to induce hypoglycemia resulted in circulating insulin concentrations higher than those that occur during meal absorption. It also remains to be established whether hyperinsulinemia reduces ghrelin concentrations directly or indirectly through changes of free fatty acid levels and/or of glucose disposal. The last possibility is supported by our demonstration of a strict inverse relationship between insulin sensitivity and plasma ghrelin concentrations (Fig. 2).

In conclusion, this study demonstrates that the novel hormone ghrelin is not required for the hormonal counterregulatory responses in defense against hypoglycemia but is inhibited by hyperinsulinemia. Our data, together with those of Brogli et al. (5), suggest the possibility of an inhibitory feedback between insulin and ghrelin in humans: insulin administration reduces ghrelin concentration (present results), whereas ghrelin administration reduces insulin secretion (5). Insulin-ghrelin negative feed-
back could explain the inverse relationship observed between circulating levels of the two hormones (6,21).

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REFERENCES