Increased GIP signaling induces adipose inflammation via a HIF-1α-dependent pathway and impairs insulin sensitivity in mice

Shu Chen, Fumiaki Okahara, Noriko Osaki, and Akira Shimotoyodome

Biological Science Laboratories, Kao Corporation, Tochigi, Japan

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Objective—In the present study, we examined the proinflammatory effect of increased GIP signaling in adipose tissue inflammation. We administered GIP intraperitoneally to misty (lean) and db/db (obese) mice and examined adipose tissue inflammation and insulin sensitivity. We also examined the effects of GIP and hypoxia on expression of the GIP receptor (GIPR) gene and proinflammatory genes in 3T3-L1 adipocytes. GIP administration increased monocyte chemoattractant protein-1 (MCP-1) expression and macrophage infiltration into adipose tissue and increased blood glucose in db/db mice. GIPR and hypoxia-inducible factor-1α (HIF-1α) expressions were positively correlated in the adipose tissue in mice. GIP expression increased dramatically in differentiated adipocytes. GIP treatment of adipocytes increased MCP-1 and interleukin-6 (IL-6) production. Adipocytes cultured either with RAW 264 macrophages or under hypoxia expressed more GIPR and HIF-1α, and GIP treatment increased gene expression of plasminogen activator inhibitor 1 and IL-6. HIF-1α gene silencing diminished both macrophage- and hypoxia-induced GIPR expression and GIP-induced IL-6 expression in adipocytes. Thus, increased GIP signaling plays a significant role in adipose tissue inflammation and thereby insulin resistance in obese mice, and HIF-1α may contribute to this process.

Methods—We administered GIP intraperitoneally to misty (lean) and db/db (obese) mice. GIPR and HIF-1α expressions were positively correlated in the adipose tissue in mice. GIP expression increased dramatically in differentiated adipocytes. GIP treatment of adipocytes increased MCP-1 and interleukin-6 (IL-6) production. Adipocytes cultured either with RAW 264 macrophages or under hypoxia expressed more GIPR and HIF-1α, and GIP treatment increased gene expression of plasminogen activator inhibitor 1 and IL-6. HIF-1α gene silencing diminished both macrophage- and hypoxia-induced GIPR expression and GIP-induced IL-6 expression in adipocytes. Thus, increased GIP signaling plays a significant role in adipose tissue inflammation and thereby insulin resistance in obese mice, and HIF-1α may contribute to this process.

Results—Glucose-dependent insulinotropic polypeptide (GIP) is a gut hormone secreted in response to dietary fat and glucose. The blood GIP level is elevated in obesity and diabetes. GIP stimulates proinflammatory gene expression and impairs insulin sensitivity in cultured adipocytes. In obesity, hypoxia within adipose tissue can induce inflammation. The aims of this study were (1) to examine the proinflammatory effect of increased GIP signaling in adipose tissues in vivo and (2) to clarify the association between GIP and hypoxic signaling in adipose tissue inflammation. We administered GIP intraperitoneally to misty (lean) and db/db (obese) mice and examined adipose tissue inflammation and insulin sensitivity. We also examined the effects of GIP and hypoxia on expression of the GIP receptor (GIPR) gene and proinflammatory genes in 3T3-L1 adipocytes. GIP administration increased monocyte chemoattractant protein-1 (MCP-1) expression and macrophage infiltration into adipose tissue and increased blood glucose in db/db mice. GIPR and hypoxia-inducible factor-1α (HIF-1α) expressions were positively correlated in the adipose tissue in mice. GIP expression increased dramatically in differentiated adipocytes. GIP treatment of adipocytes increased MCP-1 and interleukin-6 (IL-6) production. Adipocytes cultured either with RAW 264 macrophages or under hypoxia expressed more GIPR and HIF-1α, and GIP treatment increased gene expression of plasminogen activator inhibitor 1 and IL-6. HIF-1α gene silencing diminished both macrophage- and hypoxia-induced GIPR expression and GIP-induced IL-6 expression in adipocytes. Thus, increased GIP signaling plays a significant role in adipose tissue inflammation and thereby insulin resistance in obese mice, and HIF-1α may contribute to this process.

Discussion—Our results are in line with previous studies showing that increased GIP signaling in obesity and type 2 diabetes plays an important role in the pathogenesis of insulin resistance by inducing adipose tissue inflammation. To date, however, no direct evidence has been available on the role of GIP signaling in adipose tissue inflammation or insulin resistance in vivo.

Conclusion—On the basis of these findings, we hypothesized that increased GIP signaling in obesity and type 2 diabetes plays an important role in the pathogenesis of insulin resistance by inducing adipose tissue inflammation. To date, however, no direct evidence has been available on the role of GIP signaling in adipose tissue inflammation or insulin resistance in vivo.
may interact with hypoxic signaling to cause adipose tissue inflammation. However, the association between GIP and hypoxic signaling in adipose tissue dysregulation is not clear. Therefore, the aims of the present study were 1) to examine whether increased GIP signaling has a proinflammatory effect in adipose tissues in vivo and 2) to clarify the association between GIP and hypoxic signaling in adipose tissue inflammation. Because hypoxia dramatically increased GIPR gene expression in adipocytes, we also investigated whether HIF-1 signaling is involved in the hypoxia-induced GIPR gene expression in adipocytes.

Table 1. Composition of the experimental diet

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*The fatty acid composition of the triglycerides was 35.24% oleic acid, 2.00% stearic acid, 48.15% linoleic acid, and 14.61% others.

Fig. 1. Glucose-dependent insulinotropic polypeptide (GIP) treatment induces adipose tissue inflammation in mice. A–D: GIP (10 nmol/kg body wt) was administered intraperitoneally to lean (misty) and obese (db/db) mice twice daily for 1 wk. Gene expression levels of monocyte chemoattractant protein-1 (MCP-1) in (A) retroperitoneal and (B) perirenal fat, (C) IL-6 in epididymal fat, and (D) F4/80 in retroperitoneal fat were determined with qRT-PCR. Open bars, saline group; filled bars, GIP group. Data are presented as means ± SE; n = 6. *P < 0.05, **P < 0.01; N.S., not significant (Student’s t-test). E: F4/80 protein localization in retroperitoneal fat was examined with immunohistochemistry in misty and db/db mice administered either saline or GIP. Arrows indicate “crown-like-structures”.

E415 GIP INDUCES ADIPOSE INFLAMMATION VIA HIF-1α

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MATERIALS AND METHODS

GIP treatment of mice. Male BKS.Cg-Dock7m+/+.Leprdb+/- (db/db) and misty mice (6–10 wk old; Charles River Laboratories Japan, Yokohama, Japan) were housed three per cage in a room with controlled temperature and relative humidity (23 ± 2.0°C, 55 ± 10%) and a 12:12-h light-dark cycle. Six mice were assigned to each group in such a way that the average body weight of each group was equivalent. Mice were maintained for 1 or 4 wk on the designated diet (Table 1) and water was provided ad libitum.

During the experimental period, db/db mice and misty mice were intraperitoneally administered mouse GIP (AnaSpec, Fremont, CA; 10 nmol/kg body wt) twice daily (at 0900 and 1700) for 1 wk, or mouse GIP (5 nmol/kg) once daily (at 0900) for 4 wk. For negative control mice, the same amount of saline (Otsuka Pharmaceutical, Tokyo, Japan) was administered.

Mice were anesthetized after 1 or 4 wk of GIP administration by inhalation of isoflurane (Abbott Japan, Osaka, Japan). Blood samples were collected from the abdominal vein into capillary blood collection tubes (CAPIJECT with EDTA-2Na; Terumo Medical, Tokyo, Japan) and maintained on ice until plasma preparation. After centrifugation at 3,500 g for 15 min at 4°C, plasma samples were stored at −80°C until analysis. The white adipose tissues (epididymal, mesenteric, perirenal, retroperitoneal, and inguinal) were removed and also stored at −80°C.

After 1 wk of GIP or saline administration, expressions of inflammatory genes and macrophage infiltration were analyzed with quantitative reverse transcription (qRT)-PCR and immunohistochemistry, respectively.

All animal experiments were conducted in the Experimental Animal Facility of Kao Tochigi Institute. The Animal Care Committee of Kao Corporation approved the present study.

Fig. 2. GIP treatment increases blood glucose in mice. A and B: GIP (5 nmol/kg body wt) or saline was administered intraperitoneally to db/db and misty mice daily for 3 wk. The time course of blood glucose changes before (time 0) and for 2 h after insulin administration in the insulin tolerance test (ITT) is shown for (A) db/db and (B) misty mice. Data are presented as means ± SE, n = 6, and were analyzed by repeated-measures two-way ANOVA. C: Fasting blood glucose levels were measured after 4 wk of GIP administration. Open bars, saline group; filled bars, GIP group. Data are presented as means ± SE, n = 6. *P < 0.05 (Student’s t-test).

MICE WERE ANESTHETIZED AFTER 1 OR 4 WK OF GIP ADMINISTRATION BY INHALATION OF ISOFLURANE (ABBOTT JAPAN, OSAKA, JAPAN). BLOOD SAMPLES WERE COLLECTED FROM THE ABDOMINAL VEIN INTO CAPILLARY BLOOD COLLECTION TUBES (CAPIJECT WITH EDTA-2Na; TERUMO MEDICAL, TOKYO, JAPAN) AND MAINTAINED ON ICE UNTIL PLASMA PREPARATION. AFTER CENTRIFUGATION AT 3,500 g FOR 15 min AT 4°C, PLASMA SAMPLES WERE STORED AT −80°C UNTIL ANALYSIS. THE WHITE ADIPOSE TISSUES (EPIDIDYMAL, MESENTERIC, PERIRENAL, RETROPERITONEAL, AND INGUINAL) WERE REMOVED AND ALSO STORED AT −80°C.

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Fig. 3. GIP receptor (GIPR) and hypoxia-inducible factor-1α (HIF-1α) gene expressions in mice. A and B: adipose tissues (epididymal, mesenteric, perirenal, retroperitoneal, and inguinal) and pancreas were removed from misty and db/db mice after 1 wk of saline or GIP administration. Gene expression levels of GIPR and HIF-1α in (A) the adipose tissues (n = 120) and (B) pancreas (n = 24) of misty and db/db mice were determined with qRT-PCR. A Pearson’s correlation coefficient (r) was obtained to estimate the linear correlation between GIPR and HIF-1α expression.

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All animal experiments were conducted in the Experimental Animal Facility of Kao Tochigi Institute. The Animal Care Committee of Kao Corporation approved the present study.
Insulin tolerance test. An insulin tolerance test (ITT) was performed after 3 wk of GIP or saline administration. The mice were fasted for 5 h before the ITT. Glucose levels in tail blood were measured with a blood glucose self-monitoring device (ACCU-CHEK Aviva; Roche Diagnostics, Tokyo, Japan) before and 30, 60, 90, and 120 min after an intraperitoneal injection of human insulin (db/db, 1 U/kg body wt; misty, 0.5 U/kg body wt).

Gene expression analysis by qRT-PCR. Total RNA was extracted by using an RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan) or RNeasy Lipid Tissue Mini Kit (QIAGEN). Reverse transcription was performed by using a High-Capacity cDNA Kit with random primers on a Veriti 96-well Thermal Cycler (Life Technologies Japan, Tokyo, Japan). qRT-PCR was performed using a TaqMan probe (Life Technologies) in an ABI ViiA 7 Real-Time PCR System (Life Technologies). All data were normalized to acidic ribosomal protein P0 (36B4) content.

Immunohistochemical analysis. A small piece of retroperitoneal adipose tissue was fixed in 4% paraformaldehyde. After paraffin embedding, 3-μm tissue sections were deparaffinized and then treated with methanol containing 1% (vol/vol) H2O2. Antigen retrieval was performed by autoclaving for 120 min. Then the tissues were incubated with a rat anti-mouse F4/80 antibody (MCA497R, 1:50; AbD Serotec, Kidlington, UK) overnight at 4°C. Bound F4/80 antibodies were detected by using Histofine Simple Stain Mouse MAX-PO (Nichirei Biosciences, Tokyo, Japan). All light-field images were captured by using a BIOREBO BZ-9000 microscope (Keyence, Osaka, Japan).

Plasma analysis. Blood glucose was determined with a Glucose CII test (Wako Pure Chemical Industries, Osaka, Japan). Plasma GIP was measured with a rat/mouse total GIP ELISA kit (Millipore, Tokyo, Japan), and insulin was determined with a mouse insulin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan).

Monoculture of 3T3-L1 adipocytes and GIP treatment. 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, Tokyo, Japan) containing 10% FBS and penicillin-streptomycin (Life Technologies) and incubated in an atmosphere containing 5% CO2 at 37°C. Differentiation of 3T3-L1 preadipocytes

Fig. 4. Time course of gene expression during adipocyte differentiation. Premature 3T3-L1 adipocytes were differentiated with 10 mg/l insulin, 1 μmol/l dexamethasone, and 0.5 mmol/l 3-isobutyl-1-methylxanthine for 2 days, followed by 5 mg/l insulin in DMEM for another 2 days, and with DMEM without insulin for a further 12 days. Gene expression levels for (A) GIPR, (B) C/EBPα, (C) aP2, (D) C/EBPβ, (E) PPARγ, (F) SREBP-1c, (G) HIF-1α, and (H) VEGFa at indicated time points were examined with qRT-PCR. Data are presented as means ± SE, n = 3.
into mature adipocytes was performed by using the same medium containing 10 mg/l insulin (Sigma-Aldrich), 1 μmol/l dexamethasone (Sigma-Aldrich), and 0.5 mmol/l 3-isobutyl-1-methylxanthine (Sigma-Aldrich) for 2 days. The medium was then replaced with DMEM containing 5% CO2 at 37°C. Differentiated 3T3-L1 adipocytes were cocultured with RAW 264 cells (European Collection of Cell Cultures, Salisbury, UK) were maintained in DMEM containing 10% FBS and penicillin-streptomycin, and incubated in an atmosphere containing 2% O2 for 24 h. Differentiated 3T3-L1 adipocytes were detached from the plates and were transfected with 10 nM of a HIF-1α small interfering RNA (siRNA; GeneSolution siRNA, QIAGEN) or negative control siRNA (Life Technologies) by using Lipofectamine RNAiMAX (Life Technologies). Optimal transfection conditions were identified in preliminary experiments, in which the knockdown efficiency at 24 h after transfection was determined.

The siRNA-treated adipocytes were cocultured with RAW 264 cells or incubated in an atmosphere containing ≤2% O2 for 24 h. After 24 h of coculture with RAW 264 cells, the adipocytes were stimulated with GIP (1–42) for 1 h.

Western blotting. 3T3-L1 adipocytes were lysed with CellLytic M (Sigma-Aldrich). Protein concentrations were measured with a BCA Protein Assay (Thermo Fisher Scientific K.K., Yokohama, Japan). Each sample (10–20 μg) was loaded onto a 4–15% Criterion gel for electrophoresis (Bio-Rad Laboratories, Hercules, CA). Proteins were then transferred to polyvinylidene difluoride membranes and incubated with primary antibodies, followed by an HRP-linked anti-rabbit or anti-mouse IgG secondary antibody (Cell Signaling Technology Japan K.K., Tokyo, Japan). Signals were detected by using an ECL Prime Western Blotting Detection System (GE Healthcare Japan, Tokyo, Japan). The O2 concentration was monitored with an oxygen meter (OXY-1, attachment for the kit). The cells were cultured in an atmosphere containing 21% O2 for 3 or 6 h for reoxygenation. Cobalt chloride (Wako) and deferoxamine mesylate salt (Sigma-Aldrich, 50–200 μM), activators of HIF-1α, were used as hypoxia mimic agents, and cells were collected after a 24-h incubation.

HIF-1α knockdown in adipocytes by RNA interference. Differentiated 3T3-L1 adipocytes were detached from the plates and were transfected with 10 nM of a HIF-1α small interfering RNA (siRNA; GeneSolution siRNA, QIAGEN) or negative control siRNA (Life Technologies) by using Lipofectamine RNAiMAX (Life Technologies). Optimal transfection conditions were identified in preliminary experiments, in which the knockdown efficiency at 24 h after transfection was determined.

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tein-1 (MCP-1) ELISA kits were purchased from Biolegend (San Diego, CA).

**Statistical analysis.** Data are expressed as means ± SE. Student’s *t*-test was used for single comparisons. One-way ANOVA followed by Tukey’s post hoc test was used for multiple comparisons. Two-way ANOVA (repeated measures) was used for the ITT analysis. Associations between two parameters were analyzed using Pearson’s correlation coefficient. The threshold for significance was *P* < 0.05.

**RESULTS**

GIP treatment increases proinflammatory gene expression and macrophage infiltration in abdominal adipose tissue in obese (db/db) mice. In a preliminary experiment, intraperitoneal GIP administration significantly increased blood GIP levels in both misty and db/db mice without affecting blood glucose or insulin levels (data not shown). After GIP treatment for 4 wk, blood GIP levels were significantly higher in the GIP-treated misty mice than in the saline-treated misty mice and tended to be higher in the GIP-treated db/db mice than in the saline-treated db/db mice, although blood insulin and glucose levels were similar between GIP-treated and saline control groups (data not shown). Both misty and db/db mice treated with GIP for 1 wk had significantly higher levels of MCP-1 mRNA in the retroperitoneal fat than the corresponding control mice (Fig. 1A). Messenger RNA expression of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) did not differ between the groups (data not shown). GIP-treated db/db mice, but not misty mice, had significantly more MCP-1 mRNA in the perirenal fat (Fig. 1B) and IL-6 mRNA in the epididymal fat (Fig. 1C) than did the control mice. F4/80 mRNA was significantly higher in GIP-administered misty mice than in the control misty mice, whereas F4/80 expression was similar between the GIP-treated and control db/db groups (Fig. 1D). However, immunohistochemistry showed that GIP-treated db/db mice had more F4/80-positive cells with a “crown-like-structure” (26) in the retroperitoneal fat than did the control db/db mice (Fig. 1E), whereas no notable change was found in the misty mice.

**GIP treatment increases blood glucose levels in obese (db/db) mice.** db/db mice treated with GIP for 3 wk had significantly higher blood glucose levels during the ITT than did saline-treated control mice (Fig. 2A). After 4 wk of GIP treatment, fasting blood glucose levels were significantly higher in GIP-treated db/db mice than in the control db/db mice (Fig. 2C). Fasting blood insulin levels were similar between the db/db groups (data not shown). Blood glucose levels during ITT (Fig. 2B) and in the fasting condition (Fig. 2C) were similar between the GIP-treated and control misty mice. Decline of blood glucose from the initial level (∆blood glucose) during ITT was smaller in the GIP-treated misty mice than in the control misty mice (data not shown), but the difference was not statistically significant (*P* = 0.066).

**GIPR gene expression is correlated with HIF-1α gene expression in adipose tissue in mice.** GIPR and HIF-1α gene expression were significantly higher in db/db mice than in misty mice. GIP treatment for 1 wk significantly increased GIPR and HIF-1α mRNA levels in misty mice but not in db/db mice (data not shown). The GIPR mRNA level was positively correlated with the HIF-1α mRNA level in adipose tissue (Fig. 3A), but not in pancreas (Fig. 3B), when mice from all groups (i.e., both strains of mice with and without GIP treatment) were analyzed together. Levels of GIPR and HIF-1α mRNAs in the pancreas were similar between db/db and misty mice (data not shown).

**Gene expression of GIPR, HIF-1α, and VEGFa increases during adipocyte differentiation.** During the differentiation of 3T3-L1 adipocytes, GIPR gene expression slightly increased at 2 h, declined to baseline again by 24 h, and then dramatically increased and peaked on day 6 (Fig. 4A). CCAAT/enhancer binding protein (CEBP)δ gene expression peaked at 0.5 h and then declined approximately to baseline by 2 h (Fig. 4B). Gene expression of other adipocyte differentiation markers, such as adipocyte protein-2 (aP2) (Fig. 4C), CEBPα (Fig. 4D), peroxisome proliferator-activated receptor (PPAR)γ (Fig. 4E), and sterol regulatory element-binding protein (SREBP)-1c (Fig. 4F). Gene expression in adipocytes cultured with or without macrophages or macrophage-conditioned media. A: differentiated 3T3-L1 adipocytes were cultured with (CO) or without (CT) RAW 264 macrophages (1 × 10⁵ cells/well) or with conditioned media (CM) from RAW 264 macrophages. Gene expression of GIPR was determined with qRT-PCR after 24 h of incubation. B: 3T3-L1 adipocytes were cultured with or without RAW 264 macrophages (1 × 10⁵ cells/well) or treated with IL-6 (5 ng/ml), TNFα (10 ng/ml), IL-1β (1 ng/ml), or PAI-1 (10 µg/ml) for 24 h. C: differentiated 3T3-L1 adipocytes were cultured with or without RAW 264 macrophages (1 × 10⁵ cells/well), and gene expressions of MCP-1, IL-6, PAI-1, leptin, and adiponectin were determined with qRT-PCR after 24 h of incubation. Open bars, without RAW 264; closed bars, with RAW 264. Data are presented as means ± SE, *n* = 4. **P < 0.01, ***P < 0.001 (Student’s *t*-test).
4F), peaked 6–8 days after the initiation of differentiation. Like GIPR, HIF-1α, and vascular endothelial growth factor-a (VEGFα) mRNA also had two temporal peaks, at 2 h and 8 days, after the initiation of differentiation (Fig. 4, G and H).

GIP treatment of mature adipocytes increases proinflammatory adipokine production. In a preliminary experiment, GIP treatment did not cause cytotoxicity in adipocytes (data not shown). Treatment of differentiated 3T3-L1 adipocytes on day 6 with GIP significantly increased IL-6 mRNA expression in a dose-dependent manner (Fig. 5A). Treatment of the cells with 100 nM GIP significantly increased the MCP-1 mRNA level (Fig. 5B) and the protein levels of IL-6 and MCP-1 in culture media (Fig. 5, C and D, respectively).

Coculture of adipocytes with macrophages, but not with macrophage-conditioned medium, increases GIPR gene expression. Coculture of 3T3-L1 adipocytes with RAW 264 macrophages substantially upregulated GIPR mRNA levels in the adipocytes (Fig. 6, A and B). Neither conditioned medium from RAW264 cells nor proinflammatory cytokines [IL-6, TNFα, and plasminogen activator inhibitor (PAI)1] affected TNFα or IL-1β gene expression (Fig. 6C). Although the coculture significantly increased expression of proinflammatory genes (MCP-1, IL-6, and PAI1) and significantly decreased leptin and adiponectin gene expression in the adipocytes (Fig. 6C). Only culture with IL-1β significantly increased GIPR mRNA expression in the adipocytes (Fig. 6D).

GIP treatment of adipocytes cocultured with macrophages aggravates adipokine dysregulation. In 3T3-L1 adipocytes cultured with RAW 264 macrophages, treatment with GIP (100 nM) significantly increased mRNA levels of IL-6 (Fig. 7A) and PAI1 (Fig. 7B) and significantly decreased those of leptin (Fig. 7C) and adiponectin (Fig. 7D).

Hypoxia and HIF-1α activators increase GIPR mRNA expression in adipocytes. Exposure to hypoxia (2% O2) significantly augmented GIPR gene expression in 3T3-L1 adipocytes; this increase was diminished by reoxygenation in a time-dependent manner (Fig. 8A). Hypoxia significantly increased the mRNA levels of glucose transporter 1 (GLUT1; Fig. 8B) but did not affect the levels of HIF-1α mRNA (Fig. 8C). Both CoCl2 and deferoxamine, which activate HIF-1α (7, 13), significantly increased gene expression of GIPR (Fig. 8D) and GLUT1 (Fig. 8E) in a dose-dependent manner.

HIF-1α knockdown diminishes both macrophage- and hypoxia-induced GIPR gene expression in adipocytes. Culture of 3T3-L1 adipocytes with RAW 264 macrophages (Fig. 9A) or under hypoxic conditions (Fig. 9B) increased HIF-1α protein levels in the adipocytes. Downregulation of HIF-1α mRNA in the adipocytes by a specific siRNA (Fig. 9C) significantly diminished the increase in GIPR gene expression due to either coculture with macrophages (Fig. 9D) or hypoxia (Fig. 9E) as well as the increase in GLUT1 gene expression due to hypoxia (Fig. 9F). MCP-1 gene expression was not affected by HIF-1α gene silencing (data not shown).

HIF-1α knockdown diminishes GIP-induced gene expression of IL-6 in adipocytes cocultured with macrophages. Downregulation of HIF-1α mRNA by specific siRNA (Fig. 10A) decreased GIPR gene expression in the control and GIP-treated adipocytes cocultured with RAW 264 macrophages (Fig. 10B). HIF-1α gene silencing diminished the

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**Fig. 7.** GIP induces adipocyte dysregulation in macrophage coculture. Differentiated 3T3-L1 adipocytes were cultured with RAW 264 macrophages (1 × 10⁵ cells/well) for 24 h and stimulated with GIP (0, 10, or 100 nM) for 1 or 3 h. Gene expressions of IL-6 (after 1 h; A) and PAI-1 (B), leptin (C), and adiponectin (D) (after 3 h) were determined with qRT-PCR. Data are presented as means ± SE, n = 4. *P < 0.05, **P < 0.01 vs. GIP 0 nM (ANOVA with Tukey’s multiple comparison test).
increase in IL-6 mRNA expression induced by GIP treatment in the adipocytes (Fig. 10C).

DISCUSSION

This study had three major findings. First, chronic GIP treatment induced adipose tissue inflammation, as characterized by increased gene expression of MCP-1 and IL-6 and macrophage infiltration into the tissue, and thereby impaired insulin sensitivity in obese mice. Second, mature adipocytes cultured either with macrophages or under hypoxic conditions had increased GIPR and proinflammatory gene expression, and GIP treatment of the adipocytes further increased proinflammatory gene expression. Finally, HIF-1α was involved in the macrophage-induced expression of the GIPR gene and proinflammatory genes in adipocytes. These findings support the hypothesis that increased GIP signaling plays a significant role in adipose tissue inflammation and insulin resistance in obese mice and that the proinflammatory GIP signaling is mediated by a HIF-1α-dependent pathway that augments GIPR and proinflammatory gene expression in adipocytes.

To our knowledge, this is the first study to show evidence that chronic elevation of blood GIP concentrations stimulates proinflammatory gene expression and macrophage infiltration into adipose tissue and impairs insulin sensitivity in mice. These results in mice support the view of Nie et al. (24) and Timper et al. (34), who showed that GIP impairs insulin signaling by inducing adipocyte inflammation in cultured adipocytes. In contrast to the significant increase in MCP-1 and IL-6 gene expression caused by intraperitoneal GIP treatment, neither TNFα nor IL-1β gene expression was changed in our study. Consistent with this, GIP enhanced TNFα gene expression in GIPR-overexpressing adipocytes (24) and that of IL-1β in human adipocytes (34) less than one quarter as much and one half as much, respectively, as it enhanced IL-6 gene expression.
expression. Therefore, the effect of increased GIP signaling on TNFα or IL-1β gene expression may not be meaningful in vivo.

The increase of fasting blood glucose without an accompanying change in insulin levels in GIP-treated obese mice suggests that GIP treatment may affect hepatic glucose production. In fact, expression levels of gluconeogenesis-related genes in the liver, such as fructose-1,6-bisphosphatase-1 (FBP1) and phosphoenolpyruvate carboxykinase (PEPCK), were significantly increased in the GIP-treated obese mice (data not shown). Therefore, hepatic insulin sensitivity was also impaired in db/db mice by treatment with GIP. Because no functional GIPR has been found in liver so far, the results observed in liver seem to be indirect effects of GIP.

In contrast to its effects in obese (db/db) mice, GIP treatment did not increase MCP-1 gene expression in the perirenal fat or that of IL-6 in the epididymal fat in lean (misty) mice, and did not significantly (P = 0.066) impair their insulin sensitivity.

Even though GIP stimulated MCP-1 and F4/80 gene expression in the retroperitoneal fat of the lean mice, gene expression of proinflammatory biomarkers (MCP-1, IL-6, and F4/80) and adipose infiltration of macrophages into the adipose tissues were still much less in lean mice than in obese mice. These results indicate that the adipose tissue inflammation induced by increased concentrations of GIP in the blood might not be large enough to cause insulin resistance in lean mice. Whereas GIPR and HIF-1α gene expression levels were similar between GIP-treated lean and obese mice, blood glucose, insulin, and GIP levels were significantly higher in obese mice than in lean mice (data not shown). Thus, increased GIP signaling may act in coordination with increased blood glucose, insulin, or GIP levels in obese mice to stimulate adipose tissue inflammation and cause insulin resistance.

In our in vitro study, GIPR gene expression exhibited a biphasic increase during adipocyte differentiation, and peaked on day 6 after the induction of differentiation. GIP stimulated...
IL-6 and MCP-1 gene expression and dramatically increased the secretion of proinflammatory proteins from the adipocytes when GIPR expression was at its peak in the adipocytes. Nie et al. (24) showed that GIP did not increase IL-6 and MCP-1 gene expression in 3T3-L1 adipocytes due to low endogenous expression of GIPR but did stimulate proinflammatory gene expression in GIPR-overexpressing adipocytes. Our results are compatible with their findings and suggest that adequate GIPR expression is necessary for the proinflammatory GIP signaling in adipocytes.

Little has been known about the regulation of GIPR expression in mature adipocytes, although PPARγ has been shown to upregulate GIPR expression during adipocyte differentiation (17). Of the most interest, our study provided the first evidence that GIPR expression increased dramatically along with adipocyte differentiation and was further enhanced in mature adipocytes exposed to macrophages or hypoxia. This enhancement was mediated by HIF-1α signaling in the cells. GIPR expression synchronized not only with differentiation markers such as aP2, CEBPα, PPARγ, and SREBP-1c, but also with VEGFα expression in adipocytes, which suggests that increased HIF-1α signaling may promote GIPR expression along with adipocyte differentiation.

HIF-1α signaling is activated under hypoxic conditions and induces genes related to angiogenesis, erythropoiesis, and glycolysis (30). Hypoxia has been shown to impair insulin sensitivity in adipocytes (27). Adipose tissue-specific deletion of HIF-1α protects mice from adipocyte inflammation and insulin resistance induced by a high-fat diet (12, 18). In the present study, GIPR gene expression in adipose tissue was positively correlated with HIF-1α gene expression. In addition, HIF-1α knockdown in adipocytes inhibited GIPR gene expression. These results together suggest that the HIF-1α-mediated increase in GIPR expression may play a major role in adipocyte inflammation and insulin resistance.

Stimulation of HIF-1α signaling in adipocytes does not seem to involve humoral factors from macrophages, because neither the conditioned medium nor the proinflammatory cytokines derived from macrophages increased GIPR expression in the cells. Nalwaya et al. (23) showed that O2 consumption is dramatically increased in activated macrophages in vitro. In addition, contact with macrophages has been shown to dramatically increase fatty acid release from adipocytes (32), which uncouples adipocyte respiration and increased oxygen consumption (18). The cross-talk between adipocytes and macrophages has been suggested to increase O2 consumption in both cell types (18, 23). Therefore, we consider that adjacent macrophages play an important role in the progression of adipose tissue hypoxia. However, further study is needed to clarify the mechanism for the induction of adipose tissue hypoxia by macrophages.

The results of the present study suggest that a vicious cycle involving GIP signaling and macrophage infiltration aggravates adipose tissue inflammation as follows. 1) Expression of GIPR is increased along with adipocyte differentiation, which leads to increased GIP signaling in adipose tissue. 2) Increased GIP signaling in adipocytes induces proinflammatory gene expression, which leads to macrophage infiltration in adipose tissue. 3) Macrophage infiltration stimulates HIF-1α signaling in adipose tissue, which leads to a further increase in GIPR expression. This vicious cycle seems to be extremely important...
for understanding the pathophysiology of insulin resistance in obesity. Proinflammatory GIP signaling may be further exacerbated during the progression of obesity and adipose tissue inflammation. Downregulation of HIF-1α might be a beneficial strategy for interrupting the vicious cycle of proinflammatory GIP signaling in inflamed adipocytes.

The present study had some limitations. Whereas we found that HIF-1α signaling regulated GIP signaling in cultured adipocytes, we have not yet been able to provide evidence on its role in proinflammatory GIP signaling within adipose tissues. Although GIPR and HIF-1α gene expressions were positively correlated in adipose tissue, GIP administration increased adipose GIPR and HIF-1α gene expression only in lean mice and not in obese mice. Conversely, macropage infiltration in adipose tissue and impairment of insulin sensitivity were observed in obese mice but not in lean mice. Considering that obese mice have significantly higher basal GIPR and HIF-1α expressions in adipose tissue than lean mice, transcriptional upregulation of HIF-1α may be saturated in the obese adipose tissue, which results in a lack of further induction of HIF-1α gene expression by GIP. Examination of either HIF-1α protein stabilization or gene expression of biomarkers of HIF-1α signaling will be needed to clarify whether increased blood GIP stimulates HIF-1α signaling in obese adipose tissues.

Further studies are also needed to clarify the role of HIF-1α in GIP-mediated adipocyte dysregulation, since we relied on the effects of HIF-1α silencing in adipocytes, in particular the reduction of GIP-induced IL-6 expression, to understand the function of HIF-1α (Fig. 10C). Because GIPR expression was dramatically decreased in HIF-1α-silenced adipocytes cultured with macrophages, the GIP-mediated decrease in adiponectin should be prevented in HIF-1α-depleted adipocytes. However, neither MCP-1 nor adiponectin expression was changed in the same experimental conditions shown in Fig. 10C.

Naitoh et al. (22) showed that a high-fat diet increased adiponectin expression in GIP-deficient mice, but decreased adiponectin in wild-type mice, compared with those on a control diet. The diet dramatically increased visceral fat accumulation in wild-type mice but not in GIP-deficient mice. Because visceral fat accumulation increases HIF-1α expression in the adipose tissue, HIF-1α might be depleted in the adipose tissue of GIPR-deficient mice and of mice fed a control diet compared with wild-type mice fed a high-fat diet. A high-fat diet increases blood GIP compared with a control diet in wild-type mice. Accordingly, we hypothesize that GIP decreases adiponectin expression in adipose tissue, but HIF-1α depletion may prevent the GIP-mediated decrease in adiponectin expression. Further investigations regarding HIF-1α-mediated proinflammatory GIP signaling in obese adipose tissues, including experiments to determine whether GIP-induced adipose tissue inflammation would be diminished in mice depleted of adipose HIF-1α, will promote our understanding of the pathophysiology of adipose tissue inflammation and insulin resistance induced by obesity or a high-fat diet.

In conclusion, this study provides evidence that increased blood levels of GIP stimulate adipose tissue inflammation in vivo and that HIF-1α plays an important role in regulating proinflammatory GIP signaling in adipocytes. Because the blood GIP level is elevated in obesity (1, 19) and after ingestion of a high-fat diet (4, 29), a decrease in GIP signaling in obesity or in the postprandial state may be a beneficial strategy for reducing adipose tissue inflammation and improving insulin sensitivity. Our results also disclose a novel factor, HIF-1α, as an underlying contributor to GIP-induced adipose inflammation via its upregulation of GIPR. This makes HIF-1α a therapeutic target for the development of GIP-based treatments for obesity and type 2 diabetes. Further studies are in progress to clarify the molecular mechanism of HIF-1α-induced GIPR expression in adipocytes and the efficacy of regulating GIP or HIF-1α signaling in adipose tissues for improvement of glucose homeostasis.

GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: S.C., N.O., and A.S. conception and design of research; S.C. and F.O. performed experiments; S.C. analyzed data; S.C. and N.O. interpreted results of experiments; S.C. prepared figures; S.C. drafted manuscript; S.C. and A.S. edited and revised manuscript; A.S. approved final version of manuscript.

REFERENCES
