Metabolomic linkage reveals functional interaction between glucose-dependent insulinotropic polypeptide and ghrelin in humans

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Rudovich NN, Nikiforova VJ, Otto B, Pivovarova O, Gögebakan Ö, Erban A, Möhlig M, Weickert MO, Spranger J, Tschöp MH, Willmitzer L, Nauck M, Pfeiffer AF. Metabolomic linkage reveals functional interaction between glucose-dependent insulinotropic polypeptide and ghrelin in humans. Am J Physiol Endocrinol Metab 301: E608–E617, 2011. First published May 17, 2011; doi:10.1152/ajpendo.00154.2011. —The gastric peptide ghrelin promotes energy storage, appetite, and food intake. Nutrient intake strongly suppresses circulating ghrelin via molecular mechanisms possibly involving insulin and gastrointestinal hormones. On the basis of the growing evidence that glucose-dependent insulinotropic polypeptide (GIP) is involved in the control of fuel metabolism, we hypothesized that GIP and/or insulin, directly or via changes in plasma metabolites, might affect circulating ghrelin. Fourteen obese subjects were infused with GIP (2.0 pmol·kg⁻¹·h⁻¹) or placebo in the fasting state during either euglycemic hyperinsulinemic (EC) or hyperglycemic hyperinsulinemic clamps (HC). Apart from analysis of plasma ghrelin and insulin levels, GC-TOF/MS analysis was applied to create a hormone-metabolite network for each experiment. The GIP and insulin effects on circulating ghrelin were analyzed within the framework of those networks. In the HC, ghrelin levels decreased to a similar extent whether GIP or placebo was infused, whereas in the EC, ghrelin levels decreased by 19.2% vs. baseline, P = 0.018. Ghrelin levels were significantly lower during HC with GIP than with placebo, despite insulin levels not differing significantly. In the GIP network combining data on GIP, insulin and other metabolites, ghrelin was integrated into a hormone-metabolite network with a connection to a group of long-chain fatty acids. In contrast, ghrelin was excluded from the networks of experiments without GIP. GIP decreased circulating ghrelin and might have affected the ghrelin system via modification of long-chain fatty acid pools. These observations were independent of insulin and offer potential mechanistic underpinnings for the involvement of GIP in systemic control of energy metabolism.

Obesity; euglycemic hyperinsulinemic clamp; hyperglycemic hyperinsulinemic clamp; time-of-flight gas chromatography-mass spectrometry analysis

Energy homeostasis and food intake behavior are regulated by a complex system of endocrine, neuronal, and nutrient signals including an increasingly well-understood communication channel called the brain-gut axis (42). Ghrelin, an acylated gut peptide that is primarily produced by endocrine cells of the gastric mucosa (22), stimulates food intake and promotes adiposity (43). Feeding suppresses ghrelin production and fasting stimulates ghrelin release (43, 44). The exact molecular basis for controlling these processes is still not entirely understood. Parenteral glucose and/or insulin infusions clearly suppress ghrelin levels when administered for prolonged periods or at supraphysiological doses in animals and in humans (2, 14, 26, 31, 40, 47). Physiological doses of glucose and/or insulin that mimic postprandial fluctuations, however, do not seem to affect circulating ghrelin in humans (7, 24, 41). In contrast, enteral nutrients consistently suppress ghrelin levels even at low doses (2, 14, 24, 40). Moreover, selective gastric distension, chemosensation, or nutrient exposure are insufficient to induce a ghrelin response in animals (6), whereas exposure of the small intestine to nutrients (48) or glucose (9, 36) is sufficient for suppression of circulating ghrelin. Thus, it is likely that nutrient-related ghrelin suppression is based on nutrient sensing or uptake distal to the stomach (35).

Recent data indicate that the orexigenic effect of ghrelin might also be influenced by other gastrointestinal peptides such as cholecystokinin (CCK), bombesin, peptide YY3–36 (PYY3–36), and glucagon-like peptide-1 (GLP-1), suggesting relevant cross-talk among peripheral orexigenic and anorexigenic signals in the control of appetite and body weight (49).

The incretin glucose-dependent insulinotropic polypeptide (GIP), another nutrient-dependent factor secreted by intestinal enteroendocrine cells, has recently received considerable attention as a potential drug target and endogenous regulator of energy metabolism (15, 21, 50). A potentially important interaction between GIP and ghrelin was suggested from initial experiments with isolated rat stomachs, which suggested a possible direct influence of GIP on ghrelin secretion (27). In addition, descriptive association studies indicate that postprandial ghrelin concentrations are inversely correlated with postprandial GIP concentrations in healthy subjects (6). These and other data suggest GIP as an intriguing candidate for the molecular intermediary controlling postprandial ghrelin secretion: 1) GIP is secreted distal to the stomach from intestinal K cells in response to nutrient ingestion and acts to augment...
insulin secretion in the pancreas (15); 2) the stimulation of insulin secretion by GIP occurs only in the presence of elevated glucose levels (15); and 3) GIP receptors are presented in gastric mucosa (39, 45) and are downregulated in central obesity (39).

In our previously reported pilot study in healthy nonobese individuals (38), we were unable to demonstrate downregulation of ghrelin secretion by GIP, possibly explained by short-time GIP infusion and pharmacological concentrations of both glucose and insulin levels during the hyperglycemic clamp. Moreover, that study design was unable to dissect potentially independent effects of GIP from insulin on ghrelin secretion. Thus, GIP-ghrelin interactions represent an understudied but potentially important area of research (1, 8, 27, 38).

Given that GIP, like insulin, is involved in the regulation of carbohydrate and fat metabolism (15, 21, 50), indirectly synergistic and/or combinatorial actions of GIP and/or insulin via changes in the plasma metabolome may influence ghrelin secretion. Moreover, controversial results from different techniques of glucose and/or insulin administration leading to influences on intermediate metabolites, and ultimately their impact on ghrelin secretion (17, 18), may reflect the underestimated multiplicity of their effects. Hormone-to-hormone interactions frequently demonstrate complex patterns of combinatorial or and synergistic effects in a dense network of molecular information exchange (5). We therefore applied metabolomic systems analysis to search for novel endocrine and metabolite signal patterns influencing ghrelin regulation.

The objective of this study was to investigate the functional relationship between GIP and ghrelin, either dependently or independently of circulating blood glucose and insulin concentrations. To address this issue, we employed a GIP infusion test and euglycemic and hyperglycemic glucose clamp experiments combined with infusion of GIP or placebo in a crossover manner in moderately overweight male subjects. Moreover, we applied network correlation analysis of metabolic profiles to study the generated data for molecular links reflecting GIP-ghrelin interactions.

**RESEARCH DESIGN AND METHODS**

**Subjects**

This study was approved by the Ethics Commission of Brandenburg, Germany [registration no. AS 2(a)/2005]. All individuals gave written informed consent prior to the study. This study was preregistered at www.clinicaltrials.gov (NCT00774488).

Fourteen overweight male volunteers were studied (age 47.4 ± 8.4 yr, BMI 32.5 ± 2.2 kg/m², waist-to-hip ratio 1.0 ± 0.1, fat weight 31.6 ± 5.4 kg, lean weight 67.5 ± 3.2 kg, fasting blood glucose 4.9 ± 0.4 mmol/l). Subjects with elevations in liver enzymes more than twice the respective upper normal limits or with elevated serum creatinine concentrations (>1.3 mg/dl) were excluded. None of the volunteers showed evidence of metabolic disease or glucose intolerance in a 75-g oral glucose loading (11), and all reported stable body weight over at least 12 mo. None of the participants was engaged in any type of relevant exercise program. All participants, except for one subject, were nonsmokers. Subjects were instructed to maintain their normal physical activity and to consume a normal diet containing ~200 g of carbohydrate for 3 days before and 14 days during the study.

**Study Design**

A complete physical examination, an oral glucose tolerance test (OGTT) with 75 g of glucose (Roche Diagnostics, Mannheim, Germany) after an overnight fast and laboratory evaluation was performed on each patient during the screening visit. On the same day, the subject's body fat was determined using BOD POD (Life Measurement, Concord, CA) according to the manufacturer's instructions. If the subjects met the inclusion criteria (see below), they were recruited for participation in two or four of the following procedures in the single blind, cross-over, randomized design (Fig. 1): 1) infusion tests with placebo (0.9% NaCl) or GIP infusion (n = 7); 2) euglycemic hyperinsulinemic clamps at a steady-state capillary plasma glucose concentration of 4.4 mmol/l combined with placebo or GIP-infusion (n = 9); 3) hyperglycemic hyperinsulinemic clamps at a steady-state capillary plasma glucose concentration of 7.8 mmol/l combined with placebo and GIP-infusion (n = 8). Intravenous insulin infusion and diazoxide were given in combination to suppress endogenous insulin release in response to GIP under hyperglycemic clamp conditions and to reach comparable circulating insulin concentrations in the clamps with placebo and GIP infusion. Between examination days, an intermission time of at least 7 days was maintained.

![Fig. 1. Design of the study. Fourteen moderately obese subjects were studied on 4 occasions and exposed each time to 1 of 6 different conditions: infused with glucagon-like peptide-1 (GIP, 2.0 pmol·kg⁻¹·min⁻¹) or placebo, either in the fasting state, during euglycemic hyperinsulinemic clamps (EC; blood glucose concentration 4.4 mmol/l) or during hyperglycemic hyperinsulinemic clamps (HC; blood glucose concentration 7.8 mmol/l).](http://ajpendo.physiology.org/)
**Peptides**

Synthetic GIP was purchased from PolyPeptide Laboratories (Wolfenbüttel, Germany) and processed for intravenous infusion as described (30). In all subjects, GIP from the same batch (F-0818X2) was used.

**Experimental Procedures**

All studies were done in the morning in the overnight fasted state (>10 h after last meal). Two forearm veins were punctured with cannulae and kept patent using 0.9% saline (for blood sampling and for glucose, insulin, and peptide administrations, respectively). Both ear lobes were made hyperemic using Finalgon (Nonivamid 4 mg/g, Nicoboxil 25 mg/g). For all clamps, human insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) and ghrelin (Serag Wiessner, Nails, Germany) were used. In the experiments, placebo (0.9% NaCl, Fresenius, Germany) or GIP at an infusion rate of 2.0 pmol/kg⁻¹·min⁻¹ were given as a continued intravenous infusion for 240 min. All tests were conducted for 240 min. In all tests venous blood samples were drawn at −70, 0, 60, 120, 180, 210, 220, 230, and 240 min relative to the start of the infusions.

In the euglycemic hyperinsulinemic clamp experiments (16), a continuous intravenous infusion of insulin combined with saline or GIP infusion was started at 0 min of the test. In the steady-state condition of the clamp, capillary blood glucose was adjusted to 4.4 mmol/l for at least 80 min. Throughout the clamp, plasma ghrelin concentrations were monitored every 5 min and used to regulate plasma glucose by the adjustment of a variable infusion of glucose. A deviation of a single capillary glucose concentration of more than 10% during assumed steady-state conditions was defined as nonsteady state.

The hyperinsulinemic hyperglycemic clamp experiments aiming at a steady-state capillary plasma glucose concentration of 7.8 mmol/l were started by injecting 20% glucose as a bolus (bolus glucose = body weight (kg) × 0.3 g glucose) at 0 min and was maintained by infusing glucose (20% in water), as appropriate, based on glucose determinations performed every 5 min. Exogenous hyperinsulinemia was achieved by continuous infusion of 40 mU·m⁻²·min⁻¹ human insulin for 240 min to obtain comparable circulating insulin concentrations, in the clamps with both placebo and GIP infusion. For the suppression of the endogenous insulin secretion, 150 mg of diazoxide (Proglycem, Schering, Germany) was given twice (−60 min and 120 min) during the experiments. Diazoxide is a K₅ᵥ ATP channel opener that suppresses insulin secretion by hyperpolarizing β-cells (13). Somatostatin, which is generally used for suppression of endogenous insulin production, inhibits secretion of ghrelin by itself (18) and would have interfered with our experiments.

**Analytic Procedures**

All venous blood samples were immediately centrifuged and frozen at −70°C until analyzed. Capillary blood glucose concentrations were determined using a glucose oxidase method on a Dr. Müller glucose analyzer (Freital, Germany). Serum insulin and C-peptide were measured using a commercial enzyme-linked immunosorbent assay (In-sulin ELISA, C-Peptide ELISA, Mercodia, Uppsala, Sweden). Human plasma ghrelin was measured with a commercial radioimmunoassay (Phoenix Pharmaceuticals, Belmont, CA) that uses 125I-labeled bioactive ghrelin as a tracer molecule and a polyclonal antibody raised in rabbits against full-length octanoylated human ghrelin [intra-assay coefficient of variation (CV) 4%]. No cross-reactivity with human secretin, human vasoactive intestinal peptide, human galanin, human GHRH, NPY, or other relevant molecules has been reported (34). Concentrations of total GIP were determined in plasma samples containing aprotinin (Trasylol, Bayer-Germany, to inhibit proteases) by using a human GIP (Total) ELISA Kit (Linco Research). Sensitivity was 8.2 pg/ml, intra-assay CV was 3.0–8.8%, and interassay CV was 1.8–6.1%.

For metabolite profiling, six replicates for each plasma sample were measured using a time-of-flight gas chromatography-mass spectrometry (GC-TOF/MS) technique. The applied protocol allowed analytics on both polar and apolar metabolites. Prior to the analysis, an extraction procedure for metabolites in human blood plasma was developed on the basis of preliminary tests made elsewhere (20). For the extraction, plasma samples were quickly frozen and aliquoted, and 400 μl of absolute methanol prechilled to −20°C containing [13C6]sorbitol as an internal standard was added to an aliquoted plasma sample, followed by vortexing, extraction for 2 h on ice, centrifugation at maximal speed, and collecting the supernatant, which was further dried by vacuum centrifugation. Chemical derivatization, GC-MS analysis, and initial data processing were performed as described previously (28), using the following instruments: gas chromatograph 6890N, split/splitless injector with electronic pressure control up to 150 psi (Agilent Technologies, Wilmington, DE); MDN-35 capillary column, 30 m length, 0.32-mm inner diameter, 0.25 μm film thickness (Macherey-Nagel); Pegasus III time-of-flight mass spectrometer (LECO, Saint Joseph, MI). For raw data processing, an automated mass spectral deconvolution and identification system (AMDIS; National Institute of Standards and Technology, Gaithersburg, MD), and ChromaTOF chromatography processing and mass spectral deconvolution software (LECO) were used. Peak annotation was manually performed using preconstructions mass spectral and retention time index libraries from GC-TOF/MS metabolite profiles (12), and the TagFinder visualizations of time groups and clusters (minimum three correlating fragments as unique masses for quantification) (29). The resulting data consisted of intensity values for each referenced compound and measurement, respectively, normalized using an internal standard ([13C6]sorbitol).

**Calculations and Statistical Network Analyses**

All statistical analyses were performed using SPSS for Windows 16.0 (SPSS, Chicago, IL). Data are presented as means ± SE. An average of the two consecutive baseline samples was calculated (−70 and 0 min) to reduce individual variation in basal levels. Steady-state concentrations of hormones were calculated as a mean of 210-, 220-, 230-, and 240-min values. Because of the skewed distribution of the hormone parameters under study, nonparametric tests were used. Between-group comparisons were performed using the Mann-Whitney U-test and within groups comparison with Wilcoxon’s signed rank test. Differences were considered statistically significant at P < 0.05.

Correlation network analysis of the blood plasma metabolome was performed as described in detail previously (32). In brief, first, the metabolite profiles and hormone analyses were combined in dataset I, which consisted of three hormones (ghrelin, insulin, and C-peptide) and 821 chemical compounds, or “analytes”, all containing relative concentration levels at two experimental points (−70 and 240 min of experiments). Then, from dataset I, the “unchanged analytes” that showed no significant changes during the experiments by comparison of −70 min and 240 min values in the paired t-test with P < 0.05, were removed. Dataset II encased 337 analytes and three hormones. In the next step, the redundant analytes were excluded and the final dataset III for network reconstruction contained data on 133 analytes and three hormones. The Pearson correlation coefficient (r) and the mutual information distance matrix of dataset III were calculated. From this dataset, two correlation networks were built: the GIP network, combining data on GIP infusion, euglycemic and hyperglycemic clamp tests, and the control NaCl network on the data from the three corresponding placebo tests in which GIP was replaced with NaCl. For further refining of ghrelin integration into information exchange with the plasma metabolome, we disaggregated the data into six sets corresponding to a particular test (3 with GIP and 3 with
NaCl) and built six corresponding correlation networks on these sets. In the resulting networks, ghrelin connectivity was specifically analyzed. Correlation network visualizations were generated using the Pajek software for network analysis (http://vlado.fmf.uni-lj.si/pub/networks/pajek/).

RESULTS

**GIP Administration Decreases Circulating Ghrelin Independently Of Plasma Insulin Levels**

*OGTT.* Circulating total ghrelin levels reached nadir at 90 min after the oral glucose load and decreased over time by 22.3 ± 12.1% from baseline (112.0 ± 29.8 pg/ml at baseline vs. 83.4 ± 25.7 pg/ml at 120 min, P = 0.002). Fasting and postloading ghrelin levels were not correlated with insulin or blood glucose levels during the test (data not shown).

**GIP and NaCl infusion tests.** In both tests, circulating insulin levels remained unchanged and blood glucose levels were reduced. However, there were no differences between the tests in the blood glucose concentrations (NaCl infusion: 4.9 ± 0.1 vs. 4.5 ± 0.3 mmol/l at 240 min, P = 0.028; GIP infusion: 4.9 ± 0.3 to 4.5 ± 0.3 mmol/l, P = 0.043, respectively). The GIP plasma concentration during infusion was 140.03 ± 41.32 pmol/ml and significantly higher than saline infusion (4.71 ± 2.11 pmol/l, P < 0.001). Circulating ghrelin levels remained unchanged during NaCl and GIP infusion (76.5 ± 37.3 vs. 89.4 ± 49.5 pg/ml at 240 min, P = 0.13; 84.9 ± 36.4 vs. 77.3 ± 35.1 pg/ml at 240 min, P = 0.89, respectively; Fig. 2).

**Hyperinsulinemic euglycemic clamp tests.** In these experiments, steady-state ghrelin concentrations and serum insulin concentrations were not different between tests with GIP and NaCl infusion (serum insulin: 398.4 ± 88.2 vs. 379.8 ± 75.0 pmol/l, respectively, P = 0.26). The GIP plasma concentration during infusion was 123.13 ± 12.48 pmol/ml and significantly higher than saline infusion (5.07 ± 1.02 pmol/l, P < 0.001). In the presence of GIP infusions, circulating ghrelin levels decreased over time by 14.2 ± 2.4% of baseline, from a mean of 85.2 ± 28.8 pg/ml to 77.1 ± 20.3 pg/ml during the steady-state of the clamp (P = 0.008) and was significantly different from that in its absence (P = 0.028; Fig. 2). In the clamp tests with NaCl infusion, only a trend to decrease in ghrelin levels in the steady state were observed (82.0 ± 25.6 vs. 77.0 ± 23.7 pg/ml, 7.3 ± 3.9% of baseline, P = 0.059, NS). In the euglycaemic hyperinsulmineic clamp with GIP, ghrelin levels decreased significantly compared with baseline already after 60 min of the start of GIP-infusion (77.7 ± 27.1 pg/ml, P = 0.006 vs. baseline) and remained suppressed during the entire clamp until 240 min. The variability of circulating ghrelin was 31.1% throughout the euglycemic hyperinsulinemic clamps.

**Hyperglycemic hyperinsulineic clamp tests.** In the hyperglycemic hyperinsulineic clamp tests with and without GIP, neither glucose nor insulin levels during steady state of the clamps differed significantly (serum insulin: 1,121 ± 475 and 754 ± 322 pmol/l, P = 0.093, respectively). The GIP plasma concentration during infusion was 130.42 ± 13.71 pmol/ml and significantly higher compared with saline infusion (3.01 ± 0.54 pmol/l, P < 0.001). In the hyperglycemic hyperinsulinemic clamp experiments, circulating ghrelin levels at 0 min, i.e., 60 min after oral administration of diazoxide, were not different from baseline (~70 min) levels of ghrelin (data not shown). Ghrelin levels decreased from a mean of 93.8 ± 38.4 pg/ml to 73.0 ± 25.1 pg/ml during the steady state of the clamps with NaCl infusion (19.2 ± 4.7% of baseline, P = 0.028). In the clamp experiments with GIP infusion, ghrelin decreased from a mean of 100.5 ± 50.3 pg/ml to 60.6 ± 21.3 pg/ml (33.8 ± 5.6% of baseline, P = 0.018) at steady state, which was significantly greater compared with clamp experiments with NaCl infusion (P = 0.018; Fig. 2). In the hyperglycemic hyperinsulinemic clamp with GIP, ghrelin levels decreased significantly compared with baseline after 60 min of the start of GIP infusion (67.8 ± 25.6 pg/ml, P = 0.025 vs. baseline) and remained suppressed during the entire clamp until 240 min. In contrast, a significant decrease in ghrelin levels was observed primarily at 120 min of the hyperglycemic hyperinsulinemic clamp without GIP (78.6 ± 27.8 pg/ml, P = 0.024 vs. baseline). The variability of circulating ghrelin was 37.1% throughout the euglycemic hyperinsulinemic clamps.

**Ghrelin in the Hormone-Metabolite Correlation Networks**

Molecular profiling of plasma metabolites using mass spectrometry platforms obtained a plethora of metabolite changes as a result of GIP administration in the differential experimental conditions. To prioritize the novel endocrine and metabolite
signal patterns influencing ghrelin regulation, the correlations matrix comprising all of the measured metabolites and hormones in plasma was calculated using all three experimental settings with GIP and NaCl infusions. Although insulin and glucose concentrations were manipulated during clamp experiments, we included these in the correlations matrix by reason of additional information of metabolic interactions between ghrelin, insulin, and glucose in conditions of increased circulating GIP concentrations. There were 20 plasma metabolites that were found to be significantly correlated with ghrelin (Table 1). Nine of these plasma metabolites were plasma fatty acids, all of which were found to be positively correlated to ghrelin in the experiments with GIP. The strongest positive correlation was observed with two amino acids, valine and threonine, in the hyperglycemic hyperinsulinemic clamp with GIP. Moreover, we included in Table 1 the link to a detailed description of each of these metabolites in the human metabolome database (http://www.metabolomics.ca).

Comparative network analysis of ghrelin interactions with the plasma metabolome during experiments with either GIP or NaCl infusions revealed alternative correlation networks highlighting a substantial difference. Ghrelin involvement in the information exchange with the plasma metabolome was integrated into the summarized GIP network through a connection via octadecanoic (directly) and other fatty acids (Fig. 3) but stayed completely dis-integrated from the NaCl network (zero connections, data not shown). Regarding the other network integrators, glucose and insulin keep informational exchange with ghrelin through inverse correlation to a group of amino acids including valine, leucine and threonine at the first order (directly connected to glucose and insulin) and further connection to glutamic acid, serine, tyrosine, and phenylalanine. The last two amino acids also keep the integration with the glucose/insulin part of the network through connection to galactonic acid and unidentified metabolite 2165. Refined network analysis of ghrelin connectivity in GIP infusion, euglycemic hyperinsulinemic clamps with GIP (EC+GIP), and hyperglycemic hyperinsulinemic clamps (HC+GIP) networks and in the corresponding control NaCl networks was performed to estimate the strength of ghrelin integration into the informational exchange at a particular test condition (Fig. 4). The biggest number of ghrelin connections in the GIP-HC network reflects its strongest integration into the molecular information exchange at this condition, and disintegration from the control HC+NaCl network points to an integrating role of GIP for ghrelin involvement into the information exchange in the hyperglycemic clamp test. The second strongest network integration of ghrelin was be seen in the NaCl infusion during the fasting state. Both EC networks, EC+GIP and EC+NaCl, kept ghrelin dis-integrated from the molecular information exchange, which was reflected in the small connectivity with ghrelin observed in these networks.

**DISCUSSION**

Here, we present novel data showing that GIP suppressed circulating ghrelin independently of insulin effects in the hyperglycemic clamp. This was supported by the observed pronounced involvement of ghrelin in the hormone-metabolite network via coordinated changes in the pool of long-chain fatty acids. In hyperinsulinemic euglycemia, the effect of GIP on circulating ghrelin was small but still statistically significant, suggesting that the suppression of ghrelin secretion requires hyperglycemic conditions, similar to the insulinotropic action of GIP. In contrast, GIP had no effect on circulating ghrelin levels under fasting conditions.

The ability of GIP to suppress ghrelin secretion was surprising, as it indicates an indirect suppressive effect of GIP on appetite and food intake behavior. It was hypothesized 13 years ago that GIP could be involved in appetite regulation (25), GIP, but not GLP-1, increased rapidly after intraduodenal glucose infusion, and reductions in ratings of hunger as well as in energy intake and increased fullness were observed. Interestingly, coinfusion of octreotide suppressed the secretion of incretins and led to reversal of hunger sensations (25). Later studies with similar designs (9, 36) showed a dose-dependent suppression of ghrelin under intraduodenal glucose application. This effect was not related to rises in insulin or glucose concentrations. However, studies using direct intravenous GIP application showed conflicting results regarding appetite regulation (4, 10, 46).

The exact mechanisms by which GIP affects ghrelin secretion remain to be established. Besides the possibility of direct effects of GIP via binding to putative GIP receptors located on ghrelin-producing cells in the stomach (39, 45), indirect metabolite-dependent mechanisms may be involved. In support of this hypothesis, we observed the most pronounced involvement of ghrelin in the network of molecular information exchange during hyperglycemic hyperinsulinemic clamp conditions with GIP infusion. In the summarized GIP network, ghrelin was directly connected to octadecanoic acid, which had a central position in the subnetwork of fatty acids and amino acids. Insulin and glucose were the hubs of the second subnetwork and had no direct connections to ghrelin or octadecanoic acid. Such network topology, positioning fatty acids between glucose and insulin, on the one side, and otherwise positively correlating with ghrelin specifically in a GIP network may well reflect involvement of GIP in the regulation of circulating ghrelin. In contrast, and unexpectedly, we observed no connectivity of ghrelin in the metabolic network in conditions of euglycemic hyperinsulinemia (EC+NaCl experiments). Moreover, ghrelin was only weakly connected via positive correlation to the amino acid proline in the metabolite network in conditions of clamped hyperglycemic hyperinsulinemia (HC+NaCl test).

Regulation of ghrelin by GIP via modification of circulating long-chain fatty acid pools is the new and pioneering finding in our study. The possibility of free fatty acids regulating ghrelin secretion was tested in the first experiments on healthy subjects made in our laboratory eight years ago (31). Later studies gave conflicting results (17, 18, 47). With respect to an observation in rodents (33), it is possible that the type of fatty acids used in these different studies may contribute to the controversy of existing data. In addition, a differential suppression of ghrelin dependent on the concentrations of circulating FFA under hyperinsulinemia was suggested (17, 18). Moreover, our study raises questions about mechanisms by which GIP influences the pool of long-chain fatty acids. Our studies indirectly support recently published observations showing that GIP in conditions of clamped hyperglycemia enhances fatty acid re-esterification with a resulting decrease in circulating fatty acids (3) and with comparable although milder effects under eugly-
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>GIP-Inf</th>
<th>NaCl-Inf</th>
<th>EC-GIP</th>
<th>EC-NaCl</th>
<th>HC-GIP</th>
<th>HC-NaCl</th>
<th>Pathways</th>
<th>Associated Disorders</th>
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<td>Octadecanoic acid</td>
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<td>0.296</td>
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<td>0.257</td>
<td>0.138</td>
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<td>0.042</td>
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<td>Prostate cancer</td>
</tr>
<tr>
<td>Hexadecenoic acid, 9-(Z)-</td>
<td>0.454</td>
<td>0.118</td>
<td>0.208</td>
<td>0.153</td>
<td>0.688</td>
<td>0.171</td>
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<tr>
<td>Leucine</td>
<td>0.025</td>
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<td>0.151</td>
<td>0.063</td>
<td>0.687</td>
<td>0.258</td>
<td>Transcription translation valine, leucine and isoleucine biosynthesis valine, leucine and isoleucine degradation</td>
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<tr>
<td>Serine</td>
<td>−0.150</td>
<td>0.137</td>
<td>0.077</td>
<td>0.060</td>
<td>0.672</td>
<td>0.433</td>
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<td>Schizophrenia</td>
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<td>Nonadecanoic acid methylester</td>
<td>−0.279</td>
<td>−0.039</td>
<td>0.093</td>
<td>−0.049</td>
<td>0.672</td>
<td>0.339</td>
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<tr>
<td>Proline</td>
<td>0.139</td>
<td>0.286</td>
<td>0.089</td>
<td>0.237</td>
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<td>0.616</td>
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<td>−0.261</td>
<td>−0.198</td>
<td>−0.125</td>
<td>0.582</td>
<td>0.114</td>
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<th>GIP-Inf</th>
<th>NaCl-Inf</th>
<th>EC-GIP</th>
<th>EC-NaCl</th>
<th>HC-GIP</th>
<th>HC-NaCl</th>
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<th>Pathways</th>
<th>Associated Disorders</th>
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<td>Glyceric acid</td>
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<td>−0.564</td>
<td>−0.116</td>
<td>−0.208</td>
<td>0.476</td>
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<td>Xylose</td>
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<td>-0.281</td>
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<td>Pentose and glucuronate, interconversions</td>
<td>Crohn’s disease; HIV and diarrhea; kidney disease; lung cancer; prostate cancer; small intestinal bacterial overgrowth; small intestinal malabsorption, HIV, &amp; cryptosporidiosis; small intestinal malabsorption, HIV, &amp; diarrhea; small intestinal malabsorption, scleroderma, &amp; bacterial overgrowth</td>
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<tr>
<td>Arabinonic acid-1,4-lactone</td>
<td>−0.376</td>
<td>−0.671</td>
<td>−0.481</td>
<td>−0.031</td>
<td>0.314</td>
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<tr>
<td>Galactonic acid</td>
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<td>Arginine and proline metabolism, butanoate metabolism, D-glutamine and D-glutamate metabolism, glutamate metabolism, glutathione metabolism, histidine metabolism, nitrogen metabolism, porphyrin metabolism, transcription translation</td>
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<td>Glutamic acid</td>
<td>−0.627</td>
<td>−0.146</td>
<td>0.011</td>
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<td>Blood glucose</td>
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<td>−0.024</td>
<td>0.235</td>
<td>0.214</td>
<td>−0.592</td>
<td>−0.385</td>
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</table>

Pearson correlation coefficients are shaded for 2 significance levels of correlation coefficients $P < 0.05$ (light gray) and $P < 0.01$ (dark gray).
cemic conditions (4). Furthermore, consumption of long-chain fatty acids produced a marked GIP response in animals (23). This effect seems to be dependent on the saturation of the fatty acids and is possibly modulated via activation of “free fatty acid receptors” GPR40, GPR119, and GPR120 on the GIP-producing K cells in the gut (37). Taken together, the GIP-dependent decrease of the circulating long-chain fatty acid levels may be part of a physiological negative feedback mechanism of the regulation of fuel metabolism and especially GIP action: fatty acids induce postprandial GIP secretion, which leads to the increased fatty acid reesterification and fatty acid uptake in the fat tissue, with this process resulting in a decrease of the circulating fatty acid pool and decreased GIP secretion. The parallel decrease in circulating ghrelin levels by GIP may switch over the saturation signal from the gut to the brain. In rodents, the administration of a fatty acid synthase inhibitor suppressed plasma ghrelin levels but not the ghrelin mRNA levels in the stomach (19).

It is important to note that the detection of metabolites in the present study was made by a semiquantitative method and that the results of the observed changes in the fatty acid pools need further replication by a quantitative method.

The role of insulin in ghrelin regulation remains controversial. In the present study, we observed a decrease of ghrelin levels only under euglycemic clamp conditions with GIP infusion. In contrast, the effect of euglycemic hyperinsulinemia alone on circulating ghrelin was weaker and not statistically significant. Several studies have shown that supraphysiological doses of insulin can suppress ghrelin and that insulin and ghrelin show reciprocal daily profiles (2, 14, 17, 26, 31, 40, 47). However, further studies have not demonstrated a physiological role for insulin in ghrelin regulation (7, 24, 41). With respect to our hormone-metabolite network analyses, ghrelin appeared to have no involvement in the metabolomic interaction under the condition of hyperinsulinemic euglycemia. In line with previous observations regarding effects of hyperglycemia on ghrelin levels (40), we observed a significant 19% decrease of circulating ghrelin levels from basal values in hyperglycemic hyperinsulinemic experiments without GIP. Thus, direct regulation of ghrelin by insulin, for example, via insulin receptor-mediated activity in ghrelin-producing cells, may be proposed. In this case, the observed discrepancy regarding reproducibility between insulin and ghrelin in the numerous clamp studies (2, 14, 26, 31, 40, 47) may reflect the different stages of insulin sensitivity in the studied subjects.

In the present study, the largest reduction of ghrelin was observed under GIP infusion during hyperglycemic hyperinsulinemic clamp. Insulin levels, therefore, were somewhat higher in the presence of GIP, although this was not statistically significant. However, since markedly elevated levels of insulin elicited only a small decrease of ghrelin under euglycemic conditions, it is unlikely that the difference in insulin levels accounted for the difference in ghrelin levels. In our previous pilot study in healthy nonobese individuals (38), we were unable to demonstrate a downregulation of ghrelin secretion by GIP. However, methodological differences between previously published studies and the present study may be involved. In our first study (38), higher concentrations of glucose and insulin during hyperglycemic clamp were achieved, and GIP infusion was given for 1 h followed by arginine infusion. Given the typical ghrelin secretion pattern with decrease at 60 and 120 min postprandially (44), the infusion protocol in our previous

Fig. 3. Involvement of ghrelin in the GIP-dependent hormone-metabolite network combining data of 3 experiments: GIP infusion test, hyperinsulinemic euglycemic clamps with GIP, and hyperinsulinemic hyperglycemic clamps with GIP. Color code for hierarchical distance from ghrelin: direct connectors was green, the connectors of the next orders are yellow, then red. Coordinated changes are shown with a continuous line; contraordinated changes are shown with broken line.

Fig. 4. Total number of ghrelin connectivities to other hormones and metabolites in the different networks of experiments. The numbers of connectivities are given for 2 significance levels of correlation coefficients: $P < 0.05$ (light gray) and $P < 0.01$ (dark gray).
study was possibly too short to observe effects of GIP on ghrelin.

Among potential limitations of the present study is that only total serum ghrelin concentrations were measured, and these reflect only the predominant des-acylated variant of the peptide (47). Several studies have suggested that des-acylated ghrelin may regulate food intake independently of binding to GHSR1a, and have some nonendocrine function (22). Moreover, hyperinsulinemic euglycemia alone and in combination with lipid infusion had no effect on circulating acylated ghrelin levels, and differential regulation of total and acyl-ghrelin by insulin and fatty acids has been suggested (47). Thus, it is unlikely that acyl-ghrelin was relevantly changed in our experiments. Second, we made no measurement of appetite rating and/or energy metabolism in our study. Third, we used diazoxide for the suppression of insulin secretion (13) in the hyperglycemic clamp experiments. In vitro experiments showed that diazoxide decreased GIP secretion in the primary cultures of small intestine (37). The concentrations of GIP in the hyperglycemic clamp experiments were not different from those of the euglycemic clamps. Moreover, no difference was observed between the ghrelin levels before administration of diazoxide and 1 h later. Thus, the experimental setting was not affected by administration of diazoxide in our study, although we cannot absolutely exclude the fact that some plasma metabolites could be induced by the diazoxide in our experiments. Finally, the glucose clamp is an excellent method to study insulin sensitivity and insulin secretion in vivo, but this technique is possibly too static regarding the analysis of the complexity in the hormone and metabolites interactions. Further experiments using some dynamic tests such as a meal test or an intravenous glucose tolerance test with co-infusion of GIP or lipids could be useful for replication of our data.

In conclusion, results from the present study indicate that GIP suppresses circulating total ghrelin levels independently of insulin. This assumption was supported by integrative hormone-metabolite network analyses, which demonstrated coordinated changes in the long-chain fatty acid pool. The reciprocal interaction between the gastrointestinal hormones GIP and ghrelin may affect satiation signals from the gut to the brain and lead to the suppression of hunger sensation and appetite.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

GIP, GHRELIN, AND PLASMA METABOLOME