Glucose-dependent insulinotropic polypeptide induces cytokine expression, lipolysis, and insulin resistance in human adipocytes

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Glucose-dependent insulinotropic polypeptide (GIP) is an incretin hormone also acting on adipose tissue-derived inflammation. Glucose-dependent insulinotropic polypeptide (GIP) is an incretin hormone also acting on adipocytes. We investigated whether GIP affects inflammation, lipolysis, and insulin resistance in human adipocytes. Human subcutaneous preadipocyte-derived adipocytes, differentiated in vitro, were treated with GIP to analyze mRNA expression and protein secretion of cytokines, glycerol, and free fatty acid release and insulin-induced glucose uptake. GIP induced mRNA expression of IL-6, IL-1β, and the IL-1 receptor antagonist IL-1Ra, whereas TNFα, IL-8, and monocyte chemotactic protein (MCP)-1 remained unchanged. Cytokine induction involved PKA and the NF-κB pathway as well as an autocrine IL-1 effect. Furthermore, GIP potentiated IL-6 and IL-1Ra secretion in the presence of LPS, IL-1β, and TNFα. GIP induced lipolysis via activation of hormone-sensitive lipase and was linked to NF-κB activation. Finally, chronic GIP treatment impaired insulin-induced glucose uptake possibly due to the observed impaired translocation of glucose transporter GLUT4. In conclusion, GIP induces an inflammatory and prolipolytic response via the PKA-NF-κB-IL-1 pathway and impairs insulin sensitivity of glucose uptake in human adipocytes.

GIP; cytokines; lipolysis; insulin resistance; human adipocytes

GLUCOSE-DEPENDENT INSULINOTROPIC POLYPEPTIDE (GIP) is an incretin hormone released from enteroendocrine K cells in response to oral ingestion of fat or glucose (34). GIP is best known for its stimulation of insulin release from pancreatic β-cells in a glucose-dependent manner (11), transducing its biological effects via G protein-coupled receptors (GPCR) (39). Depending on experimental conditions in vitro, GIP also influences lipid metabolism in rodent (64) and human adipocytes (61) via lipogenesis (36) and lipolysis (22, 25, 38). In vivo, treatment with GIP impairs insulin sensitivity in rodents (3, 37) and augments postprandial hyperglycemia in patients with type 2 diabetes (7). Conversely, ablation of GIP signaling protects against diet-induced obesity and insulin resistance (18, 19, 42). Circulating GIP levels are increased in obesity and type 2 diabetes in animal models and humans (17, 60). Taken together, this points to a possible “diabetogenic” effect of GIP that is supposed to be mediated by GIP effects on adipocytes. Chronic high-fat diet, obesity, and type 2 diabetes are associated not only with a disturbance in gut hormone regulation but also with a state of local and systemic low-grade inflammation (55). Thereby, adipose tissue has emerged as a major source of cytokines like interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNFα) (28, 58), implicated in lipolysis (31, 50) and insulin resistance (30, 53). Lipopolysaccharide (LPS), which is increased postprandially and after oral fat intake (1), is triggering this activation of the innate immune system (4). Adipose tissue is also an important source of IL-1 receptor antagonist (IL-1Ra), an endogenous inhibitor of IL-1 action, in obese rodents and humans (33). Serum levels of IL-1Ra are markedly increased in obesity (32, 40) and are considered as predictor of type 2 diabetes (6).

Although GIP is implicated in the etiology of diet-induced obesity and related insulin resistance (29), effects of GIP on human adipocytes in the context of inflammation have not been studied. Therefore, we investigated the effect of GIP in human preadipocyte-derived adipocytes in vitro on expression and secretion of cytokines as well as its impact on lipolysis and glucose uptake.

MATERIALS AND METHODS

Human adipocyte culture and treatment. Human adipocyte tissue samples from subcutaneous abdominal fat depots were obtained from nine patients (age 50.3 ± 18.0 yr, BMI 30.4 ± 8.3 kg/m²), 4 nonobese (3 nondiabetic, 1 diabetic), 5 obese (1 diabetic, 4 nondiabetic) undergoing elective visceral surgery for nonmalignant, noninflammatory reasons or bariatric surgery. The study was approved by the local ethics committee, and informed consent was obtained from every patient. Stromal vascular cells were isolated as previously described (16) and expanded in DMEM-F12 containing 10% fetal calf serum (FCS), 5 ng/ml 2-fibroblast growth factor (FGF) (all from Lonza, Verviers, Belgium), sodium pyruvate, and penicillin-streptomycin (both from Invitrogen, Basel, Switzerland) until confluence was reached. Differentiation into adipocytes was induced by incubating confluent cells in DMEM-F12 containing 3% FCS, 50 mg/ml gentamicin solution, and 30 mM HEPES (all from Invitrogen) and supplements as follows: 250 μM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone, 0.2 nM 3,3,5-triiodo-L-thyronine, 100 mM l-ascorbic acid, 8 μg/ml insulin, 15 mM pantothenate (all from Sigma-Aldrich, Buchs, Switzerland), 5 μM transferrin (Merck, Dietikon, Switzerland), 100 nM insulin (Novo Nordisk, Küssnacht, Switzerland), and 1 μM rosiglitazone (GlaxoSmithKline, Worthing, UK).
State of differentiation was evaluated by accumulation of lipid droplets using an inverted microscope (Zeiss, Feldbach, Switzerland). After 14 days of differentiation, cells were washed twice with phosphate-buffered saline (PBS) and incubated in 5 mM glucose-DMEM-F12 containing 3% FCS, supplemented with 50 μg/ml gentamycin, 15 nM HEPES, 100 nM L-ascorbic acid, 8 μg/ml bovine, and 15 mM β-pantothenate for 48 h unless indicated otherwise. After 48 h, the medium was renewed for another 24-h period. For experimental procedures, the cells were serum starved or not, as indicated for 2 h prior to experimental treatment. Adipocytes were exposed to human GIP(1–42) (Bachem, Bubendorf, Switzerland) as indicated. For controls, equivalent concentrations of dimethylsulfoxide (DMSO; Sigma-Aldrich, Buchs, Switzerland) were added. For antagonist studies, cells were treated with the GIP receptor (GIP-R) inhibitor GIP(6–30) (100 nM-10 μM, preincubation 15 min; Bachem), H-89 (20 μM, preincubation 1 h; Merck, Dietikon, Switzerland), sc-514 (100 μM, preincubation 1 h; Merck), recombinant human IL-1Ra (1–100 μg/ml, preincubation 1 h; Amgen, Seattle, WA), and mouse anti-human IL-6Ra (1 μg/ml, preincubation 1 h; R&D Systems, Abingdon, UK). IMD0354 (1 mM, preincubation 1 h), orlistat (100 μM, preincubation 1 h), U-0126 (10 μM, preincubation 1 h), KT-5720 (1 μM, preincubation 1 h), and SP-600125 (500 nM, preincubation 1 h) were from Merck. LPS (100 ng/ml, 24 h) was from Sigma-Aldrich, IL-1β (20 ng/ml, 24 h) and TNFα (10 ng/ml) were from PeproTech (London, UK). Deoxy-D-glucose (2-1H[G; 1 μC, 15 min) was obtained from PerkinElmer (Schwerzenbach, Switzerland). Cells from passages 2 to 7 were used in all experiments.

RNA isolation and quantitative analysis of mRNA expression of preadipocyte-derived adipocytes. RNA was isolated using TRizol (Sigma-Aldrich) following the manufacturer’s protocol. Total RNA (1 μg) was subjected to reverse transcription-PCR, and cDNA was subjected to quantitative real-time PCR analysis as previously described (51). Optimal sets of primers yielding short PCR products suitable for SYBR-Green detection were designed using the Probe Finder (Roche Applied Bioscience) website (http://qpcr2.probefinder.com). The oligonucleotide primers used were as follows (forward and reverse, respectively): 5'-TCT TCA GAA CGA ATT GAC AAA CAA A-3' and 5'-GCT TTC ACA CAT GTT ACT CTT G-3' (IL-6), 5'-AGC TGA TGG CCC TAA ACA GA-3' and 5'-TCG GAG ATT CGT AGC TGG AT-3' (IL-1β), 5'-TGC CTG TTC CAT TGT GAG CCG TC-3' and 5'-TGC CCC CGG TGC CAT TGC GTG TC-3' (IL-1Ra), 5'-CTC AAC TGG ACA TCA CAG CAG-3' and 5'-AGG TCG TTG TTA GCA CAG-3' (β-actin). Real-time PCR amplification was carried out using a Light Cycler (Roche Applied Bioscience). Relative mRNA expression level was calculated using the comparative Ct method (2^-△△Ct).

Fig. 1. Treatment with glucose-dependent insulinotropic polypeptide (GIP) induces mRNA expression of IL-6, IL-1β, and IL-1 receptor antagonist (IL-1Ra) in a dose- and time-dependent manner. A: for dose-response experiments, differentiated human preadipocyte-derived adipocytes were treated with GIP (10 pM–100 nM) for 1 h. B: for time course experiments, differentiated human preadipocyte-derived adipocytes were treated with GIP (1 nM) for indicated periods of time. For all experiments, total RNA was isolated from each sample, and mRNA expressions of IL-6, IL-1β, and IL-1Ra were determined by real-time PCR and normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression levels. Results are expressed in arbitrary units (AU) with the control value taken as 1 and are means ± SE of 3–6 independent experiments, each carried out in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

Fig. 2. Classical inflammatory and lipolytic pathways are involved in GIP-mediated cytokine mRNA expression. Differentiated human preadipocyte-derived adipocytes were treated with or without PKA inhibitor H-89 (20 μM; A), IKKβ inhibitor sc-514 (100 μM; B), lipase inhibitor orlistat (100 μM; C), or IL-1Ra (1 μg/ml; D) 1 h prior to and during stimulation with GIP (1 nM, 1 h). For all experiments, total RNA was isolated from each sample, and mRNA expressions of IL-6, IL-1β, and IL-1Ra were determined by real-time PCR and normalized to HPRT expression levels. Results are expressed in AU with the control value taken as 1 and are means ± SE of 3 independent experiments, each carried out in triplicate. Immunoblots (IB) showing the content of IκBα protein and p(Ser/Thr)PKA substrate levels are given as fold of basal and were corrected for potential loading variations using β-actin. Data (G and H) are means ± SE of 3–4 independent experiments each carried out in duplicate. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; 1P < 0.001, 2P < 0.01, 3P < 0.001 vs. GIP (1 nM, 1 h).
GTA TCG T-3' and 5'-TGC TCG CTC CAC CAC AAA C-3' (GLUT1), 5'-CCC CCT CAG CAG CGA GTG A-3' and 5'-GCA CCG CCA GGA CAT TGT TG-3' (GLUT4), 5'-TCA GGC AGT ATA ATC CAA AGA TGG T-3', 5'-AGT CTG GCT TAT ATC TAT ATC CAA CAC TTC-3' hypoxanthine-guanine phosphoribosyltransferase (HPRT). Each cDNA sample tested for quantitative gene-expression was subjected to HPRT mRNA analysis. Results are expressed as the ratio of the respective gene of interest mRNA and HPRT mRNA threshold values. The reaction volume was 25 μl, and the conditions were set as suggested by the manufacturer.

**Western blot analysis.** Cells were harvested in a 20 mM Tris·HCl (pH 7.5) lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10% glycerol, protease inhibitor cocktail, Na3VO4, NaF, PMSF, and EDTA and incubated on ice for 10 min. Cell lysates were cleared by centrifugation (10,000 g, 10 min), sample buffer as well as reducing agent (NuPAGE, Invitrogen) were added, and lysates were heated up to 70°C for 10 min and centrifuged afterward. Protein concentrations of lysates were determined by a BCA protein assay kit from Thermo Fisher Scientific (Lausanne, Switzerland). Lysates containing equal amounts of proteins were subjected to 4–12% NuPAGE Bis-Tris gels
(Invitrogen) and blotted on a nitrocellulose membrane (pore size 0.2 μm; Bio-Rad, Rheinach, Switzerland) using the Mini Trans-Blot Electrophoretic Transfer System (Invitrogen). After blocking of the membrane (1 h, 20–22°C) with O DysSEY blocking buffer (Li-Cor Biotechnology, Bad Homburg, Germany), the membrane was incubated (16 h, 4°C) with primary antibodies against IκBα, phospho-(Ser/Thr) PKA substrate, phospho-HSL(Ser552), HSL, phospho-Akt(Ser73), Akt, IRS-1 (all from Cell Signaling), GLUT4 (Abcam, Cambridge, UK), washed, and incubated (1 h, 20–22°C) with goat anti-rabbit secondary antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Band intensities were detected with the Odyssey System (Li-Cor). The membranes were washed twice with TBS-0.1% Tween to remove the antibodies added and to probe them again with anti-β-actin primary antibody [Millipore (Chemicon), Zug, Switzerland] used as loading control. Results are expressed as relative protein levels standardized such that values obtained in untreated adipocytes are set to 1 unless indicated otherwise.

**Lipolysis assays.** Cells were serum starved in 5 mM glucose-DMEM-F12 for 2 h and stimulated in the same medium or were serum starved for 1 h in Krebs-Ringer buffer (KRB) with 0.1% fatty acid-free BSA (Roche, Germany) and 2 mM glucose before treatment with indicated agents. Inhibitors were added 1 h prior to GIP treatment. Glyceral content in the cell culture supernatant was determined as an index of lipolysis using a UV-method kit from R-Biopharm (Darmstadt, Germany). Free fatty acid (FFA) levels were measured using the ACS-ACOD-MEHA method from Wako Chemicals (Neuss, Germany) as previously described (63).

**Glucose uptake.** Cells were serum starved in 5 mM glucose-DMEM-F12 for 2 h before the addition of inhibitors and GIP for indicated incubation time and concentration. Cells were then treated with insulin (100 nM) for 20 min. At the end of the stimulation period, cells were exposed to 1 μC of [2-3H]Glc per well for 15 min. Then, cells were washed three times with ice-cold PBS and lysed in 0.1% sodium dodecyl sulfate. Radioactivity was measured by liquid scintillation counting. An aliquot of each sample was used for determination of protein concentration by BCA assay (Thermo Scientific, Lafayette, CO).

**Plasma membrane fractionation and GLUT4 immunoblotting.** Cells were serum starved in 5 mM glucose-DMEM-F12 for 2 h. Cells were then treated with 1 nM GIP for 6 h and in the end with 1 nM insulin for 20 min. Following stimulation, adipocytes were washed with HES buffer, scraped, homogenized, and centrifuged as previously described (24). The supernatant was further centrifuged (18,000 g, 20 min). The resulting pellet, representing the crude plasma membrane (PM) fraction, was resuspended in lysis buffer to obtain PM fraction lysate and subjected to Western blot analysis as depicted above.

**Protein measurement in cell supernatant.** Cells were serum starved for 2 h and treated with LPS (100 nM), IL-1β (20 ng/ml), and TNFα (10 ng/ml) with or without GIP (1 nM, 100 nM) for 24 h as indicated. Cell supernatants were collected and immediately centrifuged at 14,000 rpm, 4°C, for 10 min to remove cell debris. Samples were stored at −70°C until measurement. IL-6 and IL-1Ra contents in the supernatants were measured using human anti-IL-6 and human anti-IL-1Ra enzyme-linked immunosorbent assay kits (R&D Systems, Abingdon, UK), with a detection level of 0.7 pg/ml for IL-6 and 6.26 pg/ml for IL-1Ra. Intra- and interassay coefficients of variation were <10%. The coefficient of variation of the IL-6 ELISA was 0.2–16.9% and 0.6–20.3% of the IL-1Ra ELISA.

**Statistical analyses.** Data are expressed as means ± SE from a minimum of three independent experiments, each carried out in triplicate unless otherwise indicated. Data were analyzed using the GraphPad Prism program (version 5.04, San Diego, CA). For multi-group comparison, statistical significance was determined by one-way ANOVA followed by Bonferroni’s post hoc test. For comparison of two groups, statistical significance was determined by paired t-test. A P value of <0.05 was considered significant.

**RESULTS**

**GIP induces cytokine gene expression in differentiated human preadipocyte-derived adipocytes in a dose- and time-dependent fashion.** Treatment of differentiated preadipocyte-derived adipocytes with 1 nM GIP for 1 h increased IL-6, IL-1β, and IL-1Ra mRNA expression 4.2 ± 0.3-, 1.6 ± 0.1-, and 7.2 ± 1.1-fold, respectively (Fig. 1A). While mRNA expression of IL-6 and IL-1β was already maximally induced at 1 nM GIP, IL-1Ra mRNA expression was induced maximally up to 23.5 ± 3.9-fold at 100 nM GIP after 1 h (Fig. 1A).

In contrast, mRNA expressions of TNFα, IL-8, and MCP-1 were not altered (data not shown). On the basis of the dose-response studies, we used 1 nM GIP for time course experiments. IL-6 mRNA expression was induced 2.6 ± 0.5-fold at 1 h and decreased to baseline level thereafter (Fig. 1B), whereas IL-1β and IL-1Ra were maximally increased after 4 h (Fig. 1B). Since serum components like antibodies might nonspecifically activate GPCRs (20), additional serum-free experiments were performed to ensure specificity of the GIP effect. Serum-free conditions revealed an even more pronounced effect of GIP, inducing IL-6 mRNA 7.7 ± 1.0-fold (P < 0.001 vs. control) at 1 h, IL-1β mRNA to a maximum of 2.5 ± 0.2-fold (P < 0.001 vs. control) at 6 h, and IL-1Ra mRNA 12.5 ± 1.7-fold (P < 0.001 vs. control) at 6 h (data not shown).

To elaborate the specificity of the GIP effect, cells were preincubated with the competitive GIP-R antagonist GIP(6–30) (100 nM–10 μM) 15 min prior to GIP stimulation. Pretreatment with GIP(6–30) inhibited GIP-induced IL-6, IL-1β, and IL-1Ra mRNA expression (data not shown). In undifferentiated preadipocytes, the lack of GIP-R expression (57) was mirrored by the absence of a GIP effect on IL-6, IL-1β, or IL-1Ra gene expression (data not shown).

Although differentiated preadipocyte-derived adipocytes were cultured from donors differing in phenotype, we observed no differences in cell shape or responsiveness to GIP or insulin treatment in preadipocyte-derived adipocytes from obese vs. lean and diabetic vs. non-diabetic patients.

**PKA, IKKβ/NF-κB, and IL-1R activation are involved in GIP-induced cytokine expression.** Next, we investigated the underlying mechanisms of GIP-induced cytokine expression. Pretreatment with the PKA inhibitor H-89 reduced GIP-stimulated expression of IL-6 and of IL-1β to basal levels and IL-1Ra mRNA expression by 74% (Fig. 2A). Similar results were found using a different PKA inhibitor, KT5720 (data not shown). Preincubation with the IKKβ inhibitor sc-514 reduced GIP-induced IL-6 gene expression by 61% and IL-1Ra gene expression by 61%, whereas IL-1β mRNA expression was not significantly altered (Fig. 2B). Similar results were obtained using another IKKβ inhibitor, IMD0354 (data not shown). Preincubation with IL-1Ra reduced GIP-induced IL-6 mRNA expression by 68%, whereas IL-1β and IL-1Ra were not altered (Fig. 2C). Interestingly, preincubation with the lipase inhibitor orlistat reduced GIP-induced mRNA expression of IL-6 by 78%, IL-1β to basal level, and IL-1Ra by 80% (Fig. 2D). Indicative for NF-κB pathway activation by GIP, we found that GIP reduced IκBα protein expression by 20% (Fig. 2E), which was prevented by pretreatment with sc-514 (Fig. 2E) but not by preincubation with H-89 (Fig. 2F). Investigating a possible link between the PKA and the NF-κB pathway, we also measured phosphorylation status of (Ser/Thr) PKA sub-
strate. p(Ser/Thr) PKA substrate was induced 1.4 ± 0.08-fold by GIP, which was prevented by preincubation with H-89 or sc-514 (Fig. 2F).

**GIP potentiates LPS-, IL-1β-, and TNFα-induced IL-6 protein secretion.** In contrast to the effect of GIP at mRNA level, treatment with GIP alone for up to 24 h failed to induce IL-6 or IL-1Ra protein release (data not shown). GIP at 1 nM and 100 nM potentiated IL-6 protein secretion 1.7- and 2.3-fold in the presence of LPS, 1.8- and 1.8-fold in the presence of IL-1β, and 1.4- and 2.3-fold, respectively, when coincubated with TNFα (Fig. 3, A–C). IL-1Ra protein secretion was induced 6.6 ± 1.0-fold by LPS alone and 7.8 ± 1.5- and 9.2 ± 2.1-fold in the presence of 1 nM and 100 nM GIP, respectively (Fig. 3D). IL-1Ra was induced 4.0 ± 0.6-fold by IL-1β alone and 6.6 ± 1.3- and 6.5 ± 1.5-fold when coincubated with 1 nM and 100 nM GIP, respectively (Fig. 3E). TNFα alone induced IL-1Ra secretion 80 ± 30.2- and 82 ± 31.0-fold, respectively (Fig. 3F). The amount of IL-6 and IL-1Ra protein secretion induced by TNFα alone differed greatly between

**Fig. 5. GIP-induced lipolysis additionally involves IKKβ/NF-κB and MAPK modules as well as IL-1 receptor activation.** Differentiated human preadipocyte-derived adipocytes were serum starved for 2 h and preincubated with 100 μM sc-514 (A), 1 μM IMD0354 (B), 1 μg/ml IL-1Ra (C), 10 μM U-0126 (D), 500 nM SP-600125 (E), or 10 μM SB-203580 (F) for 1 h and then treated with 1 nM GIP for 6 h. Glycerol release was measured in cell culture medium as an index of lipolysis. Data are expressed in AU with the control value taken as 1 and are means ± SE of 2–3 independent experiments, each carried out in triplicate. G: immunoblots showing phosphorylation status of Ser552 and protein content of β-actin upon treatment with GIP (1 nM) for 1 h with or without preincubation with H-89 (10 μM) or sc-514 (100 μM) for 1 h. Data are means ± SE of 3 independent experiments, each carried out in duplicate. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; †P < 0.05; ‡P < 0.01; ††P < 0.001 vs. GIP (1 nM, 6 h or 1 h as indicated).
Long-term treatment with GIP impairs insulin-induced glucose uptake possibly via impaired GLUT4 translocation. Treatment of adipocytes with 1 nM GIP alone slightly increased glucose uptake 1.1 ± 0.03- and 1.2 ± 0.06-fold after 6 and 24 h, respectively (Fig. 6A). Coincubation with GIP for 20 min did not increase insulin-induced glucose uptake at insulin concentrations ranging from 1 nM to 100 nM (Fig. 6B). However, prolonged pretreatment with GIP for 6 h dose-dependently reduced insulin-stimulated glucose uptake by 67% at 1 nM GIP and by 74% at 100 nM GIP (Fig. 6C). Time-response experiments with 1 nM GIP revealed a reduction of insulin-induced glucose uptake by 64% after 2 h, by 77% after 4 h, and by 59% after 6 h (Fig. 6D). Pretreatment with the GIP-R antagonist GIP(6–30) 15 min prior to stimulation with 1 nM GIP for 6 h completely prevented GIP-induced insulin resistance, indicating specificity (Fig. 6E). Neither pretreatment with the PKA inhibitor H-89, the IKK inhibitor H-89, the IKKβ inhibitor sc-514, the IL-1 receptor antagonist IL-1Ra nor a blocking anti-IL-6 receptor antibody or orlistat was effective to protect from GIP-induced insulin resistance (data not shown).

To further unravel the mechanism underlying GIP-induced insulin resistance, we studied the GIP effect on GLUT1 and GLUT4. GIP treatment slightly increased GLUT1 mRNA expression 1.5 ± 0.18-fold after 6 h (Fig. 6G), whereas GLUT4 mRNA expression tended to be reduced over time (Fig. 6G). Further investigating GLUT4 translocation to the PM revealed an impairment of insulin-induced GLUT4 translocation upon 1 nM GIP treatment for 6 h (Fig. 6I). Interest-
EFFECTS OF GIP ON HUMAN ADIPOCYTES

A

Glucose uptake (Fold of control)

GIP 1 nM (hrs) - 6 24

B

Glucose uptake (Fold of control)

GIP 1 nM - + + + + +

C

Glucose uptake (Fold of control)

GIP 1 nM - + + + + +

D

Glucose uptake (Fold of control)

GIP 1 nM (hrs) - - 1 2 4 6

Insulin 100 nM - + + + + +

E

Glucose uptake (Fold of control)

GIP 1 nM - + + + + +

Insulin 100 nM - + + + + +

F

IB : p(Ser473)Akt

IB : Akt

G

IRS-1 mRNA expression (Fold of control)

GIP 1 nM (6h) - - - -

Insulin 100 nM - - - -

H

IRS-1 protein level (Fold of control)

GIP 1 nM (hrs) - 6

P=0.08

I

GLUT-4 mRNA expression (Fold of control)

GIP 1 nM (6h) - - - +

Insulin 100 nM - + + +

GLUT-1 mRNA expression (Fold of control)

GIP 1 nM (hrs) - 1 6 24

GLUT-4 protein level (Fold of control)

GIP 1 nM (6h) - + - +
ingly, GIP alone slightly induced GLUT4 translocation to the PM, although statistical significance was not reached (Fig. 6f).

DISCUSSION

Ablation of GIP receptor activation protects against diet-induced obesity and insulin resistance (19, 42), pointing to an important role of GIP in adipose tissue. Investigating the effects of GIP in nonmodified preadipocyte-derived human adipocytes revealed an inflammatory and prolipolytic response along with a mild increase in basal glucose uptake but impaired insulin sensitivity after prolonged exposure.

The pattern of cytokines induced was characterized by increased expression of IL-1β, IL-6, and IL-1Ra mRNA, while expression of TNFα, IL-8, and MCP-1 mRNA remained unaffected. In our study, GIP induced IL-6 mRNA expression showed a short lasting peak at 1 h, which is in line with a recent report demonstrating GIP-induced cytokine expression in a GIP receptor-overexpressing 3T3-cell line (47). In contrast to our findings, Nie Y et al. (47) observed the induction of TNFα and MCP-1 along with IL-6 in GIP receptor-overexpressing 3T3 cells. The differences in the cytokine expression pattern are most probably attributable to the different cell models used. Although GIP induced cytokine mRNA expression in our cells, protein secretion of IL-6 and IL-1Ra upon GIP was observed only in the presence of IL-1β, TNFα, or LPS. Full activation of cytokine processing and release often requires two separate hits, most probably to prevent overwhelming inflammation (15, 54). IL-6 mRNA is unstable and degraded with a half-life of about 30 min (48). This can be overcome by IL-1 or LPS (43, 65), which stabilize IL-6 mRNA (9, 46, 62), leading to translation and protein secretion of IL-6. Similarly, the presence of IL-1β or IL-1α is needed for IL-1Ra secretion in human adipocytes (8, 32). The synergistic stimulation of IL-6 and IL-1Ra released by GIP in the presence of IL-1β, LPS, or TNFα is of physiological relevance, since, in the setting of obesity, levels of IL-1β and TNFα are locally elevated in adipose tissue, inducing low-grade inflammation (27, 28). Note, LPS levels are elevated postprandially and chronically after high-fat diet (4, 14). In the settings of low-grade inflammation, GIP might further promote chronic inflammation in adipose tissue while in a noninflamed environment as in the fasting state, and in metabolically healthy individuals GIP might not exert any substantial inflammatory activity.

In addition, our data provide evidence that GIP is a regulator of IL-1Ra in human adipocytes. In line with this observation, elevated plasma levels of GIP and IL-1Ra have been observed in obesity (40, 60), which drop after intestinal bypass surgery (40, 52).

GIP activates cyclic AMP and its downstream signaling module PKA via GPCR (10, 38). In line with our previous finding that GIP increases cAMP in human adipocytes (57), inhibition of PKA abolished GIP-induced expression of IL-6, IL-1β, and IL-1Ra. In addition, GIP has been shown to activate the NF-kB pathway in a GIP receptor-overexpressing 3T3 cell line (47). Accordingly, in our human adipocytes, GIP reduced IkBα protein expression that was prevented by an IKKβ inhibitor as was GIP-induced cytokine expression. Both PKA inhibitors H-89 and KT5720 prevented GIP-induced cytokine mRNA expression. Using an antibody directed against p(Ser/Thr)PKA substrate, indicative of the activation of PKA, revealed an inhibitory effect of sc-514 on PKA substrate phosphorylation. Therefore, we conclude that GIP-induced PKA activation is partly dependent on NF-κB pathway activation in our experimental setting, indicative of a close interaction of the PKA and the NF-κB pathway.

Increased lipolysis is a feature of inflamed adipose tissue in obesity. Lipolysis is induced by cytokines like IL-1β, IL-6, and TNFα (31, 53), while FFAs emerging from lipolysis induce cytokine expression in adipocytes in a positive feedback loop. It has been demonstrated that, when released in parallel to glycerol upon lipolysis in adipocytes, FFAs leave the adipocyte to be then taken up again and used for reesterification (12). The induction of lipolysis by GIP in adipocytes has been previously reported (38). For the first time, we demonstrated that GIP-induced lipolysis involves phosphorylation of HSL at Ser552 in human adipocytes that is sensitive to orlistat. Furthermore, we showed that both H-89 and sc-514 inhibited GIP-induced lipolysis at the level of glycerol release as well as at the level of (Ser552)HSL phosphorylation, indicative of a close link between the PKA and the IKKβ/NF-κB pathways in GIP-induced lipolysis. The close interaction between lipolysis and inflammation was also demonstrated by the finding that GIP-induced cytokine expression was inhibited by orlistat. Investigating other cellular stress-activated kinases using specific inhibitors revealed that MAPK1/2 and JNK are also involved in lipolysis stimulated by GIP in human adipocytes. However, our results did not point to an involvement of p38 MAPK, since SB-203580 prevented neither GIP-induced glycerol release nor GIP-induced phosphorylation of (Ser552)HSL (data not shown). In line with the observations by Nie Y et al. (47), showing that the IKKβ/NF-κB module and stress kinases as well as the PKA pathway are partially involved in GIP-induced cytokine expression in 3T3 adipocytes , our results indicate the involvement of numerous signaling pathways in GIP-induced lipolysis. This might be explained by a prolipolytic effect of cytokines that are released upon GIP accompanying the raw GIP effect and leading to the activation of several different signaling pathways. The observation that GIP-induced lipolysis was partially reduced by IL-1Ra strengthens this hypothesis.

While the induction of glycerol release upon GIP was in line with previous reports (22, 38), the low lipolytic response upon forskolin in our cell system was most probably related to our experimental design lacking albumin supplementation (2, 21). Performing our experiments in the presence of albumin revealed a higher prolipolytic response upon forskolin and GIP. In line with previous reports (22), GIP slightly reduced forskolin-stimulated glycerol release, although significance was not reached. While forskolin induced FFA release, no FFA release was detected upon GIP treatment, indicating that lipolysis induced by GIP is accompanied by FFA reesterification, in line with previous observations (22). However, it might also be possible that the decrease in extracellular FFA levels in parallel to an increase in glycerol levels results from an increase in fatty acid oxidation (59). While GIP is inducing lipolysis and glycerol release in the absence of insulin, GIP-induced lipolysis is inhibited in the presence of insulin (38). This might explain why in vivo studies in humans, conducted in the presence of insulin, did not show a prolipolytic GIP effect (23).

The vicious cycle of lipolysis and cytokine expression contributes to insulin resistance in adipose tissue (31). While
IL-1β induces insulin resistance in adipocytes (30), the role of IL-6 is unclear. On the one hand, IL-6 is upregulated in adipose tissue in obesity and has been shown to induce insulin resistance (35); on the other hand, IL-6 is secreted from contracting skeletal muscle during exercise (49) and has been shown to improve whole body insulin sensitivity (5, 26) and to induce GLP-1 production (13). Since GIP induced the expression of the cytokines IL-6 and IL-1β as well as lipolysis, we investigated the effects of short- and long-term GIP treatment on insulin-induced glucose uptake. Although a short time exposure to GIP has been shown to augment insulin-stimulated glucose uptake in 3T3 adipocytes in vitro (56), chronic GIP treatment in vivo induces insulin resistance (3). In our experimental setting, GIP alone slightly induced glucose uptake in human adipocytes after 6 and 24 h albeit to a lower extent than previously described in murine adipocytes (56). Interestingly and in line with previous reports (44, 56), GIP alone tended to increase GLUT4 translocation. We found no additive effect of GIP on insulin-induced glucose uptake in the short term, in line with the results reported by Mohammad et al., demonstrating an augmenting effect of GIP on insulin-induced glucose uptake only at insulin concentrations up to 0.2 nM, whereas at insulin concentrations of 1 nM or higher, treatment with GIP did not augment the insulin effect (45). In addition, Mohammad et al. showed that GIP acts as an insulin sensitizer and has no significant effect on adipocyte glucose uptake in the absence of insulin in the short term. In line with observations in vivo (3, 7, 37) and in vitro (47), prolonged GIP treatment for up to 6 h inhibits insulin-induced glucose uptake. Regarding the underlying mechanisms of GIP-induced insulin desensitization, we detected a clear impairment of insulin-induced GLUT4 translocation to the PM upon long-term treatment with GIP, providing a reasonable mechanism for GIP-induced insulin resistance in human adipocytes. Accordingly, we found a tendency to decreased mRNA GLUT4 levels and a significant increase in GLUT1 upon long-term GIP treatment. Interestingly, GIP treatment for up to 6 h did not affect Akt phosphorylation at Ser473 in line with previous reports (47). Although GIP induced a downregulation of IRS-1 mRNA over time, IRS-1 protein levels were not significantly altered, indicating that IRS-1 is not essentially involved in GIP-induced insulin resistance.

A recent study in GIP-overexpressing mice reported reduced adipose tissue macrophage infiltration and expression of genes involved in inflammatory signaling (35). This is in apparent contrast to our data showing GIP-induced cytokine expression. However, GIP-overexpressing mice showed markedly reduced fat mass and body weight due to reduced energy intake (35). Therefore, it is difficult to assess whether reduced inflammation was due to a direct GIP effect or indirectly via body weight decrease in this study.

In summary, we report that GIP induces inflammation and lipolysis in human adipocytes and that the classical inflammatory and lipolytic signaling pathways are closely linked. Furthermore, we have found that GIP substantially potentiates the inflammatory effects of LPS, IL-1β, and TNFα in human adipocytes. Importantly, we have demonstrated that long-term GIP treatment impairs insulin-induced glucose uptake in human adipocytes, possibly via inhibition of GLUT4 translocation to the plasma membrane. These results demonstrate that, besides its known metabolic function, GIP is able to directly modulate inflammation and insulin sensitivity in human adipocytes. This may play a particular role in patients with type 2 diabetes, where GIP levels are increased, and, furthermore, adipocytes remain sensitive to GIP effects while the insulinotropic activity of GIP is blunted (41). The ability of GIP to stimulate cytokines in an already inflamed environment and to induce insulin resistance should be taken into consideration in the pharmacological approach of GIP analogs for treatment of patients with obesity and type 2 diabetes.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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