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Diabetogenic peptide from human growth hormone: Partial purification from peptic digest and long-term action in *ob/ob* mice*

(somatotropin/nonacidic peptide/prolonged glucose intolerance/fluorescamine)

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ABSTRACT **Studies in female *ob/ob* mice demonstrated diabetogenic properties of human growth hormone (somatotropin) and of a fragment generated therefrom by controlled digestion with pepsin; both the fragment and parent growth hormone produce long-term effects on carbohydrate metabolism; in acute glucose tolerance tests, only the fragment is active. Two nonacidic diabetogenic fractions have been separated from inactive fractions by chromatography on Bio-Gel P-6 followed by ion exchange chromatography at pH 4.3 and gel filtration on Bio-Gel P-2 and/or Sephadex G-25; these active fractions exhibited multiple NH_2-termini (Lys, Phe, Leu, and Tyr). Fraction CD has these characteristics: (*i*) It induces glucose intolerance in fasting female *ob/ob* mice when injected subcutaneously in a divided dose, 15 min before and concurrently with glucose; mice injected with sufficient peptide exhibit elevated fasting glucose levels as long as 7 months after a single glucose tolerance test. (*ii*) It is a peptide smaller than that reported to stimulate body growth, but larger than somatostatin. This peptide, as reported earlier, does not crossreact with antiserum to human growth hormone in radioimmunoassay.**

Our understanding of the relationship of pituitary growth hormone (GH; somatotropin) to diabetes mellitus is advancing rapidly now that human hormone is available, albeit in limited amounts, for such studies. Lewis *et al.* (1) have demonstrated that intact single-chain human GH is ineffective as a hyperglycemic agent when tested in a 9-hr glucose tolerance test (GTT) in dogs; limited digestion with bacterial proteinase converts the single-chain molecule to a double-chain, and the double-chain structure produces, under the same test conditions, a marked hyperglycemic response. Reagan (2) has studied effects of reduced and carbamidomethylated human GH in *ob/ob* mice; hyperglycemic activity is not seen 6 hr after injection, but a marked effect is observed when the same dose (200 μg) is administered once daily for a period of 3 days. We (3) have prepared a small fragment from human GH that induces acute glucose intolerance less than 1 hr after injection in *ob/ob* mice. Reported here are the partial purification of this hyperglycemic peptide fraction that was generated by controlled digestion with pepsin, and a preliminary characterization of its unique diabetogenic effect on carbohydrate metabolism.

MATERIALS AND METHODS

Preparation of Fragments and Testing in *ob/ob* Mice. Fragments were prepared from purified human GH by controlled digestion at pH 3.8 with pepsin, 1 part enzyme:140 parts GH by weight (3), after a 15-min preincubation in 0.05 M sodium acetate (4). The reaction was terminated by adding sufficient ammonium hydroxide to raise the pH to 7–7.5, after which the digest was made 0.5 M with respect to acetic acid (HOAc) and chromatographed on Bio-Gel P-6 in 0.5 M HOAc.

Individual fractions to be lyophilized were identified by the fluorescamine method (5). In our procedure human GH (HS-1942 rechromatographed on Bio-Gel P-6) gave 170 fluorescence units (FU)/nmol, where 1 fluorescence unit = 1% of a scale 1–100 on the Turner fluorometer model 110; a similar value, 200 FU/nmol, was obtained with synthetic angiotensin I (Beckman, 10 amino acid residues). To estimate the amount of peptide in eluates, we have used the latter value.

Genetically obese female mice of the C57BL/6J (*ob/ob*) strain were obtained from Jackson Laboratories, Bar Harbor, Maine; they were used from 9 weeks to 8 months of age. Blood glucose values are high in young adult mice of this strain (6), and this should be borne in mind when comparisons between different groups of mice are attempted. To test for diabetogenicity of peptides, we paired mice of the same age by weight and injected them with a priming dose of dexamethasone [2 μg subcutaneously (sc)]. They were then fasted for 6 hr, after which they were challenged with glucose (1 mg/g of body weight intraperitoneally) and bled from the tail 25 min later. Test solution or control diluent (HOAc 0.01 M, $NaHCO_3$ 0.25 M, bovine serum albumin 0.7%; 4:2:1) was given sc in divided doses 15 min before, and at the same time as, the glucose injection. Glucose was estimated on 10 μl of blood by the glucose oxidase method.

Purification of Peptic Digest of Human GH. A heterogeneous fraction (Fig. 1), eluting on the rising side of the final peak off Bio-Gel P-6, contained hyperglycemic activity (Table 1; hGH-P-P6-II); fractions I, III, and M and undigested human GH were inactive in our acute test. Pooled lyophilized fraction II was used as starting material for further purification using a sodium acetate gradient.

Fraction II from 14 experiments (1.8 μmol) was taken up in 0.005 M HOAc, the pH was adjusted to 7.2 with NaOH, and the peptides were applied to a column of Bio-Rex 70, Na^+ form, that had been equilibrated with 0.05 M sodium acetate, pH 7.2; 90% of the peptide sample was not retained. This eluate (15 ml) containing unabsorbed peptide was adjusted to pH 4.3 with glacial acetic acid and applied to a second column of Bio-Rex 70, Na^+ form, that had been equilibrated with pH 4.3 sodium acetate buffer (NaOAc) 0.02 M with respect to Na^+. Peptides were eluted with increasing concentrations of NaOAc at a constant pH of 4.3. Ionic strengths of the eluates and of the salt solutions were determined with a direct reading conductivity meter (Model CDM 2e, Radiometer, Copenhagen, Denmark).

Each fraction (A–E, Fig. 2) off the resin column was lyophilized separately and desalted on Bio-Gel P-2 in 0.01 M HOAc, or on Sephadex G-25 in 0.2 M HOAc. For assay and

Abbreviations: GH, growth hormone (somatotropin); FU, fluorescence unit; GTT, glucose tolerance test; sc, subcutaneously.

* A portion of the data was presented on January 23, 1975, at a workshop held at the Kroc Foundation, in *A Report* (1976), *Metabolism* 25, 105–124, eds. Kostyo, J. L. & Wilhelmi, A. E.

Table 1. Identification of fraction in 20-hr peptic digest of human GH that induces acute glucose intolerance in *ob/ob* mice

Test preparation*	Blood glucose (mg/100 ml)‡	P§	Activity as hyperglycemic agent†
Control	332 ± 12 (8)		
hGH-P-P6-I	381 ± 20 (4)	ns	−
hGH-P-P6-II	512 ± 20 (6)	<0.001	+
hGH-P-P6-III	301 ± 30 (5)	ns	−
Control	396 ± 33 (5)		
hGH-P-P6-M	374 ± 8 (7)	ns	−
Control	440 ± 12 (4)		
hGH, undigested	440 ± 35 (4)	ns	−

* Test preparations were lyophilized eluates from Bio-Gel P-6 (for numbering of fractions I–III and M, see Fig. 1) that were injected in diluent, sc, in a total dose of 75 nmol (15 × 10^3 FU)/0.70 ml. Female mice (control and test) used in each experiment were paired on the basis of age, weight, and previous assay experience.
† The glucose tolerance test, as used here, is a null-point assay that detects significant difference from the control.
‡ Mean ± standard error; number of mice in parentheses.
§ ns, not significant.

Table 2. Related peptides (synthetic and natural) tested for induction of acute glucose intolerance in *ob/ob* mice

Test preparation*	Blood glucose (mg/100 ml)	P	Activity as hyperglycemic agent
Control	390 ± 28 (6)		
25 Amino acid COOH-terminus of hGH, synthetic, 500 μg	392 ± 22 (6)	ns	−
Control	310 ± 9 (6)		
14 Amino acid somatostatin, synthetic, 100 μg	270 ± 18 (3)	ns	−
38 Amino acid Sonnenberg homologue of hGH, synthetic, 500 μg	285 ± 14 (2)	ns	−
Control	390 ± 15 (8)		
LMF†-Trygstad, 50 μg	370 ± 16 (8)	ns	−

* Test preparations were dissolved in diluent and injected sc in 0.70 ml. For other details, see Table 1.
† LMF, lipid mobilizing factor.

chemical analyses, lyophilized peptides were redissolved in 0.01 M HOAc, so that aliquots could be apportioned as required.

Determination of NH_2-Termini. An aliquot containing approximately 3 nmol of peptide was lyophilized in a Pyrex tube (10 × 75 mm), and NH_2-termini were determined by the dansyl procedure (7, 8). Dansyl-amino acids were identified under ultraviolet light after separation by ascending thin-layer chromatography on polyamide sheets in the following systems: in the first dimension, solvent I: water–88% formic acid (100: 1.5, vol/vol); in the second dimension, solvent II: benzene–glacial acetic acid (9:1, vol/vol) (7, 8). To establish unequivocally the identity of a given amino acid, purified dansyl-amino acid (1 nmol in 5 μl of 50% pyridine) was added to an equal volume of 50% pyridine in which the hydrolysate had been dissolved, and the mixture of test sample and internal standard was chromatographed.

Materials. Polyamide sheets were obtained from the Cheng-Chin Trading Co. (Gallard-Schlesinger Corp., New York). Dansyl chloride was procured from Pierce Chemical Co., Rockford, Ill., and purified dansyl-amino acids from Calbiochem.

The human GH was supplied by Dr. Alfred Wilhelmi and the National Pituitary Agency; two preparations, NIH-GH-HS1523D and NIH-GH-HS1942†, yielded comparable digestion products with pepsin. Pepsin (3100 units/mg) and glucose oxidase reagent were purchased from Worthington Biochemical Corp. The synthetic tetradecapeptide homologous in sequence to sheep somatostatin (9), and synthetic angiotensin I, were supplied by Drs. J. K. Chang and L. B. Marshall, Bioproducts, Beckman Instruments, Palo Alto.

The fluorescamine (4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′-dione) was a generous gift from Dr. W. E. Scott and Dr. S. Udenfriend of the Roche Institute of Molecular Biology; it was prepared as a stock solution (50 mg/ml) in acetone (Matheson, Coleman and Bell, spectroquality), and was stable for months when stored at 4°.

Materials for column chromatography purchased from Bio-Rad Laboratories included: Bio-Gel P-2 (200–400 mesh, exclusion limit 1800 daltons); Bio-Gel P-6 (100–200 mesh, exclusion limit 6000 daltons); and Bio-Rex 70 (100–200 mesh, sodium form). Bio-Gel P-6 columns, 1.2 × 46 cm, gave reproducible separations over months of use; at the end of each experiment they were washed with deionized water and held under water. Bio-Rex 70, a weakly acidic carboxylic cation exchanger, was prepared for use by passing it through three successive cycles of 0.5 M HCl and 0.5 M NaOH. Finally it was converted to the Na^+ form in 0.5 M NaOH, washed extensively with 0.5 M sodium acetate, as described by Rombauts *et al.* (10), and poured. Just prior to use the surface of the resin bed was stirred, and the column equilibrated with 600–800 ml of appropriate solution.

To avoid loss of peptide due to adsorption to glass surfaces, glassware including columns was siliconized, and eluant fractions were collected in polypropylene tubes.

RESULTS

Peptic digests of human GH yielded several fractions when chromatographed on Bio-Gel P-6; a typical elution profile is shown in Fig. 1. The first peak (tubes 15–18, fraction M) probably contains undigested GH, large fragments, and pepsin; it is inactive in our acute GTT (Table 1).

Hyperglycemic activity is localized in fraction II (tubes 31–33) on the rising side of the final peak. As can be seen in Fig. 1, the diabetogenic peptide is larger than is the tetradecapeptide (somatostatin) used to standardize the Bio-Gel col-

† HS-1523D and HS-1942 were native "A-type" preparations obtained from clinical grade human GH by chromatography on DEAE-cellulose. They were electrophoretically homogeneous; bioassays using the body weight gain test in hypophysectomized rats gave values of 1.7 and 2.3 international units/mg, respectively (Dr. Alfred Wilhelmi, personal communication). Phe was the major NH_2-terminus; Ala also was observed. In addition, we have tested both lots in our acute GTT at dose levels as high as 50 nmol and found them inactive. Chromatography of HS-1523D and HS-1942 on Bio-Gel P-6, under the same conditions that dissociate diabetogenic fragment from larger peptides in peptic digests of human GH, yielded 0.6 × 10^3 FU fluorescamine-positive material in the fraction II region, or <1% that obtained after digestion with pepsin. To us, it appears unlikely that contaminating peptide present in the parent GH preparation could account for the unique hyperglycemic effect obtained with fraction II from peptic digests of these two lots of human GH.

Table 3. Elevated fasting blood glucose levels in *ob/ob* mice* injected sc 7–9 months earlier either with human GH or with diabetogenic peptide

Test treatment	Blood glucose, mg/100 ml† GTT at onset of exp. Control		Test	Fasting value, 7–9 months after test treatment	P
Diluent only	340 ± 8 (10)		—	114 ± 3 (10)	
GH, 25 nmol (500 μg)/day × 10 days	350 ± 12 (4)	$P < 0.001$	479 ± 19 (4)	165 ± 12 (4)‡	<0.005
Diabetogenic peptide, 30–40 nmol§ in a single GTT	334 ± 8 (10)	$P < 0.01$	410 ± 23 (5)	147 ± 5 (5)	<0.001

* Mice fasted 6 hr prior to sampling.
† Mean ± standard error; number of mice in parentheses.
‡ *ob/ob* mice injected with 5 nmol (100 μg) of human GH/day for 10 days in a parallel experiment had glucose levels comparable to those that had received only diluent (mean = 108).
§ In these calculations, 1 nmol = 200 FU (for details, see *Materials and Methods*).

umn. Somatostatin, and other synthetic peptides, as well as Trygstad's lipid mobilizing factor (11), were inactive in our test system (Table 2).

More recently, in a survey of long-term diabetogenic effects, persistent elevated blood glucose levels have been observed in some 20 *ob/ob* mice that exhibited marked hyperglycemic responses to diabetogenic peptide in the acute GTT. In a series using paired controls, the blood glucose levels 7 months after a single GTT with diabetogenic peptide were found to average 147 ± 5mg/100 ml compared with 114 ± 3 for those given diluent only (Table 3). The parent GH also produces this effect, provided that it is injected over a period of several days at an adequate dose. The finding that long-term hyperglycemia can be obtained with repeated doses of GH is consistent with the well-known observations that metahypophyseal diabetes can be produced similarly in dogs and cats (12, 13). To our knowledge this is the first time such an effect has been observed in rodents using human GH.

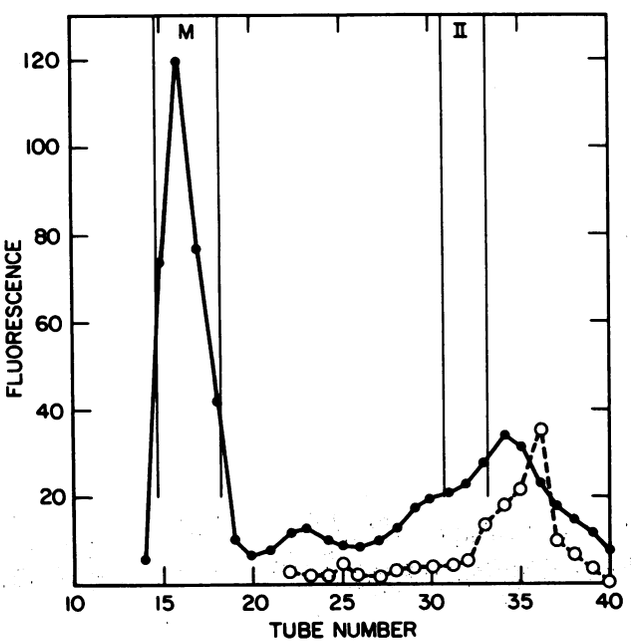

FIG. 1. Chromatography of a peptic digest of human GH, 5 mg, on Bio-Gel P-6 on 0.5M HOAc (●). Somatostatin, 400 μg, was chromatographed under the same conditions (○). One milliliter of each solution was applied to the column (1.2 × 46 cm); 1.3-ml fractions were collected; an aliquot (10 μl) was taken from each tube for fluorescence determinations. Diabetogenic activity appeared exclusively in fraction II (tubes 31–33). Fractions I (tubes 28–30), III (tubes 34–36), and M (tubes 15–18) were inactive. Number of bonds cleaved/mol was 6; peptide in fraction II was 8–10% of the total in the whole digest.

To identify the fragment responsible for the diabetogenicity of human GH, pooled fraction II was submitted to further purification on Bio-Rex 70 using increasing concentrations of NaOAc. Three peptide fractions (C_1, C_2, and D, Fig. 2), each active as a hyperglycemic agent in the GTT at dose levels as low as 4 to 5 × 10^3 FU (20–25 nmol), were isolated‡. All were heterogeneous, as shown by multiple NH_2-termini (Table 4). Lys, Phe, Leu, and Tyr were major end-groups in the three active fractions. Arg was not observed; hence, these peptides are clearly different from the COOH-terminal 25-amino acid peptide from pituitary GH described by Bornstein and coworkers (14).

Not one of the hyperglycemic fractions obtained by this particular gradient procedure could be purified sufficiently to establish, unequivocally, identity of the diabetogenic peptide. Subsequent purification included chromatography on Sephadex G-25 in 0.2 M HOAc; chromatography on Bio-Rex 70 using stepwise elution with HCl (3); chromatography on QAE-Sephadex in 0.1 M NH_4OAc, pH 8.1; and electrophoresis on silica gel in 0.1 M $NaHCO_3$, pH 8.0. We conclude that (*a*) the peptides in fractions C_1, C_2, and D are similar in size and (*b*) they have a similar charge between pH 4.3 and 8.0. A more effective isolation scheme probably will have to use procedures outside the pH range studied here.

DISCUSSION

Our previous studies (3) have shown that a fragment generated from human GH by controlled digestion with pepsin induces acute glucose intolerance in fasted genetically obese (*ob/ob*) female mice when injected sc in a divided dose 15 min before, and concurrently with, glucose. Studies reported in the present paper reveal that: (*a*) effects of the peptide may persist for several months, perhaps longer, and mice exposed to as little as 30–40 nmol in a single GTT continue to exhibit elevated fasting glucose levels. These initial studies, which have yielded self-consistent data in *ob/ob* mice, probably cannot be extended until an active replicate of the natural product has been syn-

‡ Based on the amount of peptide isolated in fraction II (8–10% of the whole) and the increase obtained in specific activity after its purification on Bio-Rex 70 (Table 4), it can be calculated that some 50-fold purification was attained.

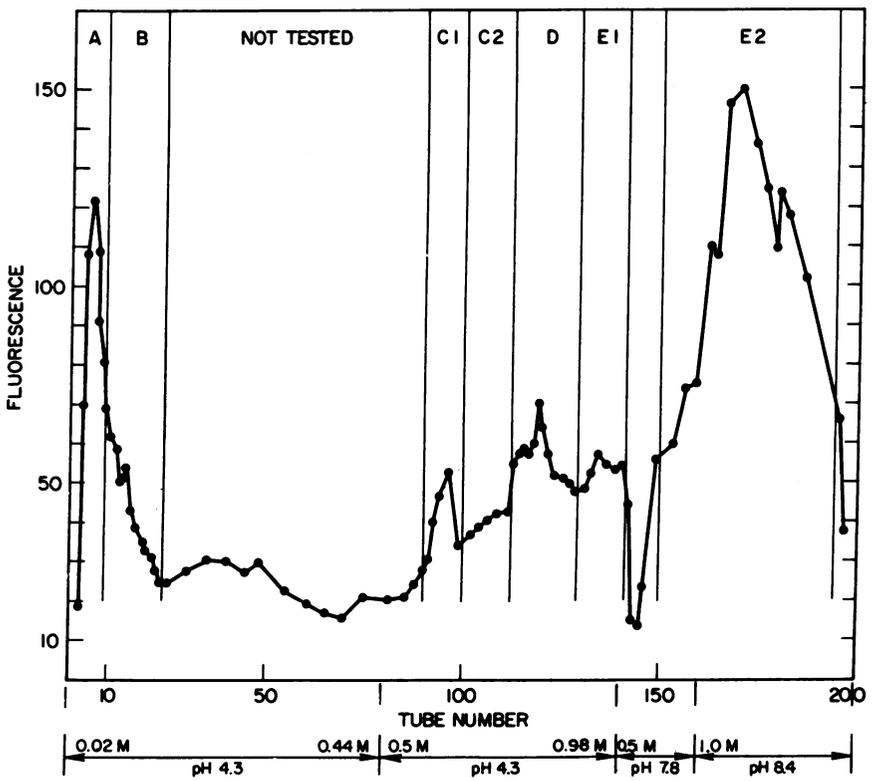

FIG. 2. Chromatography of pooled lyophilized fraction II from hGH-P-P6 on Bio-Rex 70, Na^+ form, using NaOAc buffers (pH 4.3). After application of peptides in 15 ml of 0.005 M NaOAc, a steep gradient (0.02–0.50 M) at constant pH of 4.3 was initiated and 80 tubes, 3 ml each, were collected. Fractions A and B were inactive in the GTT. Both the mixing chamber and reservoir then were emptied and a shallow gradient of NaOAc (0.50–1.0 M) was used; 60 additional tubes were collected. Fractions C_1, C_2, and D were active in the GTT. A final fraction, inactive, was eluted with 1.0 M sodium acetate (pH 8.4).

thesized, or a more abundant source of the small fragment is found. (*b*) The fragment is a nonacidic peptide, as judged from elution behavior on Bio-Rex 70 and mobility when electrophoresed on silica gel. End-group analyses indicate heterogeneity in fractions obtained with the NaOAc gradient at pH 4.3. Other approaches to purification are being developed. (*c*) It is a peptide larger than somatostatin, but smaller than the fragments reported to stimulate body growth (15, 16) or to produce hyperglycemia in mice (2) or in dogs (1). The active fraction does not crossreact with antiserum to human GH in radioimmunoassay (3).

Previously we suggested that fragments of pituitary GH may

Table 4. Summary of fractions obtained from hGH-P-P6-II by ion exchange chromatography on Bio-Rex 70, Na^+ form, with concentration gradients of sodium acetate buffers (see Fig. 2)

Fraction	Gradient (M NaOAc)	pH of eluant	Inclusive tube no.	FU of peptide recovered (thousands)*	NH_2-termini† (dansyl)	Hyperglycemic activity
A	0.02–0.04	4.3	2–9	21	Leu (Ala, Val, Gly)	0
B	0.04–0.08	4.3	10–24	18	Not determined	0
C_1‡	0.48–0.66	4.3	90–100	17	Lys, Phe, Leu, Tyr, (Val)	+
C_2‡	0.66–0.80	4.3	101–111	13	Lys, Phe, Leu, Tyr (Val)	+
D‡	0.80–0.97	4.3	112–129	29	Lys, Phe, Leu, Ala, Tyr (Val, Ile)	+
E_1	0.97–0.99	4.3	130–141	19	Ala, Tyr, Gly (Phe, Leu)	0
E_2	0.43–1.0	8.0	150–195	120	Phe, Ala, Gly (Leu)	0

* Recovered from 360×10^3 FU (1.8 μmol) of pooled fraction II put onto the column in 15 ml of 0.005 M NaOAc, pH 4.3.

† Amino acid residues in parentheses were minor, or trace.

‡ Based on gel filtration data (e.g., Fig. 1), we assume that the molecular weight of the peptide is 3000–4000; if so, glucose intolerance was produced with as little as 60–70 μg of fraction CD. Pooled fraction II was active at a dose of 250–300 μg; the parent human GH was inactive at a dose of 1.0 mg (see Table 1).

be responsible for some of the sequelae of events characteristic of diabetes mellitus in humans, and proposed a mechanism for forming these fragments *in vivo* (17). Results reported here demonstrate that a peptide generated enzymatically from human GH can have long-term effects on carbohydrate metabolism. The possibility that fragments from this hormone are responsible as well for other derangements observed in human diabetics merits further exploration.

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