Glucose-Dependent Insulinotropic Polypeptide (GIP) Induces Calcitonin Gene-Related Peptide (CGRP)-I and Procalcitonin (Pro-CT) Production in Human Adipocytes

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Context: Increased plasma levels of glucose-dependent insulinotropic polypeptide (GIP), calcitonin CT gene-related peptide (CGRP)-I, and procalcitonin (Pro-CT) are associated with obesity. Adipocytes express functional GIP receptors and the CT peptides Pro-CT and CGRP-I. However, a link between GIP and CT peptides has not been studied yet.

Objective: The objective of the study was the assessment of the GIP effect on the expression and secretion of CGRP-I and Pro-CT in human adipocytes, CGRP-I and CT gene expression in adipose tissue (AT) from obese vs. lean subjects, and plasma levels of CGRP-I and Pro-CT after a high-fat meal in obese patients.

Design and Participants: Human preadipocyte-derived adipocytes, differentiated in vitro, were treated with GIP. mRNA expression and protein secretion of CGRP-I and Pro-CT were measured. Human CGRP-I and CT mRNA expression in AT and CGRP-I and Pro-CT plasma concentrations were assessed.

Results: Treatment with 1 nM GIP induced CGRP-I mRNA expression 6.9 ± 1.0-fold (P < 0.001 vs. control) after 2 h and CT gene expression 14.0 ± 1.7-fold (P < 0.001 vs. control) after 6 h. GIP stimulated CGRP-I secretion 1.7 ± 0.2-fold (P < 0.05 vs. control) after 1 h. In AT samples of obese subjects, CGRP-I mRNA expression was higher in sc AT (P < 0.05 vs. lean subjects), whereas CT expression was higher in visceral AT (P < 0.05 vs. lean subjects). CGRP-I plasma levels increased after a high-fat meal in obese patients.

Conclusion: GIP induces CGRP-I and CT expression in human adipocytes. Therefore, elevated Pro-CT and CGRP-I levels in obesity might result from GIP-induced Pro-CT and CGRP-I release in AT and might be triggered by a high-fat diet. How these findings relate to the metabolic complications of obesity warrants further investigations. (J Clin Endocrinol Metab 96: E297–E303, 2011)

Glucose-dependent insulinotropic polypeptide (GIP) is an incretin hormone released from enteroendocrine K cells in response to oral nutrient ingestion, with fat being the main trigger (1). Obesity is associated with elevated fasting and postprandial GIP levels (2, 3). Ablation of GIP signaling protects against diet-induced obesity and insulin resistance in rodents (4–7). In addition to its role as an incretinotropic peptide (8), GIP acts on multiple target or-

Abbreviations: AT, Adipose tissue; [Ca2+]i, intracellular calcium; CGRP, CT gene-related peptide; CT, calcitonin, GIP, glucose-dependent insulinotropic polypeptide; GIP-R, GIP receptor; GLP-1, glucagon-like peptide-1; GLUT, glucose transporter; HPRT, hypoxanthine-guanine phosphorylase; LPS, lipopolysaccharide; PKA, protein kinase A; Pro-CT, procalcitonin.
gans via G protein-coupled receptors (9). Functional GIP receptors (GIP-Rs) are expressed by human adipocytes, using cAMP as second messenger (10). In rodent adipocytes, GIP directly influences fat metabolism and lipid accumulation (11). Therefore, GIP links diet-induced obesity and related metabolic dysfunctions.

Calcitonin (CT) peptides like CT gene-related peptide (CGRP-I) and procalcitonin (Pro-CT) are derived from the CALC-I gene by alternative splicing (12). They are expressed and released from adipose tissue (AT) during sepsis and systemic inflammation (13–15) and from adipocytes treated with inflammatory cytokines in vitro (14–16). Pro-CT is a widely used surrogate marker in bacterial infection and sepsis (17). In addition, Pro-CT is a marker of the chronic inflammatory activity of AT in women with the polycystic ovary syndrome (18), correlating with total fat mass, body fat distribution, and insulin resistance (18). Furthermore, a recent study reported an association of circulating Pro-CT concentrations with obesity and insulin resistance (19). Increased plasma levels of CGRP have been detected in obese vs. lean women in the fasting state (20) and after an oral high-fat load in lean subjects (20). In addition, CGRP induces insulin resistance in muscle and liver in vitro and in vivo (21–23), whereas other studies reported a direct effect of CGRP on pancreatic β-cells leading to impaired insulin secretion in humans (24), rats (25), and mice (26).

Because plasma levels of GIP (1) as well as Pro-CT (19) and CGRP (20) are elevated in the state of obesity and insulin resistance, we examined a potential link between GIP and the CALC-I gene derived peptides. We tested the hypothesis that GIP stimulates CGRP-I and Pro-CT expression and secretion in human adipocytes. We also evaluated CGRP-I and CT mRNA expression patterns in sc and visceral AT from obese vs. lean subjects and plasma levels of CGRP-I and Pro-CT after high-fat meal ingestion in morbidly obese patients.

**Materials and Methods**

**Human AT sampling, adipocyte culture, and treatment**

Human AT samples were obtained from patients undergoing elective surgery. All patients were operated for nonmalignant and noninflammatory diseases. The study was approved by the local ethical committee, and written informed consent was obtained from every patient. For AT mRNA expression studies, paired samples of sc and visceral AT were obtained from seven lean patients [three males and four females, aged 64.0 ± 15.0 yr; body mass index (BMI) 22.7 ± 2.0 kg/m2; none of seven with diabetes mellitus type 2; C-reactive protein 11.8 ± 8.3 mg/liter] and 13 morbidly obese patients [four males and nine females, aged 41.8 ± 11.4 yr; BMI 43.2 ± 3.3 kg/m2; three of 13 with diabetes mellitus type 2; C-reactive protein, 11.0 ± 6.1 mg/liter], respectively. Adipocyte-containing tissue was macroscopically dissected from nonadipose tissue and blood vessels and minced into very small pieces (5 μg). TRI reagent was added; the tissue was vortexed and needledd with a syringe (1.2 × 40 mm). After centrifugation at 14,000 rpm for 10 min at 4°C, the fat layer was removed and the fluid phase was transferred into a fresh tube. mRNA was isolated as described below.

For in vitro cell culture experiments, the stromal vascular cell fraction was isolated from sc AT samples, cultured, and differentiated for 14 d as previously described (27, 28). After 14 d of differentiation, cells were washed twice with warm PBS and incubated in 5 mM glucose DMEM/F12 containing 3% fetal calf serum, supplemented with 50 μg/ml gentamycin, 15 nM HEPES (all from Invitrogen, Basel, Switzerland), 100 nM 3′-ascorbic acid, 8 μg/ml insulin, and 15 nM d-pantothenate (all from Sigma-Aldrich, Buchs, Switzerland) for 48 h. Thereafter the medium was renewed and adipocytes were exposed to human recombinant GIP (1–42) (Bachem AG, Bubendorf, Switzerland) for indicated time points (1–24 h) and concentrations (10 pm to 100 nM). Adipocytes were treated with human GIP-R antagonist GIP (6–30) (100 nM to 1 μM, preincubation 15 min; Bachem), the adenylyl cyclase activator forskolin (10 μM, 1 h; Merck Chemicals, Nottingham, UK), the protein kinase A (PKA) inhibitor H-89 (20 μM, preincubation 1 h; Merck, Dietikon, Switzerland), and the ionophor ionomycin (1 μM, preincubation 1 h; Sigma-Aldrich). Cells from passages 2 through 7 were used in all experiments.

**RNA isolation and quantitative analysis of mRNA expression of preadipocyte-derived adipocytes and human AT samples**

RNA was isolated, 1 μg total RNA was subjected to RT-PCR and cDNA was subjected to quantitative real-time PCR analysis as previously described (16). Optimal sets of primers yielding short PCR products suitable for SYBR-Green detection were designed using the Probe Finder (Roche Applied Bioscience; http://qpcr2.probefinder.com). The oligonucleotide primers used were: 5′-GTT ACC TCC TGG GTC ATC TC-3′, 5′-CCA CCA AAT GGC CTT GAC TT-3′ (GIP-R); 5′-CCC CCT CAG CAG CGA GTG A-3′, 5′-GCA CCG CCA GGA CAT TGT TG-3′ [glucose transporter (GLUT)-4]; 5′-TGC CCA TCC AAA AAG TCC A-3′, 5′-GAA GTC CAA ACC GGT GACT TCT TT-3′ (leptin); 5′-CCC AGA AGA GAG CCT GTG ACA-3′, 5′-CTT CAC CAC ACC CCC TGA TC-3′ (CGRP-I); 5′-GCG CAG ATG AGC AGT GA-3′, 5′-TCA GAT TAC CAC ACC GCT TAG ATC-3′ (CT). Each cDNA sample tested for quantitative gene expression was also subjected to hypoxanthine-guanine phosphoribosyltransferase (HPRT)-mRNA analysis. Results are expressed as ratio of the respective gene of interest and HPRT-mRNA threshold values. Primers for HPRT were 5′-TCC GGC AGT ATC ATC AAA AGA TGG T-3′ and 5′-AGT CTT GCT TAT ATC CAA CAC TTC T-3′. The conditions were set as suggested by the manufacturer.

**cAMP measurement**

Intracellular cAMP content was determined using the cAMP Glo assay kit (Promega, Dübendorf, Switzerland) with the following modifications to the conditions set by the manufacturer: cells were starved in serum free DMEM/F12 medium 24 h before treatment. After an incubation period, the supernatant was removed and the cells were harvested in cAMP-lysis buffer, pro-
vided by the manufacturer and scraped, and the lysate was subjected to a 96-well plate. A standard curve was performed with cAMP control and cAMP-lysis buffer both provided by the manufacturer.

**Protein measurement in supernatants of preadipocyte-derived adipocytes**

After treatment the supernatants were collected, centrifuged, and immediately stored at −70 C. Supernatants were either concentrated in vacuo at room temperature for 4 h (GIP treatment alone) or concentrated for 6 h (experiments with GIP and ionomycin). The CGRP-I and Pro-CT protein in cell supernatant was determined as previously described (16, 28).

**Protein measurement in human plasma**

EDTA plasma samples from a recently performed study (29) were kindly donated by the study investigators. Briefly, fasting EDTA plasma samples from 24 morbidly obese patients (four males and 20 females, aged 40.47 ± 10.3 yr; BMI 46.12 ± 6.5 kg/m²) were obtained after an overnight fast of at least 10 h. After the fasting period, patients received a high-fat liquid test meal. We chose the time point of 180 min after meal ingestion for postprandial protein measurement. CGRP-I and Pro-CT protein were measured as described previously (16, 28).

**Statistical analysis**

Data were analyzed in triplicate using GraphPad Prism (Prism 4.0 for Windows, San Diego, CA) and expressed as means ± SEM unless indicated otherwise. For in vitro data, statistical significance was determined by ANOVA followed by Bonferroni’s post hoc test for multiple comparison analysis or by the Mann-Whitney U test when only two groups were analyzed. For analysis of mRNA expression in human AT, we used the Mann-Whitney U test. Correlation analyses were performed by using Spearman rank correlation. For analysis of CGRP-I and Pro-CT protein concentrations in human plasma samples, we used the Wilcoxon signed rank test.

**Results**

**Expression of functional GIP-R by differentiated human preadipocyte-derived adipocytes**

GIP-R mRNA expression was induced 6846 ± 1255-fold at d 7 (P < 0.01 vs. preadipocytes) and 8196 ± 962.8-fold at d 14 (P < 0.01 vs. preadipocytes) during the differentiation process (Fig. 1A). This was paralleled by the induction of other adipocyte differentiation markers like GLUT-4 and leptin (Fig. 1A). To further assess the functionality of the GIP-R in our human adipocytes, we measured GIP-induced cellular cAMP accumulation. Treatment of differentiated adipocytes with 1 and 100 nM GIP for 1 h in serum-free conditions produced a 7.1 ± 1.0-fold (P < 0.01 vs. control) and an 8.2 ± 1.4-fold (P < 0.001 vs. control) increase in cAMP accumulation, respectively (Fig. 1B). Treatment for 1 h with 10 μM forskolin served as a positive control and revealed a 9.8 ± 0.8-fold (P < 0.001 vs. basal) induction of cAMP content (Fig. 1B). Pre-incubation with 1 μM of the GIP-R inhibitor GIP (6–30) (1 μM, preincubation 15 min). Forskolin (10 μM) was added for 1 h and served as a positive control. Cellular cAMP content was determined using a luminescence assay. Results are expressed in arbitrary units with the preadipocyte values taken as 1 and are the means ± SEM of two independent experiments of two different donors each carried out in triplicate. ***, P < 0.01 vs. preadipocyte expression level of the respective gene. B, Differentiated adipocytes were serum starved for 2 h and then treated for 1 h with GIP (1 nM, 100 nM) in the presence or absence of the GIP-R antagonist GIP (6–30) (1 μM, preincubation 15 min). Forskolin (10 μM) was added for 1 h and served as a positive control. Cellular cAMP content was determined using a luminescence assay. Results are expressed in arbitrary units with the control value taken as 1 and are the means ± SEM of three independent experiments all carried out in triplicate. **, P < 0.01; ***, P < 0.001 vs. control. n.s., Not significant vs. GIP 1 nM or GIP 100 nM.

**GIP induced CALC-I gene expression in a dose- and time-dependent manner**

Treatment of human preadipocyte-derived adipocytes with increasing concentrations of GIP from 1 pM to 100 nM for 1 h revealed a significant up-regulation of the CALC-I gene.
GIP induced CALC-I gene expression was inhibited by the GIP-R inhibitor GIP (6–30)

The stimulatory effect of GIP was diminished or completely abolished by pretreatment with GIP (6–30). GIP-induced CGRP-I mRNA expression was reduced by 79% (P < 0.01 vs. GIP 1 nM, 1 h) at 1 μM GIP (6–30) and by 94% (P < 0.001 vs. GIP 1 nM, 1 h) at 10 μM GIP (6–30) (data not shown). GIP-stimulated CT mRNA expression was reduced by 96% (P < 0.05 vs. GIP 1 nM, 1 h) at 10 μM GIP (6–30) (data not shown).

The cAMP/PKA pathway and intracellular calcium ([Ca^{2+}]_{i}) are involved in GIP signal transduction

We next evaluated the signal transduction, which could be responsible for the observed effects of GIP. Addition of forskolin (10 μM) for 1 h potentiated the effect of GIP on CGRP-I and CT mRNA induction (P < 0.001 vs. GIP 1 nM, 1 h) and vice versa (data not shown), pointing to the crucial role of cAMP as second messenger. Inhibition of
PKA, the direct downstream signaling pathway module of cAMP, by H-89 (20 μM) efficiently blocked the GIP mediated stimulation of CGRP-I (P < 0.001 vs. GIP 1 nM, 1 h) and CT mRNA expression (P < 0.01 vs. GIP 1 nM, 1 h) (data not shown).

Because cAMP is often acting in synergism with [Ca2+]i signaling, we tested the effect of ionomycin, a calcium-mobilizing agent. Treatment of differentiated adipocytes with ionomycin (1 μM, 6 h) potentiated the stimulatory effect of GIP on CGRP-I (P < 0.001 vs. GIP 1 nM, 6 h) and CT gene expression (P < 0.01 vs. GIP 1 nM, 6 h) (data not shown). Costimulation with GIP (1 nM) and ionomycin (1 μM) for 24 h induced secretion of CGRP-I 1.6 ± 0.1-fold (P < 0.001 vs. control) and Pro-CT 9.7 ± 1.4-fold (P < 0.001 vs. control) (data not shown).

CGRP-I and CT mRNA levels are altered in sc and visceral AT of obese subjects

Examining paired sc and visceral AT samples, we found increased CGRP-I mRNA expression in sc AT of morbidly obese patients (P < 0.05 vs. lean subjects), whereas CGRP-I gene expression in visceral AT was not significantly altered (P = 0.2 vs. lean subjects) (data not shown). CT mRNA expression was not altered in sc AT (P = 0.6 vs. lean subjects) but was induced in visceral AT of morbidly obese patients (data not shown). Moreover, a positive correlation with BMI was found for CGRP-I mRNA expression (n = 20; r = 0.54; P < 0.05) and CT mRNA expression in visceral AT (n = 20; r = 0.45; P < 0.05). Correlation with BMI tended to be significant for CGRP-I mRNA expression in sc AT (n = 20; r = 0.43; P = 0.06) but not for sc CT mRNA expression.

Discussion

Our results demonstrate that GIP induces CALC-I gene expression and protein secretion of CGRP-I and Pro-CT in human adipocytes via GIP-R activation, involving the cAMP/PKA module and [Ca2+]i signaling pathways. Furthermore, we found increased mRNA expression of CGRP-I and CT in sc and visceral AT samples of obese compared with lean control subjects. Additionally, CGRP-I protein content was increased and Pro-CT tended to be increased in plasma samples of obese patients after a high-fat meal ingestion.

In agreement with other human adipocyte models (10, 30), we found GIP-R mRNA to be barely expressed in undifferentiated primary human preadipocytes. Induction of GIP-R during differentiation was accompanied by expression of other adipocyte specific markers like leptin. Functional analysis of GIP-R in our differentiated adipocytes confirmed the results described for GIP-R in another human adipocyte model (10). However, and in contrast to Weaver et al. (10), who reported a peak of GIP-R mRNA expression at d 6–8 and a decreased expression to 50% at d 14 of differentiation in Simpson-Golabi-Behmel syndrome preadipocyte-derived adipocytes, we found near-to-maximal GIP-R mRNA expression levels already at d 7 of differentiation with a stable expression pattern up to d 14 of differentiation process in our human adipocytes. This discrepancy might most likely be attributed to differences in the primary preadipocytes used.

Treatment with GIP directly induced mRNA expression of CGRP-I and its splice variant CT in a dose- and time-dependent manner. Regarding possible signaling pathways transducing the GIP effect on CGRP-I and CT expression and secretion, we found the cAMP/PKA pathway as well as [Ca2+]i to be involved. Both cAMP/PKA and [Ca2+]i, mediate lipopolysaccharide (LPS)-induced CGRP release in cultured neonatal rat dorsal root ganglion neurons (31). In accordance we recently demonstrated a crucial role for [Ca2+]i, in LPS-induced CALC-I gene expression and protein release of CGRP-I and Pro-CT in human adipocytes (16). In GIP-treated human adipocytes, only CGRP-I protein was secreted shortly after GIP treatment alone, whereas cotreatment with the ionophor ionomycin induced significant protein secretion of both CGRP-I and Pro-CT. This suggests that GIP-induced secretion of Pro-CT by adipocytes takes place only if cells are primed by other stimuli.

A recent study provided strong evidence for an association of Pro-CT plasma levels with obesity, insulin resistance, and the metabolic syndrome (19). Some studies provided further evidence for the detrimental effects of Pro-CT in states of systemic inflammation and sepsis (32, 33). The secretion of Pro-CT in the AT of obese patients might thereby further accelerate local proinflammatory activity in AT. Furthermore, CGRP-I has been shown to directly induce insulin resistance (21–23, 34) and to impair glucose-induced insulin secretion (24, 35). Therefore, CGRP-I and Pro-CT might mediate GIP-induced insulin resistance in diet-induced obesity and related metabolic dysfunctions.

As a physiological link to our in vitro findings, human CGRP plasma levels increase after an oral fat load (20), which is also a trigger for GIP release (1). However, another study found no systemic increase of CGRP after oral fat ingestion (36). Due to the controversy of these reports, we measured CGRP-I and Pro-CT levels in plasma samples of 24 highly obese patients before and 180 min after ingestion of a high-fat test meal. We found an increase in CGRP-I plasma levels to 1.9 ± 0.3-fold (P < 0.05 vs. fasted) and a slight increase in Pro-CT levels to 1.1 ± 0.1-fold (P = 0.07 vs. fasted; data not shown). The finding
that the Pro-CT plasma levels were not significantly increased might be attributed to the fact that concentrations of this hormone were measured only at one time point (180 min) after meal ingestion. Pro-CT plasma levels after endotoxin injection have been reported to rise at the earliest within 3–6 h (37). Therefore, we might have missed a significant increase of Pro-CT concentrations at a later postprandial time point.

The induction of Pro-CT and CGRP by GIP in human adipocytes might be also of clinical relevance in another context. Glucagon-like peptide-1 (GLP-1), another incretin hormone, is used for the treatment of diabetes mellitus type 2. GLP-1 analogs have been shown to induce CT and CGRP expression and secretion in a murine C cell line (38). Furthermore, a long-acting GLP-1 receptor agonist, liraglutide, is associated with increased risk of thyroid C cell adenomas and carcinomas in rats and mice (39). Accordingly, the Food and Drug Administration requested long-term follow-up on rates of medullary cancer in patients treated with this drug. Dipeptidyl peptidase-IV inhibitors increase plasma levels of GLP-1 and GIP in vivo (40) and are widely used for the treatment of diabetes mellitus type 2. Our findings, that GIP is inducing CALC-I gene expression in human adipocytes, should further stimulate research investigating the effect of GIP in human thyroid C cells to cast light on any potential medullary thyroid carcinoma developing in patients treated with Dipeptidyl peptidase-IV inhibitors or newly developed GIP agonists/analogs.

In summary, our study shows that GIP is directly inducing CGRP-I and Pro-CT in human adipocytes. Together with the findings that CGRP-I and CT mRNA expression levels in visceral AT were positively correlated with BMI, these results provide strong evidence for a role of CGRP-I and Pro-CT as markers for adipocyte dysfunction in obesity.

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