

Dipeptidyl Peptidase IV–Resistant [D-Ala²]Glucose–Dependent Insulinotropic Polypeptide (GIP) Improves Glucose Tolerance in Normal and Obese Diabetic Rats

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The therapeutic potential of glucose-dependent insulinotropic polypeptide (GIP) for improving glycemic control has largely gone unstudied. A series of synthetic GIP peptides modified at the NH₂-terminus were screened in vitro for resistance to dipeptidyl peptidase IV (DP IV) degradation and potency to stimulate cyclic AMP and affinity for the transfected rat GIP receptor. In vitro experiments indicated that [D-Ala²]GIP possessed the greatest resistance to enzymatic degradation, combined with minimal effects on efficacy at the receptor. Thus, [D-Ala²]GIP_{1–42} was selected for further testing in the perfused rat pancreas and bioassay in conscious Wistar and Zucker rats. When injected subcutaneously in normal Wistar, Fa/?, or *fa/fa* Vancouver Diabetic Fatty (VDF) Zucker rats, both GIP and [D-Ala²]GIP significantly reduced glycemic excursions during a concurrent oral glucose tolerance test via stimulation of insulin release. The latter peptide displayed greater in vivo effectiveness, likely because of resistance to enzymatic degradation. Hence, despite reduced bioactivity in diabetic models at physiological concentrations, GIP and analogs with improved plasma stability still improve glucose tolerance when given in supraphysiological doses, and thus may prove useful in the treatment of diabetic states. *Diabetes* 51:652–661, 2002

GIP (glucose-dependent insulinotropic polypeptide; also called gastric inhibitory polypeptide; ¹YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGGKNDWKHNITQ⁴²) is an incretin hormone released from the upper small bowel in response to luminal nutrients that amplifies insulin release from pancreatic β-cells (1). GIP modulates ion currents (2–4) and stimulates proximal and distal steps of the exocytotic cascade

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DP IV, dipeptidyl peptidase IV; EC₅₀, half-maximal stimulatory concentration; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; GRH, growth hormone-releasing hormone; HBS, HEPES-buffered saline; HPLC, high-performance liquid chromatography; IC₅₀, half-maximal inhibitory concentration; OGTT, oral glucose tolerance test; RIA, radioimmunoassay; RT, retention time; TFA, trifluoroacetic acid.

(2) by acting on a seven-transmembrane–spanning G-protein–coupled receptor coupled to stimulation of adenylyl cyclase, activation of phospholipase A₂, and increases in intracellular calcium (5,6). In addition to enhancing insulin release, GIP further acts as an insulinotropic agent by stimulating proinsulin gene transcription and translation (7,8) and upregulating plasmalemmal glucose transporters and hexokinase in the β-cell (8). There is currently interest in the potential use of incretins in the treatment of type 2 diabetes, although to date, clinical trials have been limited to glucagon-like peptide-1 (GLP-1) (9).

One of the major drawbacks with the use of either GIP or GLP-1 as potential therapeutic agents is their short duration of action, due to enzymatic degradation in vivo. The enzyme dipeptidyl peptidase IV (DP IV), a serine protease that preferentially hydrolyses peptides after a penultimate NH₂-terminal proline (Xaa-Pro-) or alanine (Xaa-Ala-) (10,11), has been shown to rapidly metabolize GIP and GLP-1 in vitro (12–14). After intravenous injections into rats, the half time of NH₂-terminal truncation of [¹²⁵I]-labeled GIP or GLP-1 was <2 min (12). Sensitive mass spectrometric techniques verified that GIP_{3–42} and GLP-1_[9–36NH₂] were the major degradation products of the incretins when incubated in human serum (15). Enhancement of the enteroinsular axis in DP IV–null mice (16) or inhibition of DP IV activity in vivo (17–20) results in improved glucose tolerance. In patients with type 2 diabetes, GLP-1_[7–36] was found to be rapidly degraded to GLP-1_[9–36] (21), indicating that a defect in DP IV action, resulting in prolonged exposure to intact GIP and GLP-1, does not contribute to the reduced incretin responses in these individuals.

In the current study, the potential of a DP IV–resistant analog of GIP as a therapeutically useful insulinotropic agent has been assessed with both in vitro and in vivo assays. In vitro studies were initially performed with Chinese hamster ovary (CHO-K1) cells transfected with the GIP receptor (wtGIPR cells) to determine the importance of the DP IV–sensitive NH₂-terminal dipeptide, Tyr¹-Ala², for receptor binding and stimulation of cyclic AMP production. It was established that substitution of a D-alanine in position 2 resulted in a peptide, [D-Ala²]GIP, with similar receptor binding and activation characteristics to those of native GIP. Because we had previously shown that this analog was among the most DP IV–

resistant when incubated with purified enzyme, as examined by mass spectrometry (22), it was selected for further study. Chromatographic studies confirmed DP IV resistance *in vitro*, and studies in the isolated perfused rat pancreas and using a bioassay in normal Wistar and lean and obese Vancouver Diabetic Fatty (VDF) Zucker rats established that this analog is able to improve glucose tolerance in these animals. It is therefore a promising therapeutic agent for the treatment of type 2 diabetes.

RESEARCH DESIGN AND METHODS

Peptides. Synthetic human GIP₁₋₄₂, [Ala¹-Tyr²]GIP₁₋₄₂, [D-Ala²]GIP₁₋₄₂, and GIP₃₋₄₂ were purchased from Hukabel (Montreal, Canada) and synthetic porcine GIP₁₋₄₂ from Bachem (Torrance, CA). Batches of GIP₁₋₄₂ (Mass+H⁺: measured, 4,984.7 g/mol; expected, 4,983.6 g/mol) and [D-Ala²]GIP₁₋₄₂ (Mass+Na⁺: measured, 4,998.6 g/mol; expected, 5,002.7 g/mol) were also synthesized (Probiobio, Halle, Germany), using methods described in detail elsewhere (22).

Animals. Male Wistar rats were purchased from the University of British Columbia Animal Care Facility (Vancouver, Canada); male VDF Zucker rats (23) were obtained from the colony maintained in the Department of Physiology, University of British Columbia. Animals were held in group housing with free access to rat food and water, with a 12-h light:dark cycle. Animals were fasted overnight (15–18 h) before experimentation. Anesthesia, where indicated, was achieved with intraperitoneal Somnotol (65 mg/kg sodium pentobarbital; MTC Pharmaceuticals, Cambridge, Canada). Animal experiments conformed to the guidelines set forth by the University of British Columbia Committee on Animal Care and the Canadian Council on Animal Care.

Peptide iodination and purification. Mono-component [¹²⁵I-Tyr¹⁰]GIP₁₋₄₂ (~300 µCi/mg) was prepared according to established protocols (12), and iodination of [D-Ala²]GIP₁₋₄₂ was based on these techniques. Briefly, 1 mCi of carrier-free iodine-125 (PerkinElmer Life Sciences, Boston, MA) was added to 1 nmol peptide dissolved in 100 µl of 0.4 mol/l phosphate buffer (pH 7.5), iodination was initiated by addition of 10 µl chloramine-T (14.2 mmol/l in 0.4 mol/l PO₄ buffer [BDH, Toronto, Canada]) for 15 s, and the reaction was quenched with 20 µl sodium metabisulfite (66.3 mmol/l in 0.4 mol/l PO₄ buffer [Fisher Scientific, Fair Lawn, NJ]). Radiolabeled peptide was separated from free iodine by gel filtration (Sephadex G-15 [Pharmacia, Uppsala, Sweden]) in 0.2 mol/l acetic acid with 0.5% BSA (RIA fraction V; Sigma Chemical, St. Louis, MO) and 2% Trasylol (aprotinin; Bayer, Etobicoke, Canada). Labeled peptides were then purified by high-performance liquid chromatography (HPLC) to yield an apparent single molecular species, as described below. Iodinated products of [D-Ala²]GIP₁₋₄₂ have not been previously characterized; five major peaks were identified on the chromatogram, and peaks 2 and 4 showed the greatest specific binding at the GIP receptor (data not shown; protocol described below). Peak 2 eluted with a similar retention time as that of [¹²⁵I-Tyr¹⁰]GIP₁₋₄₂ and appeared to be the predominant iodinated product, and thus was used in subsequent experiments.

DP IV degradation studies. Purified pork kidney DP IV was prepared as previously described (24) to a specific activity of 31.2 units/mg. The hydrolysis of iodinated peptides by purified DP IV was studied as previously described (12). In brief, radiolabeled peptides (750,000 cpm/150 µl) were incubated in HEPES-buffered saline (HBS) (40 mmol/l HEPES, 154 mmol/l NaCl, pH 7.6) with or without DP IV (10 mU) for 16 h. Samples were resolved by HPLC using a CH₃CN (Fisher Scientific)/trifluoroacetic acid (TFA) (0.1% vol/vol; Pierce Chemical, Rockford, IL) and H₂O/0.1% TFA solvent system at a flow rate of 1 ml/min, according to published methods (12). Iodinated peptides (~50,000 cpm/20 µl injection) were separated by a protocol consisting of 14 min at 32% CH₃CN/0.1% TFA and a linear gradient to 38% CH₃CN/0.1% TFA over 10 min, followed by a further 5 min at 38% CH₃CN/0.1% TFA. Fractions were collected at 15-s intervals between minutes 5 and 27 of this protocol. Data were normalized to the total radioactivity recovered in all of these fractions. Between each sample injection, the C-18 µ-Bondapak HPLC column (Waters, Milford, MA) was rinsed for 20 min in 100% CH₃CN/0.1% TFA, and any residual radioactivity was removed with three consecutive injections of 300 µl DMSO (Sigma); the sample port was rinsed with 400 µl H₂O, and the column was allowed to re-equilibrate at 32% CH₃CN/0.1% TFA for 5 min before the next injection. Under these conditions, a second peak eluting earlier than intact [¹²⁵I]GIP₁₋₄₂ has been confirmed to be [¹²⁵I]GIP₃₋₄₂ by Edman degradation analysis (12).

Receptor activation and binding affinity studies. Receptor studies were performed in a heterologous expression system: CHO-K1 cells transfected with the rat pancreatic islet GIP receptor (wtGIPR cells) (25,26). Cells were

cultured in 1:1 Dulbecco's modified Eagle's medium and Ham's F12 nutrient mixture (DMEM/F12) (Gibco BRL, Burlington, Canada) with 10% newborn bovine calf serum (Cansera, Rexdale, Canada), antibiotics (Gibco), and 0.8 mg/ml Geneticin (G418; Gibco). At 80–90% confluence, cells were harvested with trypsin/EDTA (Gibco) and seeded at 5 × 10⁴ cells per well into 24-well plates (Falcon; Becton Dickinson, Mississauga, Canada). Experiments were carried out 48 h later.

Wild-type GIPR cells (3–5 × 10⁵ cells per well) were washed twice with 37°C 15 mmol/l HEPES (Sigma)-buffered (pH 7.4) DMEM/F12 containing 0.1% BSA and allowed to preincubate for 1 h at 37°C. A 30-min stimulation of cells followed in the same buffer, additionally supplemented with 0.5 mmol/l isobutylmethyl xanthine (Research Biochemicals International, Natick, MA), at the peptide concentrations indicated in the figures (0–1 µmol/l, except where noted). Cells were then lysed in ice-cold ethanol (70%), cellular debris was removed by centrifugation, and cell contents were dried *in vacuo* (Speed Vac; Savant, Farmingdale, NY). cAMP levels were determined using a radioimmunoassay kit (Biomedical Technologies, Stoughton, MA) and expressed in terms of fmol/1,000 cells or % maximal GIP₁₋₄₂-stimulated cAMP production. Antagonism experiments were carried out according to previously published protocols (25,27,28). In these studies, cells were additionally preincubated for 15 min with varying concentrations of antagonist, followed by addition of 1 nmol/l GIP₁₋₄₂; after 30 min, cells were treated according to the above protocol.

Receptor binding studies using [¹²⁵I]GIP were completed essentially as described previously (26,28). A separate series of experiments was also carried out using [¹²⁵I]-[D-Ala²]GIP₁₋₄₂ as the radioligand, according to the same methodology. wtGIPR cells were washed twice in ice-cold HEPES-buffered DMEM/F12 with BSA (defined above) and incubated at 4°C for 12–16 h with 50,000 cpm [¹²⁵I]GIP or [¹²⁵I]-[D-Ala²]GIP in the presence of GIP or analogs (0–1 µmol/l, except where noted) in the same buffer, additionally supplemented with 1% Trasylol. After incubation, cells were washed twice with ice-cold buffer, solubilized with 0.1 mol/l NaOH (1 ml), and transferred to test tubes for counting of cell-associated radioactivity. Nonspecific binding was defined as radioactivity measured in the presence of 1 µmol/l GIP₁₋₄₂.

Perfused rat pancreas. The insulinotropic activity of [D-Ala²]GIP₁₋₄₂ relative to native GIP was assessed in the perfused rat pancreas as previously described (28,29). In brief, overnight-fasted Wistar rats were anesthetized, and the pancreas plus associated duodenum were isolated. Perfusate consisted of a modified Krebs-Ringer buffer containing 3% dextran (Sigma) and 0.2% BSA, gassed with 95% O₂/5% CO₂ to achieve a pH of 7.4. The peptides were delivered as a linear gradient (0–1 nmol/l) over a 45-min period in the presence of 16.7 mmol/l glucose. Effluent samples were collected, and insulin content was measured by radioimmunoassay (RIA) (described below).

Bioassay in conscious rats. To examine the effectiveness of GIP or analogs *in vivo*, a bioassay was developed for the conscious unrestrained rat. Male Wistar rats (~290 g) were fasted overnight and then given an oral glucose tolerance test (OGTT) (1 g glucose/kg body wt) with concurrent subcutaneous saline or peptide (8 nmol/kg injection (500 µl)). Blood samples were taken at indicated times from the tail vein using heparinized capillary tubes, and the plasma was separated by centrifugation (6,000 rpm, 12 min, 4°C) for RIA. Blood glucose was measured every 10 min using a SureStep glucose analyzer (LifeScan Canada, Burnaby, Canada). Similar experiments were subsequently carried out using age-matched 16- to 20-week-old lean (*Fa/?*) and obese (*fa/fa*) VDF Zucker rats (~335 g and 575 g, respectively).

RIA for insulin and GIP. Insulin measurement by RIA was done using GP01 insulin antisera (1:1,000,000 final dilution) under disequilibrium conditions with rat insulin (Linco Research, St. Charles, MO) as a standard. After 24 h at 4°C, 2000 cpm of chloramine-T iodinated porcine insulin (Sigma) was added to all tubes; subsequent to another 24 h 4°C incubation, antibody-bound and free radioactive insulin were separated by centrifugation with dextran-coated charcoal. This protocol has been previously reported (28).

Measurement of GIP by RIA was performed as previously described using a COOH-terminally directed antibody (30). Plasma samples were appropriately diluted in GIP RIA buffer (5% charcoal-extracted human donor plasma, 2% Trasylol, 40 mmol/l phosphate buffer, pH 6.5) and incubated with GIP antiserum RK343F (1:300,000 final dilution) at 4°C. [¹²⁵I]GIP (5,000 cpm) was added 24 h later and, after a second 24-h incubation, antibody-bound and free radiolabeled GIP were separated by PEG-8000 precipitation (12.5% wt/vol final concentration; BDH) and centrifugation.

Analytical methods. Data are presented as means ± SE. Receptor binding and cAMP data were analyzed using the nonlinear regression analysis program Prism (version 3.02; Graphpad, San Diego, CA). Student's *t* test or ANOVA followed by the Newman-Keuls multiple comparison test was performed where appropriate. *P* < 0.05 was considered significant; *F* tests confirmed that variances were equal. Integrated glucose and insulin responses were calcu-

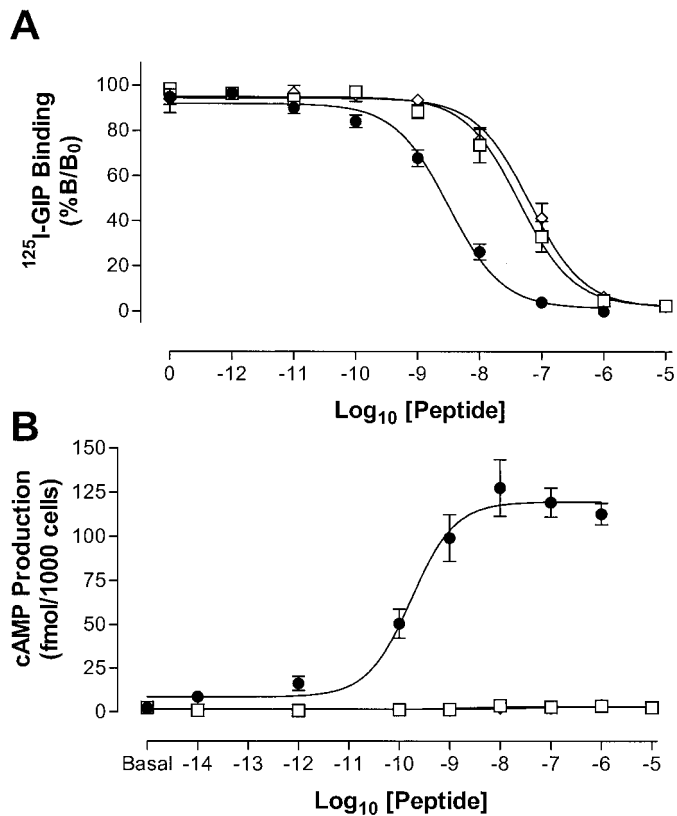


FIG. 1. Importance of the NH₂-terminus of GIP for receptor binding and activation. **A:** Competitive radioligand binding studies on wtGIPR cells. ●, GIP₁₋₄₂ (EC₅₀, 171 ± 24 pmol/l; IC₅₀, 3.56 ± 0.81 nmol/l); □, GIP₃₋₄₂ (IC₅₀, 58.4 ± 18.8 nmol/l); ◇, [Ala¹-Tyr²]GIP₁₋₄₂ (IC₅₀, 67.0 ± 20.3 nmol/l). **B:** cAMP production in receptor-transfected CHO-K1 cells (wtGIPR). Each data point represents the mean ± SE of four to six independent experiments.

lated using the trapezoidal method, using the algorithm included in the Prism software package and basal levels as a baseline.

RESULTS

Importance of GIP's amino terminus. Initial studies were targeted at determining the importance of the NH₂-terminal amino acids, Tyr¹ and Ala², for GIP receptor binding and activation of adenylyl cyclase. In competitive binding studies with wtGIPR cells, removal of the NH₂-terminal dipeptide Tyr¹-Ala² from GIP resulted in a peptide (GIP₃₋₄₂) that displaced binding of [¹²⁵I]GIP, but with a significant shift to the right in the displacement curve: half-maximal inhibitory concentration (IC₅₀) for GIP₃₋₄₂ was 58.4 ± 18.8 nmol/l; for GIP₁₋₄₂, the IC₅₀ was 3.56 ± 0.81 nmol/l (Fig. 1A). Despite high affinity for the GIP receptor, synthetic GIP₃₋₄₂ was found to have no effect on cAMP production in wtGIPR cells at concentrations as high as 10 μmol/l (Fig. 1B). Reversing the order of the first two amino acids of GIP ([Ala¹-Tyr²]GIP₁₋₄₂) resulted in a molecule with a similar binding affinity to GIP₃₋₄₂ (IC₅₀ 67.0 ± 20.3 nmol/l) that also failed to stimulate cAMP production, emphasizing the importance of the integrity of the first two amino acid residues for high-affinity binding (Fig. 1). Both GIP₃₋₄₂ and [Ala¹-Tyr²]GIP₁₋₄₂ significantly inhibited cAMP production in response to 1 nmol/l GIP₁₋₄₂, in a concentration-dependent manner. cAMP production was inhibited by 45.6 ± 5.2% (GIP₃₋₄₂) and 40.5 ± 4.6% ([Ala¹-Tyr²]GIP₁₋₄₂) and by 91.0 ± 0.9% (GIP₃₋₄₂), and

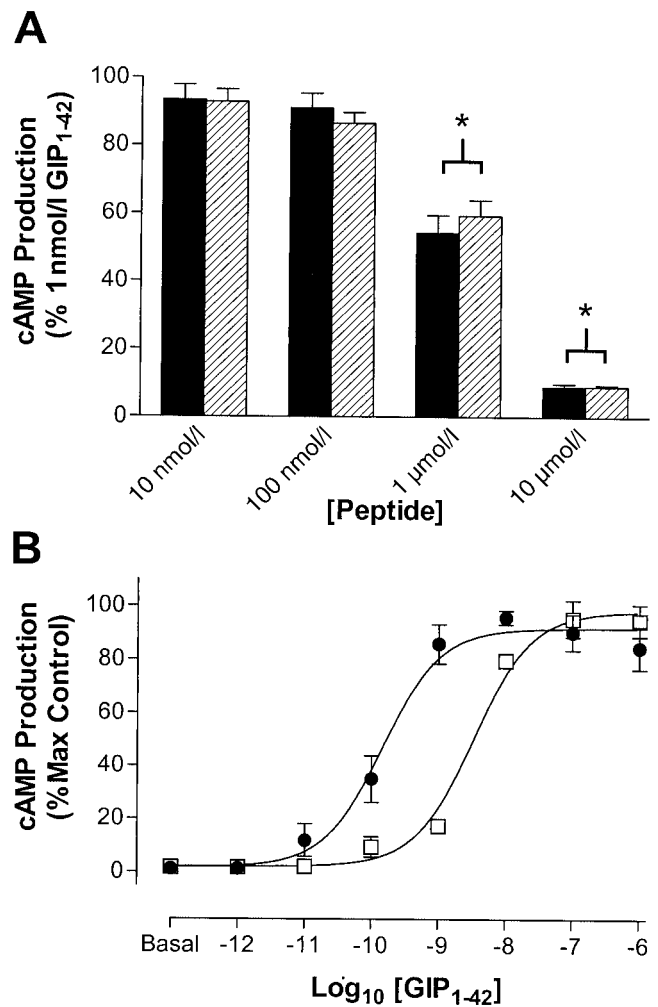


FIG. 2. Competitive inhibition of GIP₁₋₄₂-stimulated cAMP production in wtGIPR cells. **A:** Inhibition of 1 nmol/l GIP₁₋₄₂-stimulated cAMP production by varied concentrations of GIP₃₋₄₂ (■) or [Ala¹-Tyr²]GIP₁₋₄₂ (▨). **B:** Effect of 10 μmol/l GIP₃₋₄₂ on the GIP₁₋₄₂ concentration response curve. ●, GIP₁₋₄₂ control (EC₅₀, 373 ± 12 pmol/l); □, GIP₁₋₄₂ + 10 μmol/l GIP₃₋₄₂ (EC₅₀, 3.39 ± 0.29 nmol/l; *P* < 0.05). Antagonists were added 15 min before the 30-min stimulation with GIP₁₋₄₂. Each data point represents the mean ± SE of three to seven independent experiments.

90.9 ± 0.4% ([Ala¹-Tyr²]GIP₁₋₄₂) at peptide concentrations of 1 and 10 μmol/l, respectively (Fig. 2A). This antagonism appears to be a reversible competitive blockade of receptor activation, since 10 μmol/l GIP₃₋₄₂ right-shifted the half-maximal cAMP production of GIP₁₋₄₂ by ninefold when added to a concentration-response curve, but did not significantly diminish the maximal response (Fig. 2B).

For a peptide to be a suitable substrate for DP IV proteolysis, amino acid residues in the first two positions must be L-isomers and the linkage between them in the *trans* conformation. Therefore the L-amino acid in position 2 of GIP was substituted with the D-isomer. Resistance of peptides to hydrolysis by purified pork kidney DP IV was previously monitored by MALDI-TOF (matrix-assisted laser-desorption ionisation, time of flight analyzer) mass spectrometry; GIP₁₋₄₂ was shown to be degraded rapidly (*t*_{1/2} = 2.68 ± 0.16 min) by DP IV, whereas P₂ substitution with D-Ala resulted in a peptide completely resistant to DP IV degradation (22). When incubated with purified porcine DP IV, [¹²⁵I]GIP₁₋₄₂ (retention time [RT] = 14.3 ± 0.3 min)

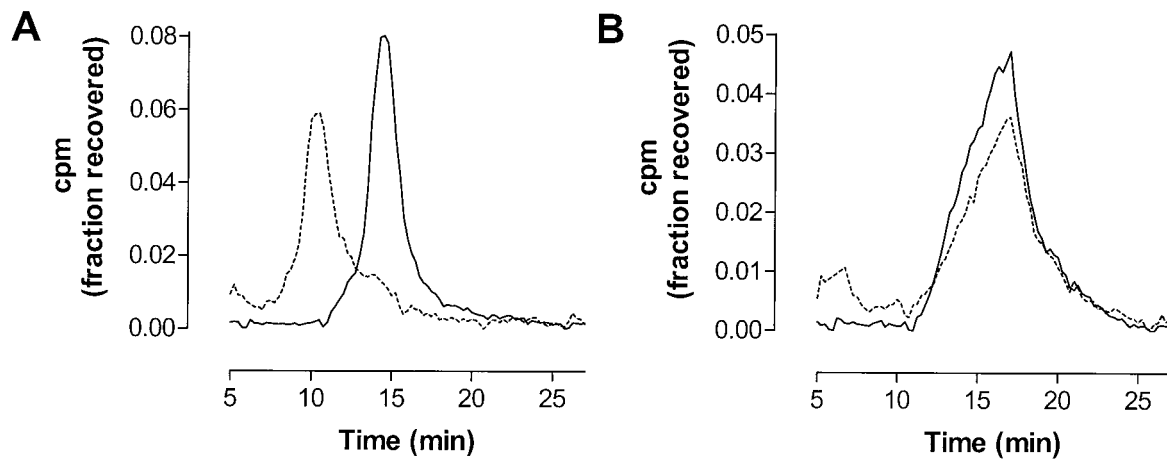


FIG. 3. HPLC separation of [^{125}I]GIP $_{3-42}$ from [^{125}I]GIP $_{1-42}$ (A) or [^{125}I]-[D-Ala 2]GIP $_{1-42}$ (B) incubated with (dashed lines) or without (solid lines) 10 nM DP IV for 16 h. Iodinated peptides were separated by a protocol consisting of 14 min at 32% CH $_3$ CN/0.1% TFA and a linear gradient to 38% CH $_3$ CN/0.1% TFA over 10 min, followed by a further 5 min at 38% CH $_3$ CN/0.1% TFA. Each trace represents the compiled results from three to four chromatograms.

was completely degraded to [^{125}I]GIP $_{3-42}$ (RT = 10.3 \pm 0.1 min), as resolved by HPLC (Fig. 3A). Similar studies using [^{125}I]-[D-Ala 2]GIP $_{1-42}$ (RT = 16.4 \pm 0.2 min) indicated that it was not a substrate of DP IV (Fig. 3B), since no peak corresponding to [^{125}I]GIP $_{3-42}$ eluted.

Receptor binding and biological activity of GIP $_{1-42}$ and [D-Ala 2]GIP $_{1-42}$ in vitro were not significantly different. In binding competition assays, regardless of whether the radioligand used was [^{125}I]GIP $_{1-42}$ or [^{125}I]-[D-Ala 2]GIP $_{1-42}$, IC $_{50}$ values were equivalent (Fig. 4). [D-Ala 2]GIP $_{1-42}$ showed nearly equal cAMP-stimulating potency to that of native GIP on wtGIPR cells (half-maximal stimulatory concentration [EC $_{50}$] values: GIP $_{1-42}$, 183 \pm 18 pmol/l; [D-Ala 2]GIP $_{1-42}$, 630 \pm 119 pmol/l; $P < 0.05$) (Fig. 5A). In view of the strong resistance to DP IV degradation and the minimal change in receptor affinity and cAMP potency with [D-Ala 2]GIP $_{1-42}$ (Figs. 3–5), its effect on insulin secretion in the isolated perfused rat pancreas was examined (Fig. 5B). In agreement with the cAMP data, integrated insulin responses to [D-Ala 2]GIP $_{1-42}$ (0.927 \pm 0.029 μmol , $n = 5$) were only slightly smaller than those with GIP $_{1-42}$ (1.130 \pm 0.037 μmol , $n = 5$; $P < 0.05$) (Fig. 5B); when we perfused the pancreases of these animals with 16.7 mmol/l glucose alone, the integrated insulin response was 0.103 \pm 0.056 μmol ($n = 4$) (28). Given the in vitro data, we hypothesized that [D-Ala 2]GIP $_{1-42}$ may have enhanced bioactivity in vivo relative to native GIP, resulting from its DP IV resistance.

In vivo bioassay of GIP and [D-Ala 2]GIP in lean and obese rats. Initial experiments were performed with conscious Wistar rats (287 \pm 6.5 g; fasting glycemia, 4.7 \pm 0.1 mmol/l; fasting insulin, 87.6 \pm 11.6 pmol/l; fasting GIP, 606 \pm 82 pg/ml; $n \geq 20$). Subcutaneous injection of 8 nmol/kg GIP $_{1-42}$ with a concurrent OGTT significantly reduced the glycemic profile relative to the saline control, and this was associated with increased circulating insulin levels (Fig. 6). Measurement of GIP levels by RIA indicated that this dose of GIP resulted in a 10-fold greater peak GIP level (control, 1.68 \pm 0.17 ng/ml; treated, 16.6 \pm 3.0 ng/ml) during the OGTT. The exogenous GIP appeared to be rapidly absorbed and to follow a similar elimination profile to that of endogenous GIP in control animals (data not

shown). In contrast, the same dosage of GIP $_{3-42}$ had no effect on postprandial glycemia or insulin release (Fig. 7). Subcutaneous injection of [D-Ala 2]GIP $_{1-42}$ resulted in a more pronounced reduction in the glycemic profile and an

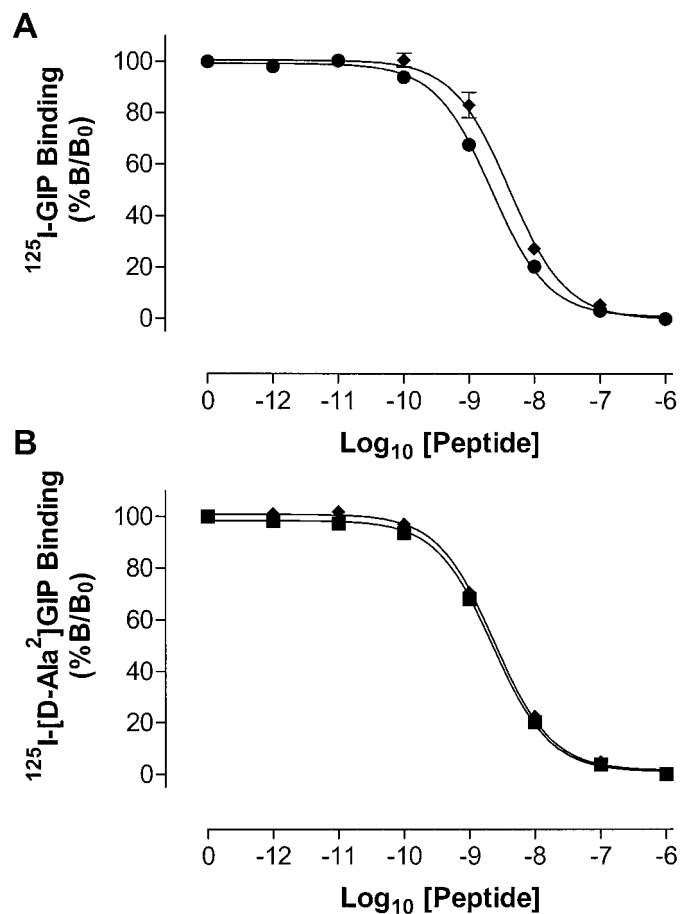


FIG. 4. Competitive radioligand binding studies on wtGIPR cells using [^{125}I]GIP $_{1-42}$ (A) or [^{125}I]-[D-Ala 2]GIP $_{1-42}$ (B) as tracer. A: \bullet , GIP $_{1-42}$ (IC $_{50}$, 2.23 \pm 0.18 nmol/l); \blacklozenge , [D-Ala 2]GIP $_{1-42}$ (IC $_{50}$, 3.48 \pm 0.20 nmol/l). B: \bullet , GIP $_{1-42}$ (IC $_{50}$, 2.33 \pm 0.18 nmol/l); \blacklozenge , [D-Ala 2]GIP $_{1-42}$ (IC $_{50}$, 2.45 \pm 0.24 nmol/l). Each data point represents the mean \pm SE of four to five independent experiments.

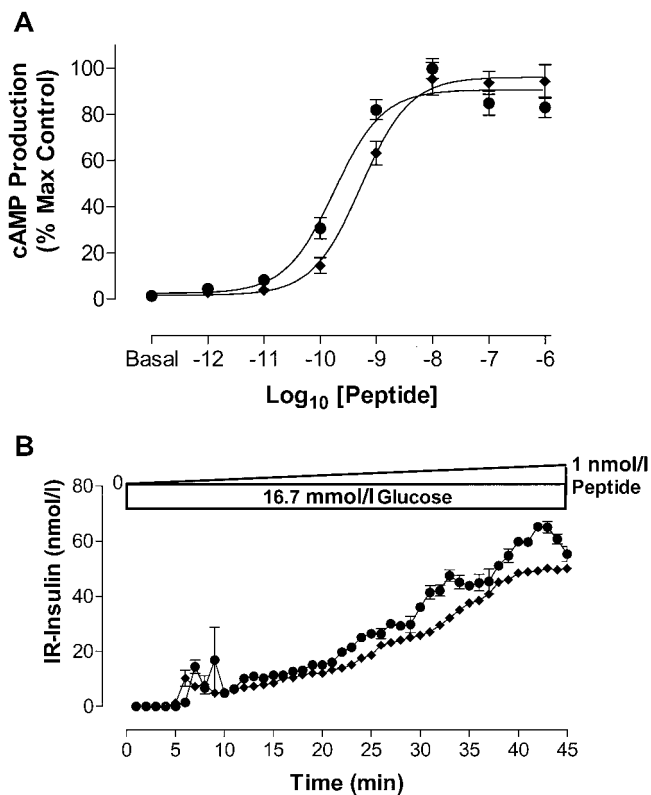


FIG. 5. Bioactivity of [D-Ala²]GIP₁₋₄₂ in vitro. **A:** cAMP production in wtGIPR cells by GIP₁₋₄₂ (●) and [D-Ala²]GIP₁₋₄₂ (◆). EC₅₀ values: GIP₁₋₄₂, 183 ± 18 pmol/l; [D-Ala²]GIP₁₋₄₂, 630 ± 119 pmol/l ($P < 0.05$). Each data point represents the mean ± SE of six to eight independent experiments. **B:** Immunoreactive (IR) insulin release from the isolated perfused rat pancreas in response to GIP₁₋₄₂ and [D-Ala²]GIP₁₋₄₂. Peptide gradient perfusions from 0 to 1 nmol/l were performed under constant high-glucose (16.7 mmol/l) conditions. Integrated responses were as follows: GIP₁₋₄₂, 1.130 ± 0.037 μmol/l; [D-Ala²]GIP₁₋₄₂, 0.927 ± 0.029 μmol/l; $P < 0.05$. Each data point represents the mean ± SE of four independent experiments.

enhanced insulin time course than that of native GIP during an OGTT (Fig. 6). Notably, both GIP and D-Ala²-modified GIP appeared to exert their glucose-lowering effects by significant enhancement of the early phase of insulin release, whereas the latter peptide displayed more protracted bioactivity. Integrated glucose and insulin profiles can be found in Table 1.

Comparison of GIP and [D-Ala²]GIP was subsequently studied in the VDF Zucker animal model of type 2 diabetes (23). Age-matched obese animals (*fa/fa*; 576.1 ± 9.1 g) displayed significantly higher fasting glycemia than their lean (*Fa/?*; 335.6 ± 14.9 g) littermates (7.3 ± 0.3 mmol/l vs. 4.8 ± 0.1 mmol/l) and, similarly, a fasting hyperinsulinemia (979 ± 109 pmol/l vs. 8.5 ± 2.4 pmol/l), typical of this animal model ($P < 0.05$, $n \geq 20$). Fasting GIP levels in these animals were not significantly different (lean, 954 ± 72 pg/ml; obese, 926 ± 110 pg/ml; $n \geq 13$), whereas after an OGTT in control animals, postprandial GIP levels (the mean of samples taken at 10, 20, and 30 min) in obese rats (1,730 ± 170 pg/ml, $n = 15$) were significantly greater than levels in lean animals (1,150 ± 120 pg/ml, $n = 19$; $P < 0.05$). Injection of 8 nmol/kg GIP₁₋₄₂ resulted in 15.4-fold (lean) and 9.6-fold (obese) greater peak GIP levels in vivo after the OGTT, relative to peak values in saline control animals. In lean animals, GIP injection produced moderate reductions in postprandial glycemic levels (16.8% reduc-

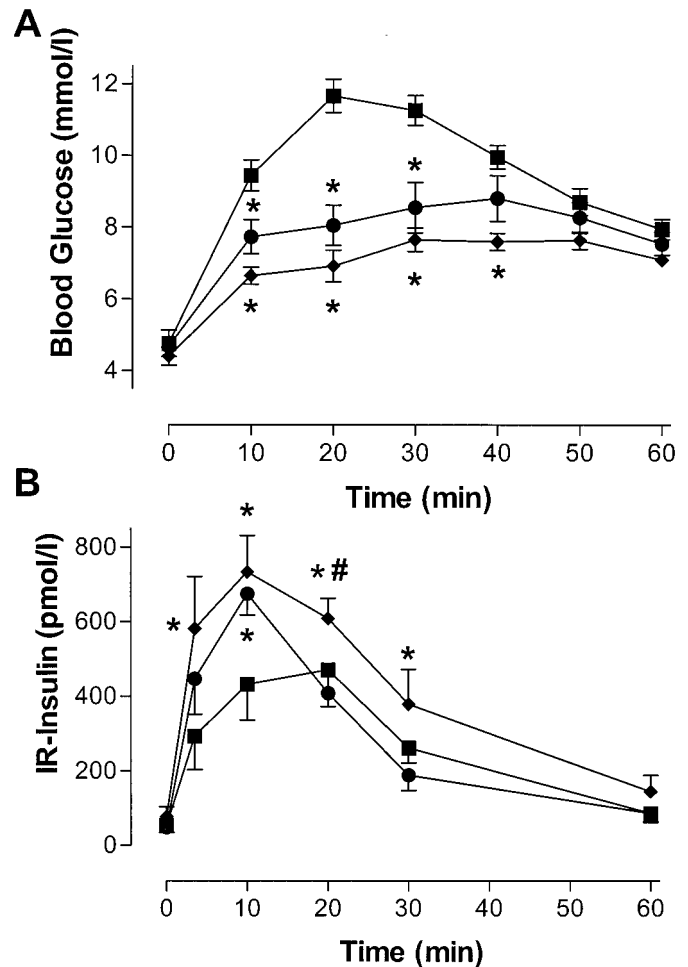


FIG. 6. Bioassay of GIP₁₋₄₂ (●) and [D-Ala²]GIP₁₋₄₂ (◆) in conscious Wistar rats, compared with a saline control (■). **A:** Whole blood glycemia measured from tail vein samples. **B:** Immunoreactive (IR) plasma insulin levels from tail vein samples. Peptides (8 nmol/kg in 500 μl saline) were injected subcutaneously at time 0, immediately following an OGTT (1 g/kg). Each data point represents the mean ± SE of eight animals. * $P < 0.05$ vs. saline control; # $P < 0.05$ between peptides.

tion compared with saline control at 40 min using fold-basal values), whereas [D-Ala²]GIP₁₋₄₂ was more potent (46.8% reduction at the same time point) (Fig. 8A). Similarly, in obese animals, comparison at the 40-min time point indicated that GIP reduced glycemia by 18.7% and [D-Ala²]GIP reduced glycemia by 41.5%, relative to the saline control (Fig. 9A). In lean VDF Zucker rats, both peptides appeared to similarly augment insulin release, with [D-Ala²]GIP resulting in more elevated insulin levels at the first time point (3.5 min) (Fig. 8B). However, in obese rats, differences in the potencies of GIP and DP IV-resistant GIP were more evident, with insulin levels remaining at near-peak values at the 60-min time point for the [D-Ala²]GIP-treated group, whereas insulin levels approached control values for the GIP-treated group after 1 h (Fig. 9B). Integrated glucose and insulin profiles for bioassay data can be found in Table 1.

DISCUSSION

Recognition of the importance of the structure of the NH₂-terminus for biological activity of peptides in the secretin-glucagon superfamily has resulted in the develop-

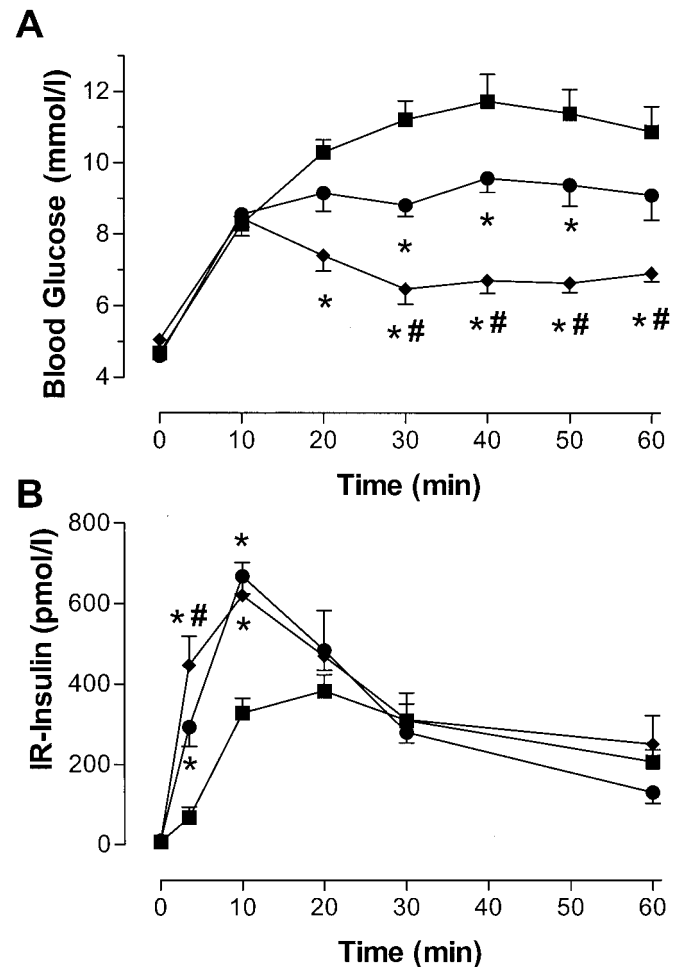
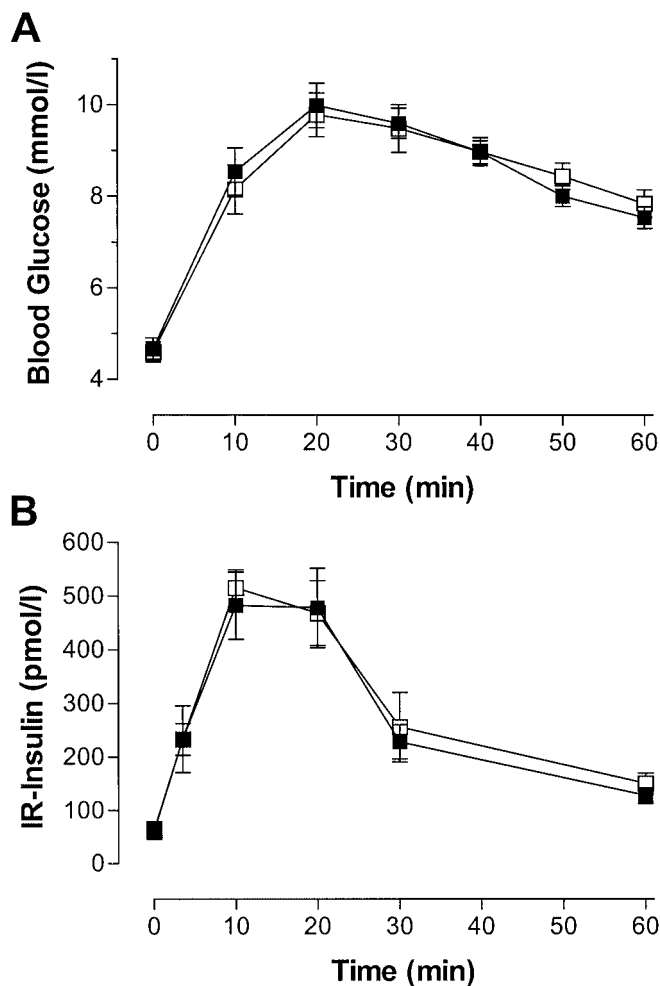


FIG. 7. Bioassay of GIP₃₋₄₂ in conscious Wistar rats. **A:** Whole blood glycemia measured from tail vein samples. **B:** Immunoreactive (IR) plasma insulin levels from tail vein samples. GIP₃₋₄₂ (□) (8 nmol/kg in 500 μl saline) or saline (■) was injected subcutaneously at time 0, immediately following an OGTT (1 g/kg). Each data point represents the mean ± SE of five or more animals.

FIG. 8. Bioassay of GIP₁₋₄₂ (●) and [D-Ala²]GIP₁₋₄₂ (◆) in conscious lean (Fa/?) VDF Zucker rats versus injection of saline (■). **A:** Whole blood glycemia measured from tail vein samples. **B:** Immunoreactive (IR) plasma insulin levels from tail vein samples. Peptides (8 nmol/kg in 500 μl saline) were injected subcutaneously at time 0, immediately following an OGTT (1 g/kg). Each data point represents the mean ± SE of six to eight animals. **P* < 0.05 vs. saline control; #*P* < 0.05 between peptides.

ment of numerous analogs with reduced in vivo catabolism and increased biological activity. Substitution of L-Tyr¹ with the D-isomer in growth hormone-releasing hormone (GRH) (31-33), L-His¹ with D-His¹ in glucagon (34) and GLP-1 (35), or D-amino acids in P₂ of glucagon (27) or GLP-1 (36,37) results in peptides with increased in

vivo potency. Although conformational changes in the molecules may play a role in increasing biological activity (34), a more prolonged biological half-life as a result of their resistance to enzymatic degradation is probably the more important factor. Frohman et al. (31,38) first showed

TABLE 1
Integrated glucose and insulin profiles in male Wistar, lean, and fat VDF Zucker rats

Animal model	Test	Glucose profile (mmol/l × 60 min)	Early insulin response (nmol/l × 10 min)	Complete insulin profile (nmol/l × 60 min)
Wistar rat	Saline	289 ± 21	2.54 ± 0.55	13.3 ± 2.3
	GIP	195 ± 26*	4.27 ± 0.49*	14.4 ± 1.2
	[D-Ala ²]GIP	145 ± 17*	4.98 ± 0.85*	20.6 ± 2.9
Lean Zucker rat (Fa/?)	Saline	315 ± 21	1.43 ± 0.20	16.0 ± 1.5
	GIP	258 ± 15*	3.23 ± 0.53*	18.6 ± 1.1
	[D-Ala ²]GIP	113 ± 13*†	4.17 ± 0.55*	21.2 ± 3.8
Fat Zucker rat (fa/fa)	Saline	503 ± 68	4.46 ± 1.58	44.9 ± 15.4
	GIP	368 ± 43*	16.8 ± 2.6*	93.5 ± 15.7*
	[D-Ala ²]GIP	226 ± 32*†	20.5 ± 1.2*	166 ± 15*†

Data represent the area under the curve (AUC) of data shown in Figs. 6, 8, and 9 and are expressed as means ± SE; **P* < 0.05 compared with saline control; †*P* < 0.05 compared with GIP test.

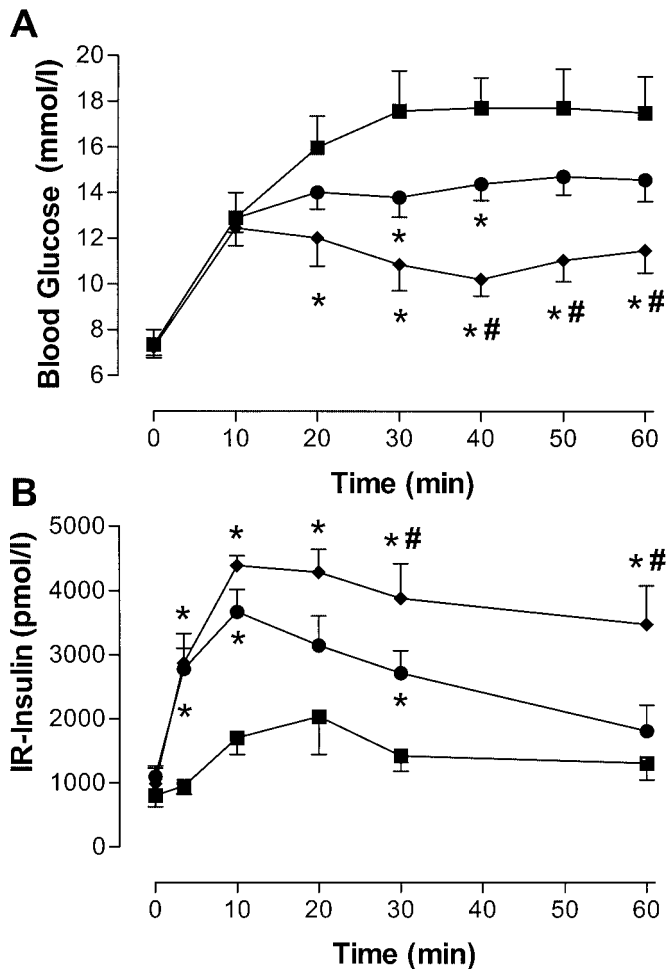


FIG. 9. Bioassay of GIP₁₋₄₂ (●) and [D-Ala²]GIP₁₋₄₂ (◆) in conscious obese (*fa/fa*) VDF Zucker rats versus saline injection (■). **A:** Whole blood glycemia measured from tail vein samples. **B:** Immunoreactive (IR) plasma insulin levels from tail vein samples. Peptides (8 nmol/kg in 500 μ l saline) were injected subcutaneously at time 0, immediately following an OGTT (1 g/kg). Each data point represents the mean \pm SE of six to eight animals; **P* < 0.05 vs. saline control; #*P* < 0.05 between peptides.

the importance of DP IV in the physiological degradation of members of the secretin-glucagon superfamily, by demonstrating that GRH is metabolized to biologically inactive GRH₃₋₄₄ by DP IV both in vitro and in vivo. Additionally, it was shown that amino-terminal substituted analogs, including des-amino-Tyr¹-, D-Tyr¹-, and D-Ala²-GRH, were resistant to DP IV cleavage (31). More recently, studies on GLP-1 have shown a similar resistance to DP IV degradation with analogs containing D-amino acids in the P₂ NH₂-terminal position (36,37). The current study was targeted at developing long-acting analogs of the second important incretin, GIP. During the preparation of this article, Flatt and colleagues (39-42) reported on [Tyr¹-Glucitol]GIP, a peptide analog modified postsynthesis, displaying both DP IV resistance and improvement of glucose tolerance in a diabetic mouse model.

Experiments were first carried out to establish the minimal structural requirements for the NH₂-terminus of GIP for receptor binding and stimulation of adenylyl cyclase. Removal of the first two amino-terminal residues (GIP₃₋₄₂), or inversion of these amino acids ([Ala¹-Tyr²]GIP₁₋₄₂), resulted in peptides that displayed reduced re-

ceptor affinity in competition binding studies and that were devoid of the ability to stimulate cAMP production in wtGIPR cells at concentrations as high as 10 μ mol/l (Fig. 1). This supports the early claim that GIP₃₋₄₂ isolated from porcine intestinal extracts lacked insulinotropic activity in the perfused rat pancreas (43). Both GIP₃₋₄₂ and [Ala¹-Tyr²]GIP₁₋₄₂ were antagonists of GIP₁₋₄₂-induced cAMP production in the micromolar range, inhibiting cAMP production by >90% (Fig. 2). Schmidt et al. (44) tested GIP₃₋₄₂ for antagonism of GIP₁₋₄₂ action on isolated rat islets and found that it was unable to reduce GIP-stimulated insulin release when administered in equal or 10-fold greater concentrations; Fig. 2 indicates that at least a 1,000-fold greater concentration of GIP₃₋₄₂ is necessary to reduce native GIP action on the cloned receptor. The claim that GIP₃₋₄₂ lacks insulinotropic activity is further supported by its lack of effect on glucose excursions and insulin profile when bioassayed in conscious Wistar rats (Fig. 7). In this bioassay, excess exogenous GIP₃₋₄₂, injected subcutaneously, was unable to block or even change the insulinotropic effect of endogenously released GIP₁₋₄₂ resulting from the oral glucose load; thus, it is extremely unlikely that GIP₃₋₄₂ plays an antagonistic role in vivo.

Given the sensitivity of the NH₂-terminus of GIP to inactivation by DP IV, we sought to generate peptide analogs resistant to this enzyme for use in vivo. Initial in vitro studies looked at modification or substitution of amino acids in positions 1-4 of GIP, using peptides based on the shorter 30-amino acid bioactive core of the hormone (1-30) (22). From this data, substitution with D-Ala in position 2 was shown to have the greatest potential for further development; preliminary trials found that this molecule was completely resistant to DP IV degradation for over 24 h and had minimal changes in receptor activity (22). When testing full-length [D-Ala²]GIP₁₋₄₂ in the current report, these results were corroborated and studies continued in animal models. Although DP IV resistance did little to the effectiveness of the analog in vitro (consistent with the lack of DP IV activity in CHO cells; S.A.H., J.A. Pospisilik, R.A.P., C.H.S.M., unpublished data), when tested in vivo, [D-Ala²]GIP reduced glycemic excursions in all animal models to a greater extent than native GIP. This was associated with enhanced early-phase insulin release in lean animals (Figs. 6 and 8; Table 1); in diabetic rats, where the first phase of insulin release is compromised, an augmentation of the entire insulin time course was observed (Fig. 9). The latter finding is of particular interest, as GIP's effectiveness in type 2 diabetes and animal models of the disease has been questioned and remains controversial. Lean Zucker rats showed significant differences in glycemic profiles between GIP and [D-Ala²]GIP, whereas both peptides appeared equally insulinotropic except at the first time point (2 min) (Fig. 8). Although the importance of the first phase of insulin release is well established, these data are also consistent with either an increased insulin sensitivity in these animals, as noted by Pederson et al. (18), and/or enhanced ability of this compound to stimulate glucose uptake in peripheral tissues (42).

Although GIP₁₋₄₂ has been shown to exhibit insulinotropic activity equivalent to that of GLP-1 (45), there have been no previous reports targeted at developing long-

acting analogs with therapeutic potential in type 2 diabetes until very recently, and these have been limited. The reason for the lack of such studies probably originates in the report of Nauck et al. (46), who observed that human GIP was almost devoid of insulinotropic activity in type 2 diabetic patients. It had been shown earlier that there was a reduced incretin response in type 2 diabetic patients, characterized by a reduction in the component of β -cell secretion resulting from oral glucose relative to that obtained with intravenous glucose (47). Additionally, the responsiveness of type 2 diabetic patients to exogenous porcine GIP, at concentrations resulting in physiological (48) or near-physiological (49) circulating levels, was blunted. It was also observed that porcine GIP₁₋₄₂ stimulated insulin secretion under fasting conditions (50,51), whereas normal control subjects did not respond, presumably because circulating fasting glucose levels in type 2 diabetic patients reach the required threshold for the insulinotropic activity of GIP. Therefore, there appears to be little doubt that insulin responses to exogenous GIP are reduced in type 2 diabetic patients. Nevertheless, the pancreas still retains some GIP sensitivity. The reason for the almost complete lack of response to human GIP observed in the study of Nauck et al. (46) may lie elsewhere. In their study, responses of normal controls to the human peptide were also extremely weak, unlike those described by the same group in an earlier study with GIP from a different commercial source (52), suggesting that the synthetic human peptide used in the type 2 diabetes study exhibited only weak biological activity. It has recently been shown by us that some (45), but not all (26), commercial preparations of human GIP exhibit very low biological activity, and it is therefore critical that further clinical trials with human GIP and GIP analogs are performed with peptides of established biological activity.

It is also important to define the origin of the resistance to GIP. One possibility is increased receptor desensitization/downregulation or altered signal-transduction pathways (6,53,54). Alternative explanations include antagonism of GIP₁₋₄₂ action by GIP₃₋₄₂, as suggested for GLP-1 (21), or a genetic defect resulting in reduced receptor expression (55). Antagonism by GIP₃₋₄₂ appears unlikely in view of its low binding affinity for the receptor and strength as an antagonist, with at least 1,000-fold higher concentrations being required to show a significant effect. However, some support for the latter hypothesis has come recently, with the finding that obese VDF Zucker rats have compromised GIP receptor expression, at both the mRNA and protein levels, in isolated islets of Langerhans (23). During an intraperitoneal glucose tolerance test, when GIP was infused at minimum threshold levels necessary to obtain a biological response in lean animals ($4 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$), obese animals were unable to respond to the same dose. This was linked to a defect in the ability to stimulate cAMP in isolated islets, and quantitative PCR and immunoblotting suggested a reduction in GIP receptor expression (23). The etiology of the diminished expression, however, has not been elucidated and may result from elevated postprandial GIP levels and subsequent desensitization/internalization/downregulation of the receptor. In the current study,

fasting GIP levels in lean and obese VDF Zucker rats were not significantly different, confirming the result of Lynn et al. (23); however, elevated levels of GIP were detected in obese animals after an OGTT, lending support for the downregulation hypothesis.

The significance of GIP as a physiological incretin has been emphasized in studies using specific GIP antagonists in rat (56) and inhibition of DP IV activity in GLP-1 receptor knockout mice (57). With the important findings that sulfonylureas improve β -cell sensitivity to GIP (58), that smaller fragments of GIP are bioactive (28,29), and in the present report, that even in animals with compromised GIP receptor expression, supraphysiological concentrations of GIP and analogs with improved plasma stability are still capable of improving glucose tolerance, the pharmacological potential of GIP in treatment of human diabetic states is preserved. To truly recognize the potential of GIP and degradation-resistant analogs, the extent of the reduced sensitivity to GIP in diabetic patients needs to be quantitatively assessed using a wide range of peptide concentrations, as has been done for GLP-1 (59), rather than single low-dosage protocols, biased to show a lack of effectiveness. No matter what underlying cause is ultimately determined to be responsible for the reduced responsiveness to GIP in human type 2 diabetes, analogs based on the DP IV-resistant form described here may be useful in stimulating insulin secretion through the residual islet capacity to respond to this incretin.

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