Insulin-Dependent Modulation of Plasma Ghrelin and Leptin Concentrations Is Less Pronounced in Type 2 Diabetic Patients

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The gastric peptide ghrelin augments and the adipocyte-derived hormone leptin reduces appetite and food intake. In the central nervous system, insulin directly decreases hunger sensation but could also act indirectly by modulating ghrelin and leptin secretion. This study examines dose-dependent effects of insulin on plasma ghrelin and leptin concentrations during hyperinsulinemic (1, 2, and 4 mU · kg^{-1} · min^{-1})-euglycemic clamp tests in six nondiabetic (control subjects) and six type 2 diabetic patients. Type 2 diabetic patients were studied before and after prolonged (12-h and 67-h) variable intravenous insulin treatment aiming at near-normoglycemia (115 ± 4 mg/dl). Nondiabetic subjects were also studied during saline infusion, which did not affect ghrelin but decreased leptin by 19 ± 6% ($P < 0.03$). In control subjects, plasma ghrelin decreased at all clamp steps (−17 ± 1, −27 ± 6, and −33 ± 4%, respectively; $P < 0.006$ vs. baseline), whereas leptin increased by 35 ± 11% ($P < 0.05$). In type 2 diabetic patients without insulin treatment, ghrelin decreased by 18 ± 7% ($P < 0.05$) only after 4 mU · kg^{-1} · min^{-1} insulin infusion and leptin increased by 19 ± 6% ($P < 0.05$). After prolonged insulin treatment and near-normoglycemia, ghrelin and leptin remained unchanged in type 2 diabetic patients during the clamps. In conclusion, insulin reduces plasma ghrelin in nondiabetic patients and, to a lesser extent, in type 2 diabetic patients before insulin therapy. These findings indicate an indirect effect of insulin via ghrelin on the suppression of hunger sensation and appetite.
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The peptides ghrelin and leptin circulate in blood and participate in energy homeostasis, feeding behavior, and regulation of body weight (1–3). Ghrelin is predominantly produced by the stomach, whereas leptin is primarily secreted by white adipose tissue (4–7). In some (6,8,9) but not all (10) human studies, fasting plasma ghrelin negatively correlated with BMI, which positively relates to plasma leptin concentrations (11–13).

Ghrelin and leptin exert antagonistic effects via their specific receptors in the central nervous system (CNS) and in peripheral tissues (14–18). In hepatocytes, ghrelin reduces and leptin augments insulin signal transduction, resulting in increased and decreased, respectively, glucose production (16,17). In pancreatic β-cells, insulin release was stimulated by ghrelin but inhibited by leptin administration (7,14,15,19).

Reduction of food intake by intracerebroventricular administration of leptin can be reversed by ghrelin co-administration in rats (18,20). Intracerebroventricular ghrelin stimulates food intake in rodents (5,18,20,21). In humans, intravenous ghrelin infusion augmented appetite, while subcutaneous leptin administration reduced hunger sensation (21,22). On the other hand, central insulin action can also decrease food intake because rodent models with disrupted or reduced intracerebral insulin receptors exhibit marked hyperphagia (23,24).

After meal ingestion, the combined increase in plasma glucose and insulin could account for the decrease in plasma ghrelin and the increase in plasma leptin in lean humans (25,26). Recent findings, however, suggest that this meal effect on ghrelin and leptin could rather be due to elevation in plasma insulin, because plasma ghrelin decreased during one-step hyperinsulinemic-euglycemic clamp tests in nondiabetic humans (27,28). In contrast to data on leptin (29,30), information concerning the dose-dependent action of insulin on plasma ghrelin is still lacking in humans. This study aimed to investigate the neuroendocrine regulation of appetite modulation by analyzing short-term changes and relationships of ghrelin and leptin in nondiabetic and diabetic patients with and without prolonged insulin therapy. This approach was chosen because both ghrelin and leptin could indirectly enhance insulin's central action on hunger sensation and appetite regulation (23,24,31,32).

Thus, we examined the dose-dependent effects of insulin during 5-h stepped hyperinsulinemic-euglycemic clamp tests on the plasma concentrations of ghrelin and leptin in *1*) type 2 diabetic patients before and after 12- and 67-h intravenous insulin infusion as well as *2*) healthy nondiabetic control subjects, who were studied again (*3*) under basal fasting conditions with saline infusion.

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AGRP, agouti-related protein; CNS, central nervous system; FFA, free fatty acid; NPY, neuropeptide Y; RIA, radioimmunoassay.

RESEARCH DESIGN AND METHODS

Study participants. Six (two women and four men) type 2 diabetic patients [aged 60 ± 2 years (range 56–68), weight 83 ± 4 kg, and BMI 29 ± 2 kg/m^2] were recruited and matched with six healthy nondiabetic subjects for sex (two women and four men), age [57 ± 2 years (range 51–62)], weight (78 ± 6 kg), and BMI (26 ± 1 kg/m^2) (each P = NS for type 2 diabetic vs. control subjects). Type 2 diabetic patients exhibited higher HbA_{1c} levels (8.6 ± 0.3 vs. 5.6 ± 0.1%, $P < 0.01$). Of note, type 2 diabetic patients did not display diabetic autonomous neuropathy or clinically diagnosed gastroparesis. In type 2 diabetic patients, any medication including oral hypoglycemic agents was discontinued for at least 3 days before the study. All participants gave informed consent to the protocol, which was approved by the institutional ethics board.

Study design. Hyperinsulinemic-euglycemic clamp tests were performed three times in type 2 diabetic patients (type 2 diabetic C1, C2, and C3 patients) and once in nondiabetic control subjects, who were studied again with normal saline infusion. The experimental protocol for the clamp studies, but not for the saline administration in nondiabetic humans, has been previously reported (33). Type 2 diabetic and control subjects were instructed to ingest an isocaloric, carbohydrate-rich diet from day −7 onwards. Type 2 diabetic patients were admitted to the hospital at least 1 day before the studies and given an isocaloric diet (25 kcal · day^{-1} · kg body wt^{-1}; 50% carbohydrate, 15% protein, and 35% fat) divided into five meals (at 7:30 A.M., 11:00 A.M., 12:30 P.M., 4:30 P.M., and 7:00 P.M.) until day 6. At the Metabolic Unit, type 2 diabetic patients underwent three hyperinsulinemic-euglycemic clamps (34) within the 6-day study period: *1*) on day 1 for analysis of baseline studies (C1), *2*) on day 3 after a 12-h overnight insulin infusion (C2), and *3*) on day 6 after a 67-h insulin infusion (C3). The 12-h overnight insulin infusion before clamp two (C2) was performed from 9:00 P.M. on day 2 until 9:00 A.M. on day 3 in type 2 diabetic patients. An intravenous insulin infusion (1 unit/ml Actrapid; Novo-Nordisk, Bagsvaerd, Denmark) at variable rates (mean 1.7 ± 0.2 units/h) was used to maintain plasma glucose at 113 ± 2 mg/dl. The 67-h insulin infusion before clamp three (C3) was performed from 2:00 P.M. on day 3 until 9:00 A.M. on day 6 in type 2 diabetic patients. An intravenous insulin infusion at variable rates (mean 2.5 ± 0.8 units/h) was again used to keep plasma glucose at 117 ± 6 mg/dl. Nondiabetic subjects were studied on two separate study days, once under the above described clamp conditions (control subjects) and again during saline infusion (saline-infused subjects) to compensate for the volume load associated with the clamp test.

Clamp protocols. All participants were studied after a 10-h fast. Two catheters (Vasofix; Braun, Melsungen, Germany) were inserted into the antecubital vein of the left and right arm for blood sampling and infusions, respectively. Three-stepped hyperinsulinemic-euglycemic clamps (34) were performed between 9:00 A.M. and 2:00 P.M. with primed-continuous infusion of insulin (Actrapid) at rates of 1.0, 2.0, and 4.0 mU · kg^{-1} · min^{-1} for 100 min each, respectively. In type 2 diabetic patients, the insulin infusion decreased plasma glucose down to 100 mg/dl (Fig. 1*A*). From then on, plasma glucose was maintained at ~100 mg/dl in type 2 diabetic patients by infusing variable rates of a 20% glucose solution throughout the clamp studies (Fig. 1*A*). In nondiabetic patients, plasma glucose remained at 98 ± 3 and 96 ± 3 mg/dl in control subjects and those receiving saline infusion, respectively (Fig. 1*A*). On all clamp study days in type 2 diabetic patients, the first and second meal was omitted to compensate for the amount of infused glucose on these days.

Sampling conditions. To obtain EDTA plasma, which served as matrix for the measurements of plasma insulin, leptin, and ghrelin, blood was filled into tubes containing potassium EDTA (50 μl liquid/ml blood) as anticoagulant. For determination of plasma free fatty acids (FFAs), blood was filled in other tubes with potassium EDTA (50 μl liquid/ml blood), to which the lipolysis inhibitor orlistat was added immediately (35). Thereafter, all plasma samples were kept on ice for ~10 min, centrifuged (10 min, 5,000 rpm, 4°C), and the supernatant plasma immediately separated for further storage at –20°C. Serum as another matrix for ghrelin measurement was allowed to clot for ~1 h at room temperature and subsequently centrifuged (10 min, 5,000 rpm, 4°C) in order to separate the supernatant serum, which was stored at –20°C.

Plasma/serum metabolites and hormones. Plasma glucose concentrations were measured using the glucose oxidase method (Glucose Analyzer II; Beckman, Fullerton, CA). Plasma concentrations of immunoreactive insulin (Pharmacia, Uppsala, Sweden) and leptin (Human Leptin RIA kit; Linco Research, St. Charles, MO) were measured by commercially available radioimmunoassays (RIAs) (12,33). Plasma FFAs were measured with a microfluorimetric assay (Wako Chem, Richmond, VA) (33). Plasma and serum ghrelin concentrations were determined by a recently described in house RIA (10). For generation of ghrelin antiserum, a synthetic COOH-terminal fragment of ghrelin (amino acids 15–28, 14Tyr; Biotrend, Köln, Germany) was coupled to hemocyanin and administered to rabbits. The serum obtained from the animals was diluted by 1:1,200. By using the iodogen technique, the tracer was

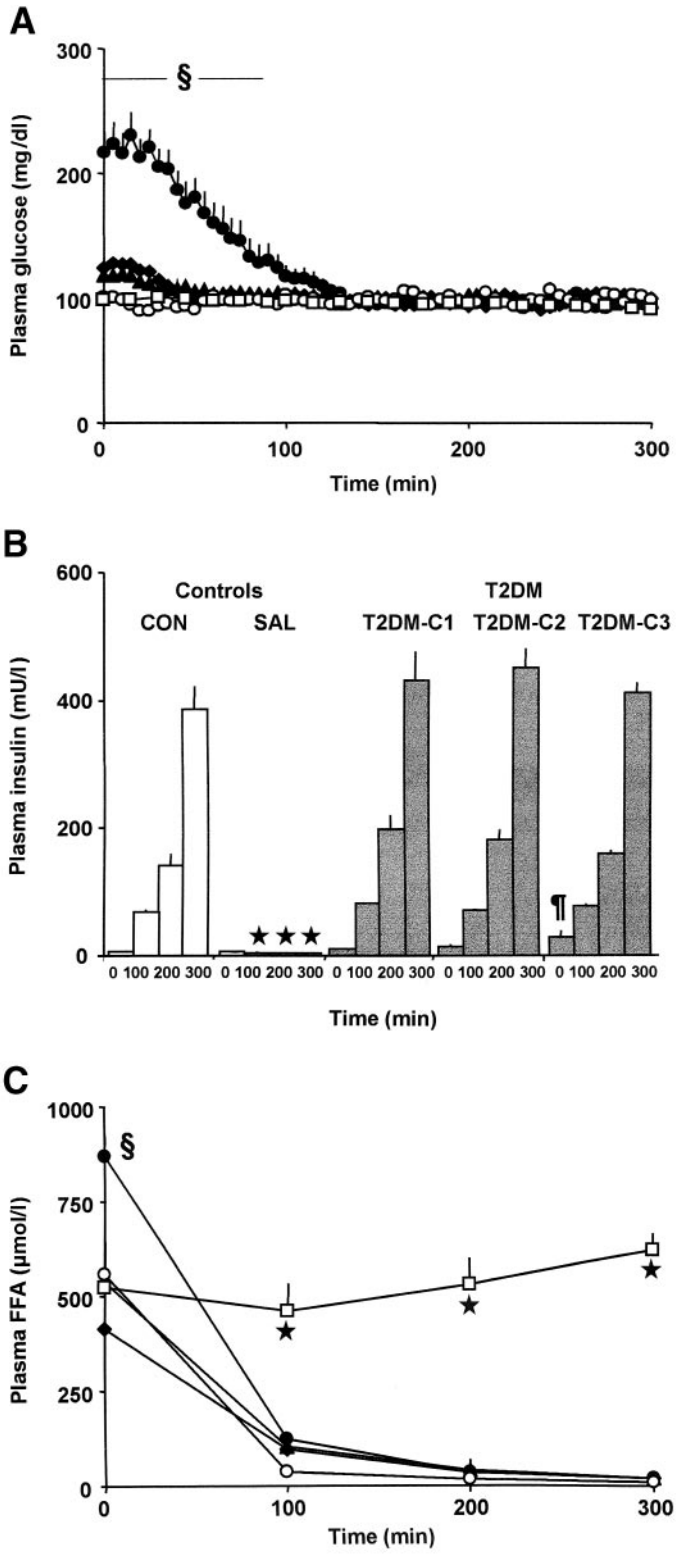

FIG. 1. Plasma glucose (*A*), insulin (*B*), and FFA (*C*) concentrations (means ± SE) during saline infusion in healthy control subjects (SAL: $n = 6$, □) and during stepped (1, 2, and 4 mU insulin · kg^{-1} · min^{-1}, each for 100 min) euglycemic-hyperinsulinemic clamps in nondiabetic control subjects ($n = 6$, ○) and type 2 diabetic (T2DM) patients ($n = 6$) before (C1, ●) and after 12 h (C2, ◆) and 67 h (C3, ▲) of variable insulin infusion. §$P < 0.05$ for C1 vs. all other groups; ⋆$P < 0.0001$ SAL vs. all other groups; ¶$P < 0.05$ C3 vs. C1 and control subjects (ANOVA with Bonferroni post hoc test). Individual mean values of type 2 diabetic patients and control subjects were presented in part previously (33).

TABLE 1
Fasting concentrations of plasma ghrelin, leptin, and insulin and ratios to BMI in nondiabetic volunteers during saline infusion ($n = 6$) and under euglycemic-hyperinsulinemic clamp tests (control subjects, $n = 6$) as well as in type 2 diabetic patients ($n = 6$) before clamp 1 (C1), clamp 2 (C2), and clamp 3 (C3)

	Saline-infused subjects	Control subjects	Type 2 diabetic patients		
			C1	C2	C3
Ghrelin (pmol/l)	231 ± 55	242 ± 53	211 ± 14	203 ± 24	219 ± 30
Ghrelin per BMI (pmol · l^{-1} · kg^{-1} · m^2)	9.7 ± 2.4	8.7 ± 2.4	7.3 ± 0.5	7.0 ± 0.8	7.5 ± 0.8
Leptin (μg/l)	5.6 ± 0.8	5.6 ± 0.9	8.3 ± 1.5	9.7 ± 1.5	10.1 ± 1.7*
Leptin per BMI (μg · l^{-1} · kg^{-1} · m^2)	0.22 ± 0.03	0.23 ± 0.04	0.28 ± 0.05	0.33 ± 0.05	0.35 ± 0.05*
Insulin (mU/l)	6 ± 1	7 ± 1	9 ± 1	16 ± 2	25 ± 6†

Data are means ± SE. *$P < 0.03$ vs. C1 (paired t test), †$P < 0.05$ vs. C1 and control subjects (ANOVA with Bonferroni post hoc test).

purified on a C18 high-performance liquid chromatography column. Antibody-bound fraction and free peptide were separated on 2% dextran-charcoal. A solution of full-length ghrelin (Bachem, Heidelberg, Germany), which remained stable at −20°C, was appropriately diluted to obtain a standard curve, which strongly correlated with comparable dilutions of circulating ghrelin samples. The ghrelin antibodies exhibited no cross-reactivity with leptin or related peptides, such as motilin and growth hormone–releasing hormone. The detection limit was 34 pmol/l, and the inter- and intra-assay CVs were 4.1 and 2.6%, respectively. Of note, this RIA measures total circulating ghrelin including both acylated and des-acyl ghrelin (2).

Calculations and statistical analyses. All data are given as means ± SE. M values (in mg glucose · kg^{-1} · min^{-1}) as a measure of insulin-mediated glucose disposal were calculated as described (33,34). Ratios of hormones to the BMI were calculated as reported (12). Intra-individual comparisons within each group were analyzed with the two-tailed paired Student's t test or ANOVA for repeated measures with post hoc Bonferroni test. Comparisons between two different groups were done with the two-tailed unpaired Student's t test. Comparisons of more than two variables were calculated with ANOVA following post hoc Bonferroni test. Linear correlations are Pearson-product moment correlations. Differences were considered statistically significant at $P < 0.05$. All statistical analyses were performed using Microsoft Excel 2000 or SPSS 10.0.7 for Windows (SPSS, Chicago, IL; http://www.spss.com/).

RESULTS

Plasma glucose, insulin, and FFAs. Before the start of the first clamp in the diabetic patients (C1), plasma glucose was approximately two times higher ($P < 0.05$) than after 12 and 67 h of insulin infusion as well as the respective concentrations of the control groups (Fig. 1*A*). Plasma glucose decreased in C1 within 90 min and from then on remained comparable with that in type 2 diabetic patients after insulin therapy and that in the control group.

Immediately before clamp 3, the type 2 diabetic patients exhibited higher plasma insulin concentrations (25 ± 6 mU/l; $P < 0.05$ vs. C1, control, and saline-infused subjects) due to the use of insulin infusion (Fig. 1*B* and Table 1). In all clamp studies, plasma insulin increased similarly to ~70, ~170, and ~400 mU/l at infusion rates of 1, 2, and 4 mU · kg^{-1} · min^{-1} insulin, respectively (Fig. 1*B*). In saline-infused subjects, plasma insulin decreased by 29 ± 5, 36 ± 6, and 37 ± 9% after 100, 200, and 300 min of saline infusion, respectively (each $P < 0.004$ vs. baseline). Plasma insulin concentrations increased from baseline until the end of the clamps by 72 ± 12-fold in control subjects and by 55 ± 5-fold in the C1 patients (P = NS for control vs. type 2 diabetic C1 subjects), while they increased by only 33 ± 3-fold and 19 ± 4-fold in type 2 diabetic C2 and C3 patients, respectively ($P < 0.05$ type 2 diabetic C1 vs. C2 and C3 patients; $P < 0.0001$ for control vs. type 2 diabetic C2 and C3 subjects).

Plasma FFAs were higher at time zero in type 2 diabetic before insulin administration ($P < 0.04$ vs. type 2 diabetic C2 and C3 and control and saline-infused subjects) but similarly decreased in type 2 diabetic C1, C2, and C3 and control subjects during the clamp tests, as reported in part (33) (Fig. 1*C*). In contrast, plasma FFAs continuously increased by 20% during saline infusion ($P < 0.002$ vs. baseline in type 2 diabetic patients and control subjects).

Plasma and serum ghrelin. At baseline, plasma ghrelin concentrations were not different between groups (Table 1). Fasting serum ghrelin concentrations (control subjects 324 ± 79 pmol/l and saline-infused subjects 364 ± 79 pmol/l) were ~47% higher ($P < 0.0001$) than the corresponding plasma concentrations and strongly correlated with each other ($r = 0.974$, $P < 10^{-16}$). While plasma ghrelin remained unchanged during saline administration, insulin infusion of 1, 2, and 4 mU · kg^{-1} · min^{-1} reduced plasma ghrelin in control subjects from baseline by 17 ± 1, 27 ± 6, and 33 ± 4%, respectively (each $P < 0.006$ vs. baseline) (Fig. 2*A*). When comparing control subjects with saline-infused subjects, plasma ghrelin was lower by 30 ± 11, 36 ± 1, and 44 ± 9% during the clamp test than during saline administration (each $P < 0.05$ for control vs. saline-infused subjects) (Fig. 2*A*). In type 2 diabetic patients without previous insulin treatment (C1), only high-dose insulin infusion (4 mU · kg^{-1} · min^{-1}) resulted in a significant decline in plasma ghrelin (−18 ± 7%, $P < 0.05$ vs. baseline), whereas after 12- and 67-h insulin therapy (C2 and C3) plasma ghrelin was not significantly changed during the clamps.

Plasma leptin. At baseline, plasma leptin and the leptin-to-BMI ratio were similar in control, saline-infused subjects, and type 2 diabetic C1 and C2 patients, but ~20% higher ($P < 0.03$ vs. C1) in the patients after 67 h insulin treatment (C3) (Table 1). During saline infusion, plasma leptin decreased slightly (−19 ± 6%, $P < 0.03$ vs. baseline) but increased during the clamp tests in healthy subjects (+35 ± 11%, $P < 0.05$ vs. baseline) (Fig. 2*B*). In type 2 diabetic patients before insulin treatment, plasma leptin concentrations were higher (+19 ± 6%, $P < 0.05$ vs. basal) at the end of the clamp. However, after both 12 and 67 h insulin treatment in type 2 diabetic patients, plasma leptin concentrations did not differ at the end of the clamps when compared with the respective baseline values.

Correlation analyses

Ghrelin and insulin. When correlating data of all clamp steps, the individual percent changes in plasma ghrelin at 100, 200, and 300 min were negatively associated with the simultaneous plasma insulin concentrations in the nondiabetic control subjects ($r = -0.597$, $P < 0.009$) (Fig. 3*A*) but not in type 2 diabetic subjects, resulting in a concen-

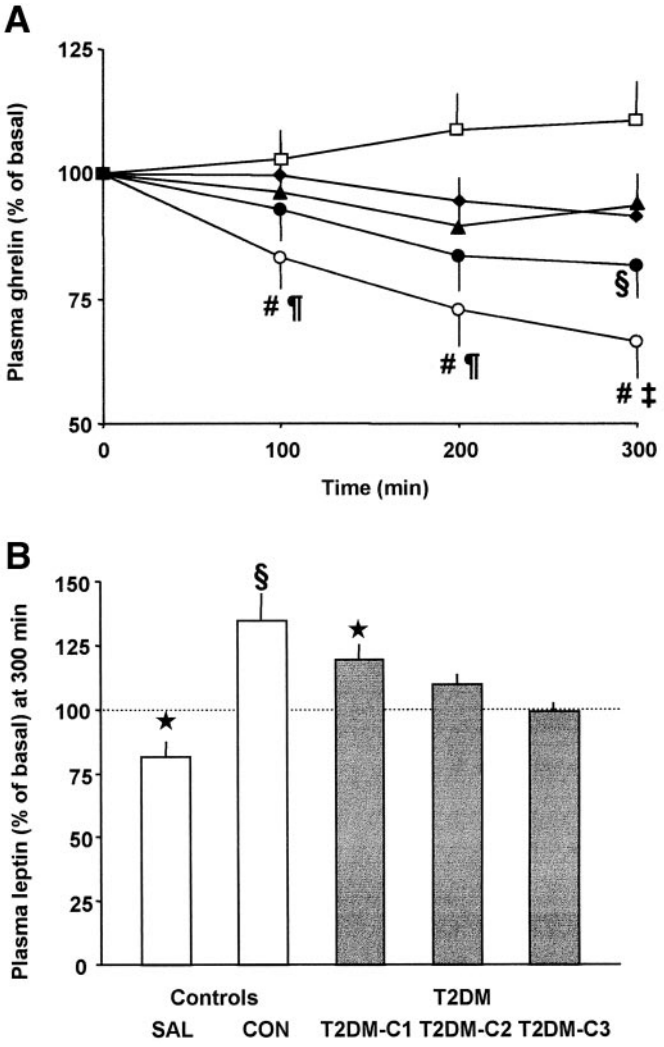

FIG. 2. ***A*****: Time course of plasma ghrelin (means ± SE, % of basal values) during saline infusion (SAL, □) or three-stepped (1, 2, and 4 mU insulin · kg^{-1} · min^{-1}) clamps in control subjects (CON, ○), type 2 diabetic (T2DM) C1 (●), C2 (◆), and C3 (▲) subjects. #$P < 0.006$, §$P < 0.05$ vs. basal (paired t test); ¶$P < 0.05$, ‡$P < 0.002$ for control versus SAL subjects (paired t test).** ***B*****: Plasma leptin (means ± SE, % of basal values) at the end of the 4 mU insulin · kg^{-1} · min^{-1} clamp step in SAL, control, C1, C2, and C3 subjects. ⋆$P < 0.02$, §$P < 0.05$ vs. basal (paired t test).**

tration-dependent reduction of plasma ghrelin by insulin. The correlation of the plasma ghrelin changes with clamp insulin infusion rates in control subjects ($r = -0.575$, $P < 0.02$) reflects a dose-dependent effect by insulin on plasma ghrelin decrease (individual data not shown).

Ghrelin and* M *values. In all participants, the percent change in plasma ghrelin from baseline at 300 min was negatively associated with the M values during the intervals from 280 to 300 min ($r = -0.531$, $P < 0.008$) (Fig. 3*B*) of the clamp tests. In control and type 2 diabetic C1 subjects combined, the plasma ghrelin-to-BMI ratios strongly correlated with M values during the 1 mU · kg^{-1} · min^{-1} clamp step (0- to 20-min interval $r = 0.906$, $P < 0.001$; 20–40 min $r = 0.856$, $P < 0.001$; 40–60 min $r = 0.752$, $P < 0.005$; 60–80 min $r = 0.717$, $P < 0.009$; and 80–100 min $r = 0.711$, $P < 0.009$) (individual data not shown).

Ghrelin and FFAs. In the nondiabetic subjects combined (control and saline-infused subjects), fasting ghrelin concentrations ($r = 0.641$, $P < 0.04$) (Fig. 3*C*) were positively related to fasting plasma FFA concentrations.

Ghrelin and leptin. Fasting concentrations of plasma

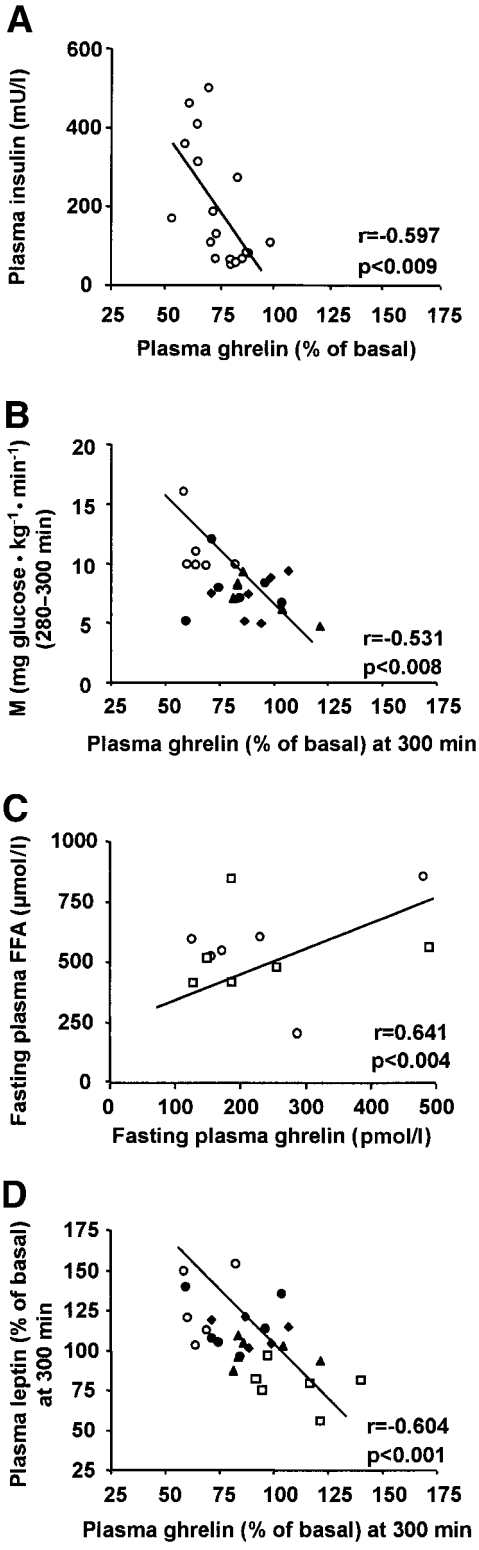

FIG. 3. Correlation analyses (Pearson's method) of plasma ghrelin with plasma insulin during the clamp (*A*) (○, control subjects: $r = -0.597$, $P < 0.009$), M **values (*B*) ($r = -0.531$, $P < 0.008$; ○, control subjects; ●, type 2 diabetic C1; ◆, type 2 diabetic C2; ▲, type 2 diabetic C3), fasting plasma FFA (*C*), and plasma leptin (*D*) (all participants: $r = -0.604$, $P < 0.001$; ○, control subjects; □, SAL; ●, type 2 diabetic C1; ◆, type 2 diabetic C2; ▲, type 2 diabetic C3) at 300 min.**

leptin ($r = 0.466$, $P < 0.02$) but not ghrelin ($r = -0.083$, P = NS) were related to the BMI of all participants (individual data not shown). At the end of each protocol (300 min), percent changes in plasma ghrelin inversely correlated with those in plasma leptin ($r = -0.604$, $P < 0.001$) (Fig. 3*D*).

Ghrelin and age. In the nondiabetic subjects (control subjects), fasting concentrations of plasma ghrelin tended to relate negatively to age ($r = -0.799$, $P = 0.057$) (individual data not shown).

DISCUSSION

The present study found that insulin decreases plasma ghrelin dose-dependently and stimulates leptin secretion in healthy humans. In type 2 diabetic patients without previous insulin treatment, supraphysiological insulin concentrations are required to significantly decrease plasma ghrelin. After 12 and 67 h insulin therapy in type 2 diabetic patients, insulin does not affect plasma ghrelin and leptin during the clamps. The changes in plasma ghrelin during the clamps are negatively related to whole-body insulin sensitivity. Finally, changes in plasma ghrelin negatively correlate with those in plasma leptin at the end of the hyperinsulinemic clamps.

Plasma ghrelin. In nondiabetic humans, plasma ghrelin decreased during the hyperinsulinemic clamps, whereas plasma ghrelin remained unchanged during saline administration. It is of note that somatostatin was not infused during any of these examinations, because somatostatin inhibits ghrelin secretion (36). As plasma insulin was markedly higher in control than in saline-infused subjects and the degree of glycemia was comparable, the reduction in plasma ghrelin is most likely due to hyperinsulinemia. From this it appears that selective hyperinsulinemia is sufficient to decrease plasma ghrelin, which is in contrast to findings that hyperglycemic-hyperinsulinemic conditions after meal ingestion are required for ghrelin reduction (37).

Two other studies in healthy volunteers have examined insulin's effects on plasma ghrelin by performing short-term one-step hyperinsulinemic clamps (27,28). Despite the use of different insulin doses [40 and 160 mU $\cdot$ m^{-2} $\cdot$ min^{-1} corresponding to 1 and 4 mU $\cdot$ kg^{-1} $\cdot$ min^{-1}, respectively (34)], plasma ghrelin decreased similarly by between 20 and 30% from baseline values in these studies (27,28). These data would suggest the absence of a dose-dependent effect of insulin on plasma ghrelin modulation. In contrast, we found that insulin dose-dependently decreased plasma ghrelin in healthy subjects after three 100-min clamp steps. Thus, it appears that insulin could be one of the regulators of ghrelin secretion.

In contrast to nondiabetic subjects, the type 2 diabetic patients before insulin treatment did not exhibit such a decline in plasma ghrelin at the first clamp step during which insulin concentrations (~70 mU/l) were comparable with those in the postprandial state. In these patients, plasma ghrelin markedly decreased at supraphysiological insulin concentrations (~400 mU/l) only. After prolonged insulin treatment, plasma ghrelin did not decrease during any of the three clamp steps. Because plasma ghrelin strongly stimulates appetite in humans and rodents (5,18,20), the failure of insulin to decrease plasma ghrelin after prolonged insulin therapy could contribute to the well-known side effect of weight gain during long-term insulin treatment (38).

The explanation for the failure of insulin to decrease plasma ghrelin after prolonged insulin therapy could result from decreased efficacy of insulin to suppress ghrelin secretion or from the observed differences in plasma insulin between baseline and clamp conditions. In type 2 diabetic patients without insulin treatment, plasma insulin was ~50-fold higher at the end than before the clamp tests, while in type 2 diabetic patients after prolonged insulin therapy plasma insulin only increased by up to 33-fold until the end of the clamp because of the higher prevailing insulin concentrations at baseline. From this it appears that the failure of high-dose insulin after prolonged insulin therapy in plasma ghrelin decrease could be due to the lower difference in plasma insulin between baseline and clamp conditions.

As the diabetic patients did not receive a saline infusion, a suppressive effect of insulin on plasma ghrelin compared with saline cannot be completely ruled out for the type 2 diabetic patients during the lower insulin infusion rate. Of note, plasma ghrelin in type 2 diabetic patients was not significantly different from that in control subjects during saline infusion.

The mechanism by which insulin might influence ghrelin release is unclear. The insulin-signaling cascade has been identified in the gastrointestinal tract, which can therefore be regarded as an insulin-sensitive tissue (39,40). Therefore, the insulin-mediated decrease in plasma ghrelin could result from inhibition by insulin of ghrelin release in the stomach cells. Alternatively, insulin not only reduced plasma ghrelin during the clamp tests, but also plasma FFAs, whereas during saline infusion both ghrelin and FFAs did not decrease. Thus, it cannot be ruled out that the decline in ghrelin was in part due to the insulin-induced decrease in plasma FFAs in the nondiabetic subjects.

Serum ghrelin. In most previous studies, ghrelin was measured in plasma only (1,3,9,21,25,27,28,37,41). However, serum was also, but very rarely, used as a matrix for ghrelin measurement (10,42). We measured both plasma and serum ghrelin concentrations in the nondiabetic subjects to allow for comparison of the matched sample matrices. Although dilution with the anticoagulant (potassium EDTA) would only account for a reduction of plasma ghrelin by 5–10%, ghrelin was found to be 47% higher in serum than in plasma. Our observations are in line with a recent study reporting 25% higher serum ghrelin concentrations, which were also strongly related to plasma ghrelin (43). Interestingly, concentrations of other proteins, such as troponin T and I are also higher in serum than in plasma (44). It is conceivable that the observed differences between plasma and serum might therefore be due to the assay system as recently suggested (43,44).

Plasma leptin. In the nondiabetic subjects, plasma leptin increased after the hyperinsulinemic clamp but decreased during saline infusion. This is in line with a previous report showing that plasma leptin is dose-dependently increased under comparable clamp conditions (1, 2, and 5 mU $\cdot$ kg^{-1} $\cdot$ min^{-1} insulin) and also declined by ~20% after saline administration (29). In the present study, the increase in leptin during the clamp tests was, however, less pro-

nounced in type 2 diabetic patients without insulin therapy and, interestingly, not present after they had been treated with insulin.

Fasting plasma leptin was reported to be higher or lower in type 2 diabetic patients (12,45). When comparing the nondiabetic with the type 2 diabetic subjects before insulin therapy, fasting plasma leptin (either in absolute concentration or when expressed as leptin-to-BMI ratio) was not statistically different (12). However, leptin was higher after insulin therapy in type 2 diabetic patients, which confirms that long-term insulin infusion is required to increase fasting leptin concentrations as previously reported for healthy humans (30).

The direct relationship between fasting plasma leptin and BMI is in agreement with several other studies (12,45,46), while some (6,8,9,41) but not all (10) authors described a negative association between fasting plasma ghrelin and BMI. In the present study, fasting plasma ghrelin was not related to BMI.

Of note, differences in body composition and particularly high BMI have been reported to regulate plasma ghrelin concentrations. Morbidly obese humans with a mean BMI of 43 kg/m^2 exhibited 60% lower fasting ghrelin concentrations but no suppression of ghrelin after ingestion of a test meal in contrast to lean subjects (25). Our type 2 diabetic patients tended to have higher BMI values (29 vs. 26 kg/m^2, NS) than nondiabetic control subjects but presented with similar fasting plasma ghrelin concentrations. Nevertheless, in a more overweight control group exhibiting BMI values identical to that of the type 2 diabetic group, the insulin-mediated suppression of plasma ghrelin might have been less pronounced due to an impact of body composition or BMI on ghrelin regulation (25).

Interestingly, the ratio of fasting plasma ghrelin to BMI was closely associated with insulin-mediated glucose disposal at physiological insulin concentrations (1 mU $\cdot$ kg^{-1} $\cdot$ min^{-1} clamp). In addition, in the nondiabetic subjects combined, fasting plasma ghrelin positively related to fasting plasma FFAs. Furthermore, the negative correlation between fasting ghrelin and age in nondiabetic subjects exhibited borderline significance and is in agreement with a recent study reporting an age-related decline of fasting plasma ghrelin concentrations (41). This suggests that factors other than obesity, such as FFA availability, insulin sensitivity, or age, predict plasma ghrelin (10).

Counteracting actions of ghrelin and leptin. The observation that short-term insulin administration during the clamp tests decreases plasma ghrelin and elevates plasma leptin explains the inverse correlation between insulin-mediated changes in plasma ghrelin and leptin. Such a negative relationship has been reported for fasting plasma concentrations of ghrelin and leptin in insulin-sensitive and insulin-resistant obesity-prone humans (8). This might be because the fasting state with low insulin concentrations serves to augment ghrelin secretion from the stomach and simultaneously to diminish leptin release from adipocytes (1).

Central effects. Both circulating ghrelin and leptin cross the blood-brain barrier into the CNS by specific carrier systems (11,47). In the CNS, ghrelin induces appetite by increasing expression of neuropeptide Y (NPY), a potent stimulator of hunger sensation, and subsequently agouti-related protein (AGRP), while leptin inhibits the synthesis of NPY and AGRP (18,20,48,49). Likewise, insulin also reduces NPY expression in the CNS as demonstrated by intracerebroventricular insulin infusion in diabetic rats (32). Thus, the marked increase of food intake termed as "diabetic hyperphagia" was reduced by 50% by insulin administration in the CNS of these rodents, indicating that central insulin deficiency contributed to, but was not solely responsible for, the hyperphagia in diabetes (31,32). Thus, hunger sensation underlies additive hormonal regulation other than that from insulin, in which ghrelin and leptin could play a relevant role (31).

Whole-body insulin sensitivity. During the clamp tests, percent change in plasma ghrelin is negatively related to insulin-stimulated glucose disposal in both type 2 diabetic and control subjects, indicating that the relative suppression of plasma ghrelin by insulin is directly associated with whole-body insulin sensitivity. In this context it is of note that postprandial insulin concentrations suppressed circulating ghrelin in lean, but not in obese, nondiabetic humans (25). Resistance to insulin action as seen in type 2 diabetes and obesity is a common characteristic of the metabolic syndrome. Insulin resistance might not only relate to glucose and lipid metabolism in muscle, liver, and fat (33,50), but also affect the suppression of ghrelin release in the postprandial state. It appears from our findings that impaired suppression of ghrelin by insulin might be another feature of the metabolic syndrome, which could contribute to increasing food intake and/or weight gain.

In conclusion, insulin reduces plasma ghrelin in healthy humans and, to a lesser extent, in type 2 diabetic patients before insulin therapy. This insulin-mediated effect depends on individual whole-body insulin sensitivity and is therefore less pronounced in the insulin-resistant type 2 diabetic patients. Moreover, intravenous insulin treatment of type 2 diabetes abolishes insulin's regulation of plasma ghrelin and leptin. These findings indicate an indirect effect of insulin via ghrelin and leptin on the suppression of hunger sensation and appetite.

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