The Diabetogenic Activity of Fragments of Human Growth Hormone in Obese (*ob/ob*) Mice

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SUMMARY

The genetically obese (*ob/ob*) mouse was used to investigate the effects of highly purified human growth hormone (hGH), reduced and S-carbamidomethylated hGH (RCAM-hGH), plasmin-digested RCAM-hGH (PD-RCAM-hGH), and peptide fragments isolated from the plasmin digests of RCAM-hGH on fasting blood glucose concentration and glucose tolerance. Mice treated for three days with hGH exhibited both increased fasting blood glucose concentrations and aggravated intolerance to administered glucose. Similar changes in the fasting blood glucose concentration and glucose tolerance were observed after treatment of mice with RCAM-hGH and PD-RCAM-hGH. Moreover, two noncovalent complexes of peptides of RCAM-hGH, one consisting of residues 1-134 bound to 141-191 (Dal) and the other consisting of almost equal amounts of 1-134 and 42-134 bound to 141-191 (Dc2), appeared to retain the full activity of the native hormone in that they increased fasting blood glucose concentrations and aggravated glucose intolerance in the *ob/ob* mice. A peptide consisting of residues 95-134 of RCAM-hGH appeared to retain some diabetogenic activity, while a peptide consisting of residues 20-41 was devoid of activity. Tolerance tests performed six hours after a single injection of either hGH or fraction Dal indicated that this time interval was not sufficient to elicit the diabetogenic response. Thus, these studies indicate that neither intact disulfide bridges nor intact primary amino acid sequence of the hGH molecule are required for the expression of its diabetogenic activity. **DIABETES *27*:883-88, September, 1978.**

INTRODUCTION

Studies of the action of growth hormone (GH) preparations on carbohydrate metabolism have demonstrated a delayed anti-insulin-like or diabetogenic action of the hormone.[1,2] While these effects of GH preparations are well known,[3] the exact chemical nature of the agent responsible for the diabetogenic response has not been firmly established. Although the suggestion that the diabetogenic action of GH preparations is due to contaminants[4] has been supported by the isolation of pituitary diabetogenic factors chemically distinct from GH,[5-7] it has not been possible to dissociate completely diabetogenic activity from highly purified GH preparations. In fact, recent structure-function studies on GH tend to support the alternative hypothesis that the diabetogenic activity is an intrinsic property of the GH molecule. Enzymatically modified forms of GH as well as fragments of the GH molecule isolated from enzymatic digests retain not only the diabetogenic activity[8-14] but also a number of other biologic actions characteristic of the GH molecule, as determined in a variety of in vivo and in vitro assay systems.[13-15]

The purpose of the present study was to determine if the diabetogenic action of human GH is retained after reduction and alkylation of its disulfide bridges and after digestion of the reduced and alkylated hormone (RCAM-hGH) with the enzyme plasmin. Further, peptide fragments of the hormone molecule have been isolated and tested for diabetogenic activity. The obese mouse (*ob/ob*) was chosen for this study because of the responsiveness of this animal to the hyperglycemic action of GH preparations.[16,17] With the exception of recent reports by Lostroh and

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Krahl,[8,9] detailed studies have not appeared in the literature to extend the original observations of Mayer and co-workers[16,17] that treatment of *ob/ob* mice with GH elevated their basal circulating concentrations of glucose and caused a greater intolerance to administered glucose.

MATERIALS AND METHODS

Animals. Female, C57BL/6J mice homozygous for the *ob* gene (*ob/ob*) were purchased from the Jackson Memorial Laboratory, Bar Harbor, Maine. The mice were fed ad libitum and were maintained in plastic cages under conditions of constant temperature with a normal 12-hour light cycle. The mice were used for experiments when they were three to nine months of age.

Determination of the effect of test materials on glucose tolerance. Glucose tolerance tests (GTT) were conducted in the following manner on groups of three to four mice that had been matched for sex, age, and weight. Each mouse was injected subcutaneously (s.c.) for three consecutive days with 0.1 ml. saline. On the fourth day, each mouse was given 2 μg. dexamethasone s.c. and fasted for six hours. After the fast, 20-μl. blood samples were obtained from the tail, and each mouse was then injected intraperitoneally (I.P.) with glucose (1 mg. per gram body weight). Blood samples were obtained every 20 or 30 minutes over the next 180 minutes. The 20-μl. blood samples were diluted to 2.0 ml. with distilled water and deproteinized by the addition of equal volumes (1.0 ml.) of 2 per cent (W/V) zinc sulfate and 1.8 per cent (W/V) barium hydroxide. Triplicate 1.0-ml. aliquots of the filtrates were stored frozen (−20° C.) until assayed for glucose concentration. Aliquots (20 μl.) of standard glucose solutions covering the range of 0 to 800 mg./100 ml. were treated in the same manner as the blood samples. The filtrates were thawed and mixed with 1.0 ml. of freshly prepared Glucostat reagent (Worthington Biochemical, Freehold, New Jersey) followed by exactly 30 minutes incubation at 37° C. The enzymatic reaction was stopped by addition of 0.3 ml. of 0.5 N sulfuric acid, and the absorbance at 410 nm. was determined in a Gilford Stasar II spectrophotometer. Glucose concentrations in the assay tubes were determined directly from the standard curves. The mean blood glucose concentration for the group of three or four mice at each time point was then plotted against time. Seven days after the beginning of saline treatment, the same group of mice was treated for three days with highly purified human growth hormone (hGH or one of the test preparations) followed, on the fourth day, by a GTT performed as described above. The data obtained after treatment with the test substances were plotted along with the data obtained on the same group of mice during the control period one week earlier to permit direct comparison. In some experiments, saline or test material was injected only at the beginning of the fast, that is six hours before the GTT, to detect possible acute effects of the test materials. In the latter design, the control period and test period were separated by one day. These assays were used to test native hGH and a series of modified hGH preparations for the ability to cause hyperglycemia and glucose intolerance.

Preparation of modified forms of hGH. A detailed description of the preparation of and the chemical and biologic characterization of the modified forms of hGH used in these experiments has been presented.[15,18-21] Highly purified preparations of hGH were reduced with dithiothreitol, and the resulting half-cystine residues were alkylated with iodoacetamide to give the reduced and carbamidomethylated form of hGH (RCAM-hGH). The RCAM-hGH was then digested with human plasmin (AB KABI, Stockholm, Sweden) at pH 8.0 for 20 to 24 hours at 37° C. Fractionation of the plasmin digests of RCAM-hGH (PD-RCAM-hGH) yielded several preparations, which are identified as follows:

Da and **Dc**: two of the major fractions isolated by ion exchange chromatography of the digests. These fractions were initially thought to be mixtures of peptides derived from the amino-terminal portion of the GH molecule;[19] however, they were subsequently found (see below) to consist primarily of amino-terminal peptides noncovalently bound to carboxyl-terminal peptides.[20] These fractions were submitted to gel filtration on Sephadex G-50 to isolate the following subfractions:

Da1: a noncovalent complex of peptides 1-134 and 141-191 of RCAM-hGH isolated from Da; **Dc2**: a noncovalent complex of large peptides but, from end-group and amino acid analyses, it appears to be composed of nearly equal amounts of peptides 1-134 and 42-134 bound to peptide 141-191 isolated from Dc; **Da2**: a peptide comprising residues 20-41 of RCAM-hGH isolated from Da; and **Dc3**: a peptide comprising residues 95-134 of RCAM-hGH isolated from Dc.

RESULTS

To ensure that manipulation of the mice during the

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assay procedure did not influence the experimental results, a group of mice was treated with saline during both the control period and the experimental period. As can be seen in panel A of figure 1, the glucose tolerance curves obtained during both the control and experimental periods of such an experiment were similar; comparable results were obtained in four other similar experiments not shown. In contrast, when the mice were treated with highly purified hGH during the three-day experimental period, a dramatic decrease in their tolerance to administered glucose was observed (panels B and C of figure 1). Treatment for three days with 0.1 mg. hGH per day resulted in a slight but consistent elevation in the fasting blood glucose concentration and higher peak glucose values (data not shown). Greater intolerance was observed after treatment for three days with higher doses of hGH (panels B and C of figure 1). The hGH-dependent intolerance to glucose required considerable time for development, as evidenced by the tolerance curves shown in panel D of figure 1. Mice treated with 0.5 mg. of hGH six hours before the GTT did not show elevated fasting blood glucose concentrations or aggravated glucose intolerance.

Treatment of *ob/ob* mice with 0.2 mg. of RCAM-hGH per day using the 'three days of injection' schedule was found to cause effects similar to those produced by 0.2 mg. of hGH per day (data not shown). Thus, disrupting the disulfide linkages of the native hGH molecule did not destroy the diabetogenic activity of the hormone. The plasmin digests of RCAM-hGH were then tested at a dose of 0.2 mg. per day for three days to determine if the intact amino acid sequence of hGH was necessary for diabetogenic activity. As shown in figure 2, this dose of the PD-RCAM-hGH (like intact hGH) caused a dramatic elevation in the fasting blood glucose concentration and an upward shift in the glucose tolerance curve, indicating that the digests contained a fragment(s) of the hGH molecule that retained diabetogenic activity.

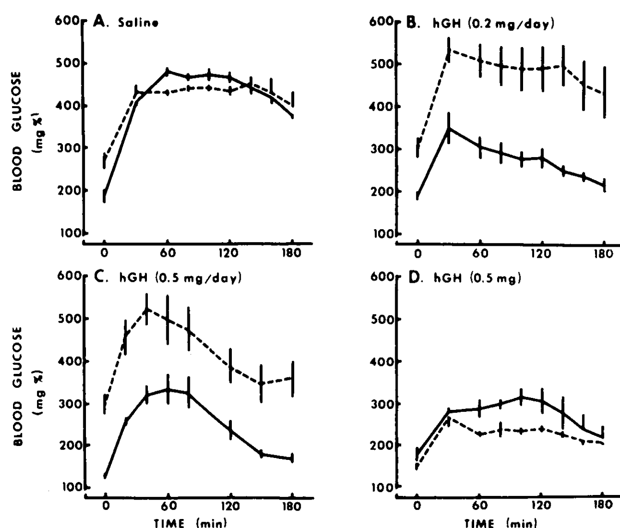

FIG. 1. Effects of saline or hGH treatment on the glucose tolerance of fasted *ob/ob* mice. Groups of *ob/ob* mice were first tested for glucose tolerance after treatment with saline (control period, ——) and then treated with saline or hGH and retested for glucose tolerance (experimental period, ---). Panels A, B, and C show glucose tolerance curves obtained after three days of treatment with saline or hGH. Panel D shows glucose tolerance curves obtained six hours after saline or hGH treatment. Each point represents the mean ± S.E.M. of four mice.

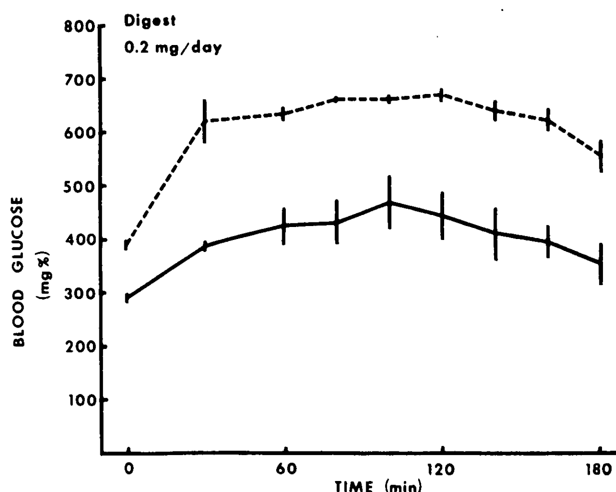

FIG. 2. Effects of treatment for three days with saline (control period, ——) or treatment for three days with plasmin-digested hGH (experimental period, ---) on the glucose tolerance of fasted *ob/ob* mice. Each point represents the mean ± S.E.M. of five mice.

Fraction Da isolated from the PD-RCAM-hGH was also found to be active in further aggravating the glucose intolerance in the *ob/ob* mice when injected for three days at a dose of 0.2 mg. per day (figure 3). Fraction Dal was also found to be diabetogenic. Treatment of *ob/ob* mice for three days with 0.2 mg. of Dal per day resulted in elevated fasting blood glucose concentrations and an upward shift in the glucose tolerance curve (left panel of figure 4). This effect appeared to be comparable to that obtained with an equivalent dose of hGH. As shown in the right panel of figure 4, *ob/ob* mice treated with 0.5 mg. of Dal six hours before the GTT did not show elevated fasting blood glucose concentrations or upward shifts of the glucose tolerance curve. The fact that the peak blood glucose concentration reached during the GTT six hours after Dal treatment was lower than that reached

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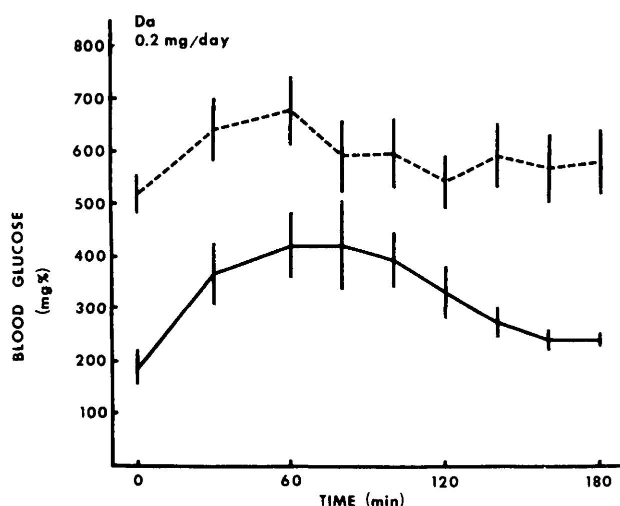

FIG. 3. Effects of treatment for three days with saline (control period, ——) or treatment for three days with fraction Da (experimental period, ---) on the glucose tolerance of *ob/ob* mice. Each point represents the mean ± S.E.M. of four mice.

six hours after saline treatment remains to be investigated. Fraction Dc2, likewise, appeared to be as diabetogenic as the native hormone when injected for three days at a dose of 0.2 mg. per day (table 1).

Two peptides derived from the amino-terminal portion of the hGH molecule were tested for diabetogenic activity. The fasting blood glucose concentrations and the tolerance curves obtained with a group of four mice after treatment with either saline or the Da2 fraction (residues 95-134), at a dose of 0.2 mg. per day, produced slight elevations of the fasting blood glucose concentration and the tolerance curves above

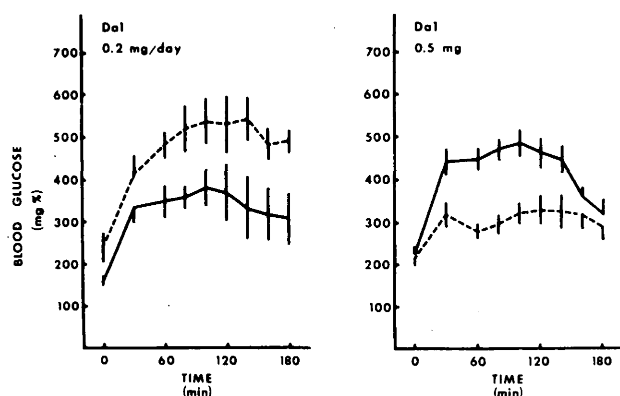

FIG. 4. Effects of treatment with saline (control period, ——) or treatment with fraction Dal (experimental period, ---) on the glucose tolerance of fasted *ob/ob* mice. The left panel shows data obtained after treatment for three days, and the right panel shows data obtained six hours after treatment. Each point represents the mean ± S.E.M. of four mice.

the values obtained after saline treatment in four of seven mice. However, only the fasting blood glucose concentration was significantly increased (table 2). Similar results were obtained with two of three mice treated with 0.4 mg. of peptide 95-134 per day (data not shown).

TABLE 1

Effect of Dc2 on the glucose tolerance of *ob/ob* mice*

Time after glucose injection	Blood glucose concentration (mg./100 ml.) Saline-treated	Hormone-treated
0	209 ± 19†	314 ± 5‡
30	310 ± 19	440 ± 12‡
60	320 ± 19	409 ± 12‡
120	344 ± 19	380 ± 18
180	268 ± 17	367 ± 29§

*Glucose tolerance curves were obtained on four mice after treatment for three days with either saline or the Dc2 fraction (0.2 mg. per day).

†Mean ± S.E.M. for four mice.

‡Indicates $P < 0.01$ as compared with saline-treated (Student *t* test).

§Indicates $P < 0.05$ as compared with saline-treated (Student *t* test).

DISCUSSION

The results of the present study demonstrate that disruption of the disulfide bonds of the hGH molecule does not abolish the diabetogenic action of the hormone. Furthermore, interruption of the primary amino acid sequence of hGH by digestion of the reduced and alkylated hormone with the enzyme plasmin did not destroy its diabetogenic action. In addition, some peptide fragments isolated from the digests retained their diabetogenic activity. The noncovalent complex of peptide 1-134 bound to residues 141-191 (Dal) retained the full diabetogenic activity of native hGH in the *ob/ob* mouse. The noncovalent complex, containing nearly equal proportions of peptide 1-134 and peptide 42-134 bound to residues 141-191 (Dc2), also appeared to be as diabetogenic as the native hGH and the Dal complex. The results obtained with peptide 95-134 suggest that this portion of the molecule may retain some of the diabetogenic activity, while the results obtained with peptide 20-41 at a dose equal to about 1 mg. of hGH per day strongly suggest that this portion of the molecule is not diabetogenic. The latter point is supported by the observation that the Dc2 preparation, in which only about 50 per cent of the peptide complexes have residues 1-41, appeared to be as active as the Dal fraction in this assay.

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TABLE 2

Effect of Da2 (peptide 20-41) and Dc3 (peptide 95-134) on the glucose tolerance of *ob/ob* mice*

Time after glucose injection	Blood glucose concentration (mg./100 ml.) Saline-treated	Hormone-treated
	Da2	
0	261 ± 21†	282 ± 8
30	439 ± 31	409 ± 19
60	464 ± 32	429 ± 39
120	516 ± 38	533 ± 59
180	407 ± 44	461 ± 74
	Dc3	
0	179 ± 7‡	245 ± 14§
30	320 ± 20	383 ± 27
60	357 ± 29	426 ± 51
120	354 ± 39	459 ± 60
180	279 ± 42	366 ± 52

*Glucose tolerance curves obtained on four mice after treatment for three days with either saline or the test preparation (0.2 mg. per day).

†Mean ± S.E.M. for four mice.

‡Mean ± S.E.M. for seven mice.

§Indicates $P < 0.01$ as compared with saline-treated (Student *t* test).

The results of these experiments strongly support the hypothesis that the diabetogenic action of GH preparations is a property of the hGH molecule. It seems highly unlikely that other pituitary diabetogenic factors unrelated to GH would survive the isolation procedures needed to obtain the hGH fragments used in these studies in sufficient amounts to be responsible for the effects observed.

Data suggesting that fragments of the hGH molecule retain the diabetogenic property of the native GH molecule have already appeared. We have reported previously that plasmin digests of native hGH produced glucosuria in partially pancreatectomized, dexamethasone-treated rats when injected subcutaneously for four days.[13] Moreover, a fraction isolated from such plasmin digests of hGH, which consisted of residues 1-134 attached to residues 141-191 by the disulfide bond between residues 53 and 165, also exhibited the diabetogenic activity of the native hormone in partially pancreatectomized, dexamethasone-treated rats.[14] Lewis et al. have recently reported that preparations of enzymatically modified hGH, with intact disulfide bonds but missing either amino acid residues 139-147 or residues 139-149 from the large disulfide loop, caused hyperglycemia in dogs.[11] Interestingly, their preparations appeared to be more potent than was native hGH in causing hyperglycemia in dogs. Lostroh and Krahl[8,9] have isolated a peptide from pepsin digests of human and sheep GH that produced not only an acute decrease in glucose tolerance when injected in a divided dose 15 minutes before, and concurrently with, the glucose, but also caused long-term hyperglycemia persisting for several months. Finally, a synthetic peptide that is homologous to residues 172-191 of hGH has been reported to have hyperglycemic activity in normal rats.[21] Thus, it is clear that the hGH molecule possesses diabetogenic activity, but the region of the molecule responsible for this activity is not firmly established.

ACKNOWLEDGMENTS

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