Insulin-potentiating Action of a Synthetic Amino-terminal Fragment of Human Growth Hormone (hGH 1-15) in Streptozotocin-diabetic Rats

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SUMMARY

The hypoglycemic activity of the synthetic peptide fragment of human growth hormone, hGH 1–15, is insulin dependent, although it does not alter the circulating levels of plasma insulin in normal and diabetic rats. In severe diabetes induced by streptozotocin, the peptide had no effect on the basal levels of blood glucose, but enhanced insulin sensitivity during intravenous insulin tolerance tests in 16-h-fasted rats. Radioreceptor binding studies show increased binding of insulin by hepatic plasma membranes prepared from rats pretreated with hGH 1–15. These results suggest that the biologic action of the peptide is, at least in part, on insulin receptors of plasma membranes.

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The administration of pituitary growth hormone at 3 mg/100 g body wt has been shown to induce a hypoglycemic response in normal fasting rats.[1] Bornstein et al.[2] have suggested that this action may be attributed to the amino-terminal region of the molecule. Various partial sequences of the amino terminus of human growth hormone (hGH) have been synthesized: hGH 1–10 and 6–10 by Beyerman,[3] hGH 1–24 and 25–51 by Chillemi and Pecile,[4] hGH 1–35 by Niall and Tregear,[5] hGH 44–77 by Lostroh and Krahl,[6] hGH 1–53 and 17–23 by Noble et al.[7,8] No study, however, has yet been carried out to examine whether these amino-terminal hGH peptides have any hypoglycemic activity. Previous studies in our laboratory with synthetic hGH 1–20, 1–15, 1–13, 1–10, 3–13, 6–13, and 6–11 have shown that peptides containing the sequence Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala (hGH 6–13) stimulate glucose uptake in isolated rat diaphragms in the presence of insulin.[9] Recent work reveals that hGH 8–13 is also active in the same bioassay but that shortening of the peptide abolishes the biologic activity (unpublished data, Pullin, Ng, and Bornstein).

We previously reported[10] that the synthetic peptide hGH 1–15* induced hypoglycemia without increase of the circulating level of insulin in venous blood of laboratory animals under the conditions of an intravenous glucose tolerance test; and, based on findings obtained in vitro using the isolated rat diaphragm, that this action was dependent on the presence of insulin. These findings were extended by the demonstration that hGH 1–15 enhanced in vivo and in vitro activity of the insulin-dependent enzyme of muscle glycogen synthase.[11] Again, the in vitro data showed that the action of the peptide was insulin dependent but in vivo proof was lacking.

This paper describes experiments in which the action of the peptide has been investigated in vivo using insulin-deficient streptozotocin-diabetic rats, and, in view of the fact that the primary action of insulin is to bind to specific cell plasma membrane receptors,[12] the effect of preinjection of the peptide on insulin binding by isolated hepatic plasma membrane of rats.

MATERIALS AND METHODS

Peptide synthesis. The synthetic peptide hGH 1–15 was synthesized by the solid phase technique,[13] cleaved and deblocked with anhydrous hydrogen fluoride, and purified by the following technique. The synthetic hGH 1–15 was initially purified on a column of Biogel P-4 (5.0 × 17.0 cm) in 0.1 M acetic acid-0.01 M mercaptoethanol. The fractions comprising peptide material were detected spectrophotometrically at 260 nm, pooled, and lyophilized. Further purification was carried out by cation chromatrography on a column of carboxymethylcellulose (2.5 × 40.0 cm) in 0.01–0.10 M ammonium acetate-0.01 M mercaptoethanol, pH 4.5, and by gel filtration on Sephadex G-25 SF

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* The amino-terminal fragment of human growth hormone, hGH–15, is NH_2–Phe–Pro–Thr–Ile–Pro–Leu–Ser–Arg–Leu–Phe–Asp–Asn–Ala–Met–Leu–OH.

(1.7 × 60.0-cm column) in 1 M acetic acid-0.01 M mercaptoethanol.

The purity of the final preparation was checked by amino acid analysis of acid hydrolysates,[14] and results showed Phe, 2.0; Pro, 1.9; Thr, 0.9; Ile, 0.9; Leu, 3.0; Ser, 0.8; Asn and Asp, 1.9; Arg, 1.1; Ala, 0.8; and Met, 0.8. On high-voltage electrophoresis in pyridine acetate buffer (pH 3.5, Whatman 3MM, 2000 V, 90 min) the purified hGH 1–15 showed a single spot, R_{Lys} 0.30, with some tailing, probably caused by the low solubility of this peptide. Thin layer chromatography was run on silica gel 60 (Merck) in three systems: (1) ethyl acetate/pyridine/acetic acid/water, 5:5:1:3; (2) 1-butanol/pyridine/acetic acid/water, 30:20:6:24; and (3) n-propanol/pyridine/ethyl acetate/acetic acid/water, 5:4:4:1:6. The purified hGH 1–15 showed single spots in all systems, with the following R_f values, respectively: (1) 0.65, (2) 0.55, and (3) 0.56.

Animals. Diabetes mellitus was induced in rats by the intravenous injection of streptozotocin[15] [2-deoxy-2-(3 methyl-3-nitrosoureido)-D-glucopyranose] (Upjohn Company, Kalamazoo, Michigan) freshly dissolved in 0.01 M citrate buffer, pH 4.3, into male Wistar rats (200 ± 10 g body wt) at a dose of 100 mg/kg body wt. An equivalent group was given citrate buffer only. Both groups were then maintained on a standard pellet diet with water ad libitum. On the fifth day after injection, the animals were fasted for 16 h and blood was taken under light pentobarbital anesthesia for glucose and venous plasma immunoreactive insulin estimations. The mean blood glucose was 22.6 ± 1.6 mmol/L in streptozotocin-treated rats and 5.1 ± 0.7 mmol/L in untreated controls. Plasma insulin likewise differed from streptozotocin-treated to untreated animals (2.1 ± 0.8 μU/ml to 27.3 ± 7.2 μU/ml). Those animals with blood glucose levels higher than 18 mmol/L and plasma insulin below 3 μU/ml were considered diabetic and insulin deficient. All such animals showed polyuria, glycosuria, and weight loss as well as hyperglycemia and hypoinsulinemia.

In vivo studies. The effect of hGH 1–15 on basal blood glucose and plasma insulin levels was determined on 16-h-fasted animals. The peptide hGH 1–15 (2 mg/kg body wt) was given intravenously, and blood samples (25–100 μl) were collected from the tail vein of pentobarbital-anesthetized animals at various time intervals as shown in the figures and tables.

Intravenous insulin tolerance tests (IVITT) and intravenous glucose tolerance tests (IVGTT) were performed on both control and diabetic groups as previously described.[16] In all such tests, the mode of administration was 0 time:peptide or solvent, 5 min:insulin or glucose; the first glucose sample was taken at 0 + 10 min. All tests were carried out 5 days after the administration of streptozotocin or buffer, as by this time the physiopathologic state had stabilized.[17]

Glucose and insulin determinations. Glucose levels were estimated immediately on the collection of blood by the glucose oxidase method using a Yellow Spring YS1, Model 23AM, glucose analyzer. The plasma was then separated by centrifugation and stored at −20°C. Plasma immunoreactive insulin (IRI) was then estimated using the method of Herbert et al.,[18] the antigen and antibody being obtained from the Amersham-Searle Corporation, Amersham, England.

Preparation of hepatic plasma membranes. The animals were fasted for 16 h and divided into two groups. One group was injected intravenously with 2 mg/kg body wt of hGH 1–15 in saline and the control group with saline only. Two hours after injection both groups were killed after blood was collected for glucose analysis, and hepatic plasma membranes prepared following the method of Ray.[19] Blood glucose levels of both the control and treated groups were 6.3 ± 1.5 mmol/L.

The plasma membrane preparations of the control and treated animals were analyzed for their protein patterns with high-voltage electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS gels). The apparatus as described by Reid and Bieleski[20] was used and the experimental procedure of Laemmli and Favre[21] was employed. The results (Figure 1) reveal that the preparations of both plasma membranes were virtually identical in their protein patterns. Radioreceptor binding for human growth hormone of the plasma membranes was investigated by the method of Herington et al.[22] by Dr. A. Herington, Medical

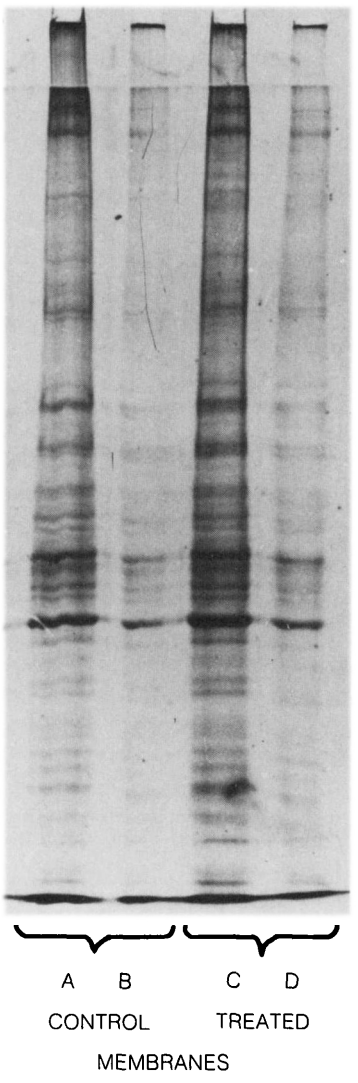

FIGURE 1. Comparison of protein profiles of hepatic plasma membranes prepared from control and hGH 1–15-treated rats. Electrophoresis was performed in 0.1% sodium dodecyl sulfate in 8% polyacrylamide slab gels. The two different amounts of total protein used for each type of membranes were 20 μg (B and D) and 100 μg (A and C).

TABLE 1
Effect of hGH 1-15 on fasting levels of blood glucose in normal and severely diabetic rats

Animals	Time after injection of hGH 1–15 (min)						
	0	5	10	15	30	45	60
	Blood glucose (mmol/L)						
Normal (6)	5.6 ± 0.8	5.4 ± 1.2	5.6 ± 1.0	5.4 ± 1.3	5.2 ± 1.0	5.0 ± 0.6	5.1 ± 1.4
Severely diabetic (6)	20.8 ± 3.4	21.4 ± 2.7	21.5 ± 1.9	21.6 ± 2.0	21.4 ± 1.9	20.9 ± 1.9	20.1 ± 2.0

All values are expressed as mean ± SD.
Figures in parentheses indicate the number of animals studied.

Research Centre, Prince Henry Hospital, Melbourne. For both the control and pretreated membranes, the specific bindings for hGH were similar. This further confirms that the variability of the membrane preparations was negligible.

Binding studies. Studies of binding of ^{125}I-insulin to hepatic plasma membranes were conducted at 30°C in 50 mM Tris-HCl buffer, pH 7.5, containing 0.5% bovine serum albumin.[23] Aliquots of plasma membrane (350 μg/ml) were preincubated with various concentrations of unlabeled insulin for 15 min before the addition of 1 ng/ml of ^{125}I-insulin for a further 30 min incubation. Immediately after incubation, the membrane-bound hormone was recovered by centrifugation of triplicate samples and radioactivity estimated using a Philips Automatic Gamma Analyzer, Model PW4520.

Analysis of data. All experimental data are expressed as mean ± SD, and differences in means were analyzed by the Student's *t* test. A P-value of <0.05 was accepted as statistically significant.

RESULTS

The effects of hGH 1–15 on basal glucose and plasma insulin levels are shown in Tables 1 and 2. It is seen that administration of the peptide has no effect on either the glucose levels or the plasma insulin levels of fasting normal or diabetic rats.

The data arising from glucose tolerance tests show that a significant increase of glucose uptake is observed in the normal animals, but that the peptide is devoid of any activity in the diabetic animals (Figure 2).

Insulin tolerance tests show that the peptide significantly potentiates the action of exogenously administered insulin in both normal and diabetic animals, the effect apparently being quantitatively larger in the diabetic animal (Figure 3), supporting the concept of potentiation of insulin action as being the action of the peptide. However, the peptide has no activity per se in the insulin-deficient diabetic animals, as previously shown.

Receptor binding studies. The maximal binding of ^{125}I-insulin by hepatic plasma membranes from both control- and hGH 1–15-injected rats is shown in Figure 4. It is seen that the hGH 1–15-treated membranes bound more ^{125}I-insulin at all concentrations of the hormone, the experimental and control curves being significantly different over an insulin concentration range of 1×10^{-4} μg/ml to 1×10^{-1} μg/ml. This difference cannot be regarded as a result of insulin degradation, since no differences were observed in measurement of intact ^{125}I-insulin by TCA precipitation after 2.5 h incubation in the presence and absence of 350 μg of membrane/ml.

Scatchard analysis[24] of the binding data from Figure 4 indicates that both the hGH 1–15-treated and control curves are curvilinear and essentially parallel to each other (Figure 5), implying that the differences in binding are due to an increased number of active receptors in the hGH 1–15-treated membranes. This activity, in view of the short time needed to demonstrate the peptide potentiation of insulin actions, is probably due to a change in the ratio of high affinity to low affinity receptors, as 10–20 min is hardly long enough for significant new synthesis of such binding sites.

DISCUSSION

The data obtained from experiments with insulin-deficient diabetic rats clearly indicate the absolute requirement for insulin for the peptide to reduce blood glucose levels. Experiments with normal animals show that hGH 1–15 is not an insulinogogue and that it is not capable, at the dosage used, of potentiating the effect of fasting levels of insulin, but is active when insulin secretion is provoked by glucose (Figure 2) or exogenously administered (Figure 3). The considerably larger quantitative response observed in potentiation of the same dose of insulin in diabetic animals (Figure 3) may be due either to the far higher blood glucose level providing a more favorable gradient for transport into the cell or the increase in the total number of receptors ob-

TABLE 2
Effect of hGH 1-15 on fasting levels of plasma insulin in normal and severely diabetic rats

Animals	Time after injection of hGH 1–15 (min)					
	0	30	60	90	120	150
	Plasma insulin (μU/ml)					
Normal (5)	30.0 ± 6.3	31.4 ± 5.0	30.8 ± 7.8	27.0 ± 6.2	29.6 ± 6.8	27.8 ± 7.2
Severely diabetic (5)	2.8 ± 2.3	2.6 ± 1.8	2.8 ± 1.9	2.2 ± 1.5	2.4 ± 1.5	2.5 ± 1.6

All values are expressed as mean ± SD.
Figures in parentheses indicate the number of animals studied.

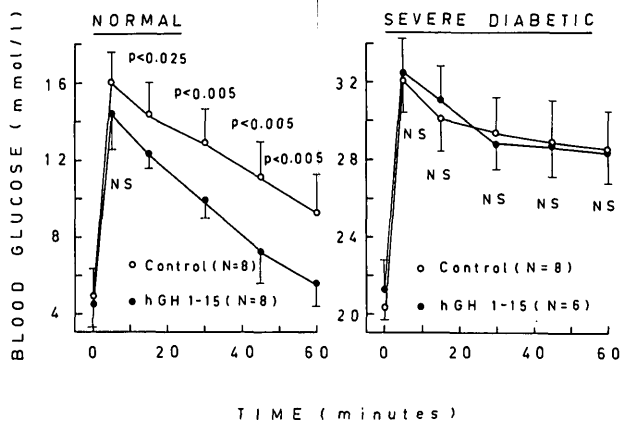

FIGURE 2. Effect on hGH 1–15 in normal and severely diabetic rats during intravenous glucose tolerance tests. The peptide was administered intravenously 5 min before the glucose injection. Blood glucose levels were done as shown in the figure. Each point represents the mean blood glucose values (± SD) of the number of animals as indicated in parentheses (N). The statistical difference between control (○) and test (●) groups of the normal animals was significant from 15 to 60 min. No significant difference (NS) was detected at any time between control (○) and the test (●) groups of the diabetic animals.

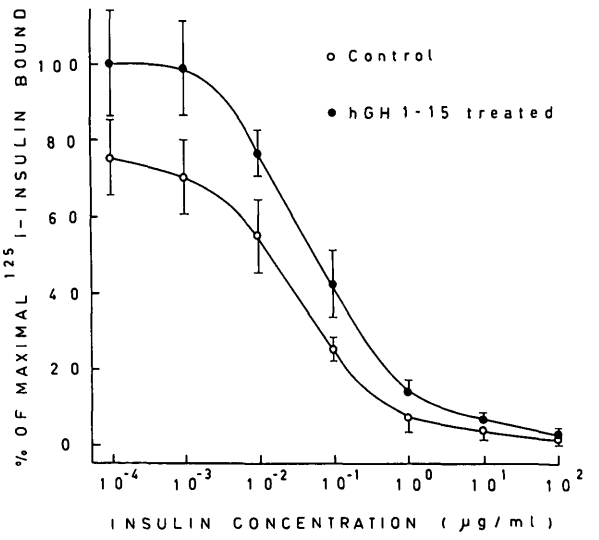

FIGURE 4. Comparison of percentage of maximal ^{125}I-insulin binding to hapatic plasma membranes from control (○) and hGH 1–15-treated (●) rats. Data are corrected for nonspecific binding and represent mean ± SD of nine determinations. The curves are significantly different ($P < 0.05$) at insulin concentrations from 10^{-4} to 10^{-1} μg/ml.

served in streptozotocin-diabetic rats,[25] or a combination of both factors.

The radioinsulin receptor studies strongly support the hypothesis that the mechanism of action of hGH 1–15 is by the potentiating action of insulin. The prime event in the initiation of insulin action is the binding of the hormone to a highly specific plasma membrane receptor. This concept is strongly supported by the finding that autoantibodies to insulin receptors, which bind highly specifically to their antigen, activated the insulin-dependent enzyme, glycogen synthase, in adipocytes.[26] This study shows (Figures 4 and 5) that pretreatment of animals with hGH 1–15 results in increased specific binding of insulin to hepatic plasma membranes. It supports the potentiation hypothesis either as a consequence of increase in the number of receptors or their affinity for insulin, providing an amplified biologic signal, which then results in an increased uptake of glucose and activates the insulin-dependent enzyme, muscle glycogen synthase.[11]

Milman and Russell[1] originally reported that purified growth hormone produced hypoglycemia in normal rats but was ineffective in chronically depancreatized diabetic animals. The results observed in Table 1 for hGH 1–15 are essentially similar, particularly if it is remembered that growth hormone administration provokes insulin secretion.[27] This parallelism between growth hormone and one of its partial sequences suggests that the hypoglycemic action of pituitary growth hormone may be due to an active center within the hGH 1–15 sequence, but cannot be regarded as proof of the hypothesis. It is premature to compare the data obtained using peptide hGH 1–15 or its minimum active sequence hGH 8–13 with the effects observed by others using

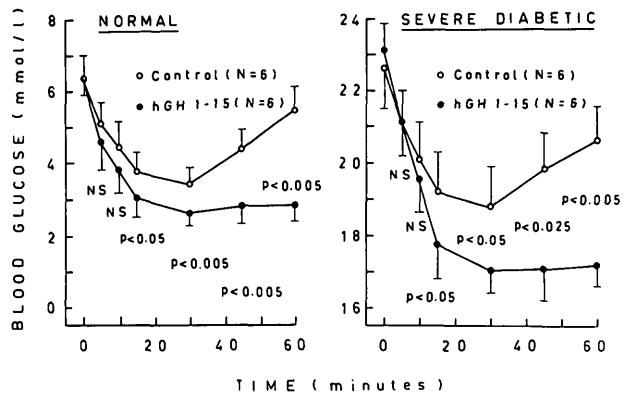

FIGURE 3. Effect of hGH 1–15 in normal and severely diabetic rats during intravenous insulin tolerance tests. Each point represents mean ± SD of blood glucose at time indicated. Changes of blood glucose between the control groups, which had only saline before insulin (○), and the test groups, which received peptide before insulin (●), were statistically evaluated according to Student's *t* test. The P values are shown at each time point. NS means no statistical significance. Both normal and diabetic animals who received only the peptide hGH 1–15 showed no change of blood glucose levels for the same period of time (Table 2). Numbers of animals studied for each group are indicated in parentheses (N).

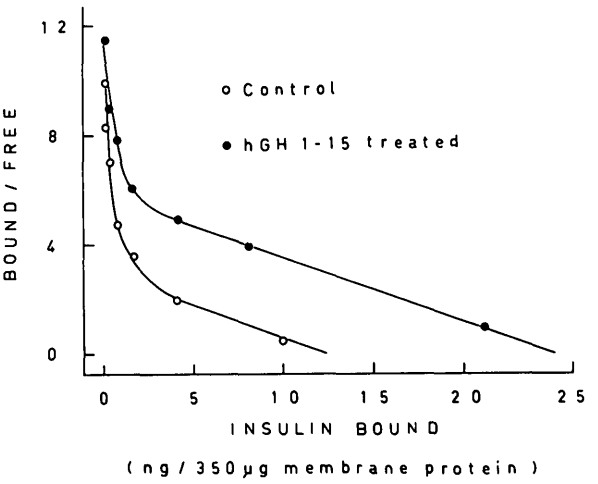

FIGURE 5. Scatchard analysis of data derived from the same series of experiments as in Figure 3. Bound/Free ratio is plotted as a function of bound insulin to hepatic plasma membranes from control (○) and hGH 1–15-treated (●) rats. Data have been corrected for nonspecific binding.

intact bovine growth hormone: Goodman[28] attributed the hypoglycemic action entirely to effects in adipose tissue, and Fineberg and Merimee[29] attributed these actions entirely to hepatic mechanisms. Work in this laboratory clearly indicates that the peptide hGH 6–13 has a hepatic action inhibiting glycogen phosphorylase[30] and more recent preliminary data indicate clear-cut activity in peripheral tissues.

In conclusion, the experimental data presented suggest that the action of polypeptide hGH 1–15 is probably due, at least in part, to the increase of the binding of insulin to its receptors, thus amplifying the biologic signal which leads to the actions of the hormone. No evidence as yet can be positively presented as to whether hGH 1–15 is a natural peptide or must be regarded as a pharmacologic agent of some therapeutic potential.

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