0013-7227/88/1226-2940$02.00/0
Endocrinology

Vol. 122, No. 6
Printed in U.S.A.

Increased Sensitivity of Adipose Tissue to Insulin after *in Vivo* Treatment of Yellow A^{vy}/A Obese Mice with Amino-Terminal Peptides of Human Growth Hormone*

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ABSTRACT. Treatment of obese yellow A^{vy}/A mice with the human GH (hGH) peptide hGH-(1–43) enhanced the *in vitro* sensitivity of their adipose tissue to insulin. Insulin-stimulated glucose oxidation, as determined by measurement of $^{14}CO_2$ production, was enhanced 106% after administration of hGH-(1–43) at a dosage of 1 μg/day for 3 days. A significant increase in CO_2 production was detected with as little as 100 ng peptide/day for 3 days. A single injection of 10 μg increased sensitivity to insulin 2–5 times. This enhancing effect of insulin action could not be seen in lean agouti A/a animals nor could it be demonstrated by *in vitro* addition of hGH-(1–43) to adipose tissue. Synthetic hGH-(1–43) was used for these studies, but initial physiological work was done with peptide isolated from pituitary glands. At equimolar doses, intact hGH, a trypsin digest of either hGH or BSA, carbidomethyl cysteine-hGH-(146–191), and hGH-(32–46) were inactive. Carbidomethyl cysteine-hGH-(1–139) and hGH-(1–15) showed the enhancing property, but were only about 10% as active as hGH-(1–43). HGH-(1–43) did not increase serum insulin concentrations in the obese mice. We conclude that when administered *in vivo* to obese mice, hGH-(1–43) enhances the sensitivity of adipose tissue to the action of insulin, an indication that the peptide may play a role in carbohydrate metabolism. (*Endocrinology* **122:** 2940–2945, 1988)

SOON after isolation of the 20K variant of human GH (hGH) (hGH 20K) (1), Frigeri *et al.* (2) reported that the variant did not stimulate glucose oxidation in rat adipose tissue as does the major form of the hormone (hGH 22K). Lack of insulin-like activity in adipose tissue was confirmed by Smal *et al.* (3), who showed that hGH 20K was not lipogenic in adipocytes and that this failure to stimulate lipogenesis was correlated with the absence of binding of the variant to membrane receptors. These observations suggested that the sequence missing from hGH 20K (residues 32–46) (4) was involved in the insulin-like activity of hGH 22K (5). Several reports now clearly indicate that this region of the amino-terminal portion of hGH can indeed influence glucose utilization, even though the mechanism is not understood. Activity was found when hGH-(32–46) was given to rodents, dogs, and primates (6–11). Reported effects include enhancement of the action of administered insulin, increase in glucose uptake by liver and muscle, and increase in the sensitivity of adipose tissue to insulin. Although we were unsuccessful in finding hGH-(32–46) in pituitary extracts, hGH-(1–43), a longer peptide that contained much of the 32–46 sequence, was isolated (12) and was found to be more potent than the shorter peptide in enhancing insulin-stimulated action. We now use synthetic hGH-(1–43) for metabolic studies. Because hGH-(1–43) is a component of pituitary glands (at least 12 μg/gland), and because there is increasing evidence that low mol wt forms of hGH circulate in blood in significant concentrations (13), we believed a study of the physiological actions of hGH peptides to be important. The activity of hGH-(1–43) reported here indicates that this peptide may play a role in carbohydrate metabolism.

Received September 3, 1987.
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* This work was supported in part by NIH Grants DK-314117 and BRSG S07-RR-05876, the Adler Foundation (Rye, NY), and the Donald S. Anson Memorial Fund.
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Materials and Methods

Experimental design

Obese yellow mice or their lean agouti littermates were injected once only or once daily for 3 days with a peptide or saline; 60 min after the last dose the animals were killed, and the adipose tissue was removed and tested for its sensitivity to the action of insulin. Sensitivity was assessed by the amount

of $^{14}CO_2$ produced from ^{14}C-labeled glucose by known quantities of insulin.

Animals and peptide administration

Yellow obese A^{vy}/A mice, 50–60 g BW, and their lean agouti A/a F-1 hybrid littermates, 35–45 g BW, were used in these studies (14). The ages of the mice ranged from 6 to 12 months. These (BALB/c × VY) F-1 hybrid mice were produced by mating BALB/c-AnNCr1BR females purchased from Charles River Breeding Laboratories (Wilmington, MA) with VY/WffC3H/NCTR-A^{vy}/a males raised at the National Center for Toxicological Research. Diet (Wayne Lab Blox) and tap water were given *ad libitum*. The ambient temperature was 22 ± 2 C, and a 12-h light, 12-h dark cycle was maintained. Although male yellow mice were used for the studies reported here, females can also be used with the same results. Rats were of the Sprague-Dawley strain, 150–170 g BW.

hGH-(1–43) and hGH-(1–42)-NH_2 were dissolved in 10 mM HCl (0.1 ml/mg peptide) and then diluted with saline to the desired concentration. The other peptides were dissolved in 0.5 M $NaHCO_3$ and diluted with saline. Administration was done by ip injection. For the cannulation studies described below, where the effect of peptide on serum insulin was studied, the test substance was given intrajugularly. This route of administration was also tried in yellow mice and was found to be neither more nor less effective than the ip route.

Cannulation

The details of the cannulation technique have been described previously (15). On the day of the experiment, animals were left completely undisturbed in their isolation chambers. Blood samples (0.5 ml) were withdrawn via the indwelling catheter at the desired times, and heparinized blood was centrifuged immediately at 4 C. Plasma was removed for glucose and insulin determinations; blood cells were returned to the animals 20 and 70 min after the first bleeding. We are indebted to Dr. William B. Wehrenberg for carrying out the cannulations and blood sampling when he was at the Salk Institute (La Jolla, CA).

Glucose oxidation

The method used was essentially as described by Rodbell (16), except that a Krebs-Ringer HEPES buffer, pH 7.4, containing glucose (KRHG) was used. HEPES (20 mM) was used to stabilize the pH of the KRHG buffer. Pieces of adipose tissue from yellow and agouti mice were obtained from the distal epididymis and randomized. One or two animals provided sufficient tissue for one experiment. After rinsing with saline, pieces of 20–30 mg were weighed and transferred into Multiwell Disposo Trays (Linbro Co.), which contained KRHG buffer and were maintained at 37 C. Immediately after all pieces were weighed, they were distributed in 20-ml plastic vials (a minimum of eight pieces per vial) containing KRHG buffer with 350,000 dpm D-[U-^{14}C]glucose and the desired amount of insulin. The sensitivity of the adipose tissue to insulin varied with age of the mice. A test run was always necessary when a new group of mice was to be used in order to determine the amount of insulin needed for a significant response. At the end of a 2-h incubation at 37 C, 1 M Hyamine X-10 was added to filter paper (Whatman no. 1, Clifton, NJ) suspended in cups within the flasks. The incubation was stopped by the addition of 0.25 ml 1 N H_2SO_4 to the medium, and an additional 0.5-h incubation was allowed to collect $^{14}CO_2$ on the filter paper. Radioactive $^{14}CO_2$ was determined by liquid scintillation counting, with counting efficiency measured by the external standards technique.

Reagents and peptides

Crystalline porcine insulin was a gift from Dr. R. N. Chance, Elli Lily, Inc. (Indianapolis, IN). D-[U-^{14}C]Glucose had a specific activity of 250–350 mCi/mmol. Synthetic hGH-(1–15), -(32–46), -(1–42)-NH_2, and -(1–43) were made at the Salk Institute. Initially, hGH-(1–43) that had been isolated (12) from pituitary extracts was used, but with availability of active synthetic peptide, subsequent work was done with it. Preparation of hGH 22K and the 1–139 and 146–191 fragments has been described (17). A trypsin digest of hGH was made by treating hGH 22K with (L-1-tosylamide-2-phenylethyl chloromethyl ketone)trypsin at an enzyme to hormone ratio of 1:50 in 0.05 M NH_4HCO_3 for 16 h at 25 C. The trypsin was inactivated by 10^{-3} M diisopropylfluorophosphate, and the sample was lyophilized. A trypsin digest of BSA was made in the same manner.

The RIA for serum insulin was done according to the method of Morgan and Lazarow (18).

Statistical methods and calculations

Statistical comparisons were done with one-way analysis of variance. Throughout this report we present ΔCO_2 values (change in the average CO_2 production of a test sample from the saline value) to eliminate the variation experienced in basal values between experiments, where age of animals influenced tissue sensitivity to insulin. Basal CO_2 production by the adipose tissue from obese mice varied from 34–84 dpm/h·mg, a variable dependent upon age. Care had to be taken to match animals carefully to minimize this variation. We saw no significant difference between basal values in saline- and peptide-treated mice. A special experimental design was used for the data of Table 1. A description is included in the footnote of this table. Each experiment was performed at least three times. Coiro *et al.* (19) did a careful study of this procedure and came to the conclusion that the percent increase is a valid way of expressing the data.

Results

Action of hGH peptides on adipose tissue

Table 1 shows that *in vivo* treatment of yellow mice with hGH-(1–43) enhanced the sensitivity of their adipose tissue to the action of insulin *in vitro*. The mice received three daily ip injections of peptide before removal of the adipose tissue 60 min after the last injection. When 2500 μU/ml insulin were used in the incubation medium, a dose of 0.2 nmol (1μg)/day peptide for 3 days

TABLE 1. Effect of *in vivo* administration of hGH peptides on *in vitro* insulin-stimulated glucose oxidation by adipose tissue from obese mice

Peptide	Amount (nmol/day)	ΔCO_2 (%)	P^a
hGH 22K	2	−1 (8)	NS
Trypsin-hGH 22K	2	−4 (8)	NS
F_1	2	50 (8)	<0.04
F_2	2	−2 (8)	NS
hGH-(1–15)	2	38 (16)	<0.04
hGH-(32–46)	2	12 (16)	NS
hGH-(1–43)	0.002	10 (8)	NS
hGH-(1–43)	0.2	106 (16)	<0.03
hGH-(1–43)	2	120 (8)	<0.01

The insulin concentration in the tissue medium was 2500 μU/ml. F_1, Cys-Cam-hGH-(1–139); F_2, Cys-Cam-hGH-(146–191). Animals had been given three daily injections of peptide, and tissue was removed 1 h after the third injection. Numbers in parentheses indicate the number of pieces of tissue used to test each peptide or to serve as a control. Three separate assays were carried out on each test substance. Each assay used randomized tissue from a single obese mouse. In any one assay only three test substances and saline were examined. For calculations, the average saline value, therefore, was the same for each of three samples tested.

[a] Test substance *vs.* saline. NS = ($P > 0.05$).

given to obese mice produced a near-maximum effect amounting to a 106% enhancement of CO_2 production above that seen with saline-treated animals.

Table 1 also shows that other portions of the hGH amino acid sequence were not as active as hGH-(1–43) in enhancing insulin sensitivity of adipose tissue. Intact hGH itself was inactive, as was a trypsin digest of the hormone and the COOH-terminal peptide, carbamidomethyl cysteine (Cys-Cam)-hGH-(146–191). The most active fragments other than hGH-(1–43) were hGH-(1–15) and Cys-Cam-hGH-(1–139), which were only 5–10% as effective as hGH-(1–43). In this test, hGH-(32–46) showed no activity. Not shown in Table 1 are the results obtained with a total trypsin digest of BSA. At a dose of 90 nmol, the digest had no significant effect on sensitivity of the adipose tissue to insulin.

Figure 1 demonstrates further the insulin-enhancing action of hGH peptides. Obese mice were treated with saline, hGH-(1–43), or hGH-(1–42)-NH_2. Sixty minutes after injection of the substances, the animals were killed, and adipose tissue was removed and tested for sensitivity to insulin-stimulated glucose oxidation. In the control (saline-treated) mice, 1 mU insulin caused a 30–40% increase in CO_2 production above basal values. Insulin at half this dose was ineffective, *i.e.* the CO_2 value obtained was not significantly different from the basal value, which is shown as the zero baseline in Fig. 1. Treatment of the mice with either hGH-(1–43) or hGH-(1–42)-NH_2 shifted the response curves to the left to an extent that indicated the tissue was then 2–5 times more sensitive to insulin.

Table 2 shows the increase in sensitivity to insulin

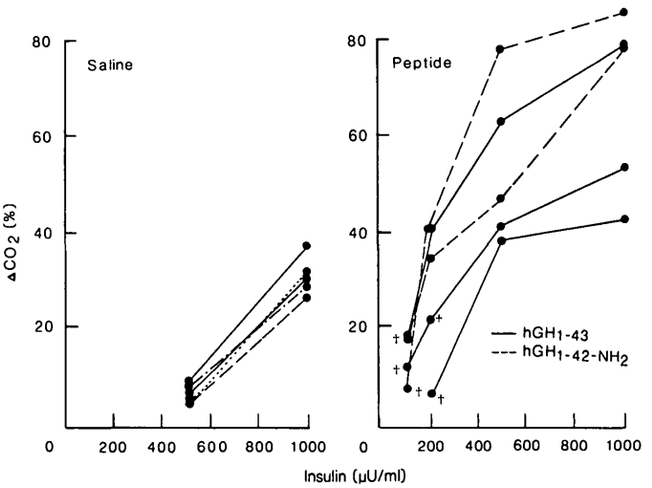

FIG. 1. Enhancement of sensitivity to insulin action in adipose tissue from obese mice treated with hGH peptides. Basal CO_2 production by the tissue was taken as zero. Animals had been given a single injection of peptide, and tissue was removed 1 h later. Each of the five lines represents CO_2 produced by tissue from a single animal. For each *data point*, eight pieces of tissue were used. For the tissue from saline-treated animals (*left panel*), CO_2 produced by 500 μU/ml insulin was not significantly different from the basal value; the 1000 μU/ml insulin values were significant at $P < 0.05$. The *right panel* is for tissue from peptide-treated animals. All *data points* for peptide-treated animals were significantly different from the basal value ($P < 0.05$ or better), except for those marked with a †.

TABLE 2. Response produced by varying doses of hGH-(1–42)-NH_2 enhancing insulin-stimulated glucose oxidation by adipose tissue from obese mice

Amount of peptide (nmol/day)	ΔCO_2 (%)	P
Saline	56	
0.002	64	NS
0.02	109[a, b]	<0.03
0.2	115[a]	<0.04
2.0	126[a]	<0.01

Peptide was given for 3 days. The last injection was made 1 h before removing the adipose tissue for measurement of stimulation of glucose oxidation by 2000 μU/ml insulin. Tissue pieces from two animals were randomized for use in this experiment. Two additional separate experiments were done with similar results, *e.g.* essentially maximum effect was obtained with the 0.02-nmol dose. The ΔCO_2 values are increases above the basal value. NS = $P > 0.05$. P values are for comparison of peptide treatment with saline treatment.

[a] These three values are not significantly different from one another.

[b] $P < 0.04$ compared to the 0.002 nmol value.

produced by varying doses of a hGH peptide. The data are for hGH-(1–42)-NH_2, but similar results were obtained with hGH-(1–43). Treatment with as little as 0.02 nmol (100 ng) peptide/day for 3 days produced a significant effect. Note that the percent increase in CO_2 production by this peptide was similar to that observed with hGH-(1–43) (Table 1).

Many attempts were made to detect direct *in vitro*

actions of hGH-(32–46) and hGH-(1–43) on adipose tissue, but none was found. Incubation of tissue with the peptide 10 min to 4 h before the addition of insulin had no effect on CO_2 production, nor did preincubation of tissue alone for 1–4 h before the addition of peptide and insulin affect sensitivity to insulin. Incubation of tissue for 2 h, followed by a 1-h treatment with peptide before insulin addition, was also ineffective. These results contrast with an earlier report (20) which indicated an *in vitro* action for hGH-(32–46).

Need for an obese animal

Treatment of the lean agouti-colored littermates of the obese yellow mice with hGH-(1–43) produced no effect on the insulin sensitivity of the adipose tissue. This is shown in Table 3. Note also the greater sensitivity of this adipose tissue to insulin compared to tissue from obese mice, as shown in Fig. 1. With tissue from the obese animals, insulin alone at a concentration of 2500 μU/ml caused barely a 60% increase in glucose oxidation above the basal value. A more detailed study of the concentration of insulin in blood and the sensitivity of tissue to insulin in these lean agouti and obese yellow mice has appeared previously (21). In that report it can be seen that basal CO_2 production by adipose tissue from lean agouti mice was not significantly different from basal values for age-matched obese animals.

Effect on serum insulin

The serum insulin values of obese mice given 2 nmol of either hGH-(32–46) or hGH-(1–43) did not increase within a 1-h period after injection of the peptides. Serum insulin values for the males were in the range of 180–200 μU/ml; for females, the range was 100–120 μU/ml. Similar negative results were obtained when freely moving cannulated rats were used to avoid the stress of handling and bleeding. Under these conditions, hGH-(32–46) did not significantly increase the serum insulin concentrations even when given at a dosage of 90 nmol/rat. Administration of hGH-(1–43) (10–80 nmol) or hGH (23 nmol) to those rats also had no effect on either plasma glucose or insulin. Plasma glucose values remained near 105 mg/dl; the insulin concentration was near 80 μU/ml after treatment with the peptides.

TABLE 3. Effect of hGH-(1–43) on insulin-stumulated glucose oxidation by adipose tissue from lean agouti mice

Treatment	Insulin (μU/ml)	
	100	500
Saline	195 ± 9	223 ± 25
hGH-(1–43) (2 nmol)	175 ± 8	249 ± 25

The values represent the mean ± SEM percent ΔCO_2 for eight observations. A dose of 2 nmol was given daily for 3 days.

Discussion

Two of the observations reported here deserve special comment. The first is that a physiological effect was produced by the active hGH peptides only after *in vivo* administration. Addition of the peptide *in vitro* to the adipose tissue during or before insulin stimulation was without effect in enhancing *in vitro* CO_2 production from glucose. Possible explanations could be that peptides themselves, when administered *in vivo*, react with cellular membranes to increase insulin receptor number or affinity, or that the peptides stimulate production of another substance *in vivo*, which then acts on the adipose tissue to enhance insulin sensitivity. Both of these explanations are amenable to experimentation and are under investigation.

The other observation is that hGH-(1–43) was active in obese A^{vy}/A mice, but not in lean A/a animals. Because the obese mice are hyperinsulinemic and exhibit insulin resistance (21), the peptide must function in enhancing insulin sensitivity in this physiological state. The potential clinical importance of this observation in treating insulin-resistant diabetics is of major interest to us.

To explain the lack of activity of intact hGH in enhancing insulin sensitivity, we suggest that at least some of the physiological actions of hGH are produced by proteolytic cleavage products (22) and that exogenous hGH does not undergo the same proteolytic processing as endogenous hGH. This suggests that production of hGH-(1–43) occurs in the pituitary gland and not as a postsecretory event. Development of a RIA for hGH-(1–43) will help to study this possibility. The amount of hGH-(1–43) isolated from the pituitary was 12 μg/gland. This is a minimum value, because no account is taken of losses during development of the isolation procedure. For comparison, there is about 50 μg FSH/gland and about 5 mg hGH/gland.

Evidence for an insulin-potentiating peptide from hGH was first reported by others (23–25). The extreme amino-terminal portion, the first 15 residues or parts of that sequence, was the region investigated. In our assay system hGH-(1–43) was at least 10 times as active as hGH-(1–15), but it will be important to determine relative activities in other assays, such as those used for the study of the action of hGH-(1–15). The specificity of the action of the amino-terminal peptides to increase insulin sensitivity is indicated by the inactivity of the carboxy-terminal peptide (residues 146–191), which has approximately the same mol wt as hGH-(1–43), by the lack of activity in the trypsin digest of BSA, and by the lack of activity of the trypsin digest of hGH, which would contain many segments of the hGH structure. The reason for the inability of hGH-(32–46) to act on adipose tissue of obese mice is not clear, but it may be an indication

that various portions of the amino-terminal sequence of hGH have different degrees of activity, depending on the type of tissue used. We have evidence (7) (to be published in full) that this may indeed be the case. In that study both hGH-(32–46) and hGH-(1–43) were active in enhancing insulin-stimulated glucose metabolism in liver and muscle of obese mice. This, then, offers an explanation for the enhancement of exogenous insulin by hGH-(32–46) (8, 10, 11) and the increased uptake of glucose by muscle (8, 9) under the effect of this hGH fragment in normal animals (6–11) under totally *in vivo* conditions, where adipose tissue most likely plays a minor role, compared to liver and muscle, in maintaining overall glucose balance. The reason why adipose tissue of agouti mice is unaffected by hGH-(1–43) may be related to similar differences in reactivity of tissues. In the normal animals, when the hGH peptides were active (6, 8–11) the measurements were made on intact animals, a situation in which all tissues could influence the observed results.

A factor also to be considered when activities of *in vivo* administered peptides are compared is the rate of clearance. The larger hGH-(1–43) may remain in the circulation longer than hGH-(32–46). This point will be investigated when specific RIAs are developed for the peptides.

Stevenson *et al.* (10) reported that in the euglycemic dog there was an increase in serum glucagon after administration of hGH-(32–46). To interpret the results, the investigators suggested a direct action of the hGH peptide on glucagon secretion. Another interpretation, however, is that the rise in glucagon levels was in response to an enhancement by the peptide of glucose utilization, an event generally recognized as a stimulus to glucagon secretion. Stevenson and associates also showed that hGH-(32–46) caused an increased glucose uptake during infusion of the sugar, and they attributed the greater utilization to elevated insulin levels produced by an augmentation of glucose-induced insulin secretion by the peptide. Our results cannot be explained by such an effect. Notably, Stevenson *et al.* (26) had reported previously that another hGH peptide, hGH-(32–38), increased glucose utilization without increasing serum insulin. To explain this second effect, it was suggested that there had to be two mechanisms for peptide action: one directed at target tissue, the other at the pancreas. In contrast, a hypothesis that proposes a single mode of action is that treatment with the peptides alters tissue receptors to permit greater insulin binding, and as a result, there is enhanced activity. This idea was suggested by our observation of increased sensitivity of adipose tissue to insulin after treatment with hGH-(1–43). The action of hGH-(32–38) (26) can also be accommodated by this concept. The increase in serum insulin in dogs treated with hGH-(32–46) cannot be reconciled at this time with the hypothesis; so, clearly, additional work is needed to determine if such an increase in insulin secretion can be explained by enhancement of insulin action by hGH-(32–46) rather than by a direct action of the peptide on the pancreas to stimulate insulin release. A more detailed discussion of the possible importance of the 32–46 sequence to the insulin-like activity of GH can be found elsewhere (27).

Acknowledgment

We thank Dr. W. P. VanderLaan for many valuable discussions.

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