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THE 20,000 DALTON STRUCTURAL VARIANT OF RECOMBINANT DNA-DERIVED METHIONYL HUMAN GROWTH HORMONE HAS EARLY INSULIN-LIKE EFFECTS IN HYPOPHYSECTOMIZED RATS

F. C. Tinsley, E. L. Grinnan, S. H. Baker, J. G. Powell, K. G. Bemis, and C. J. Shaar

Lilly Research Laboratories, Eli Lilly and Company
Lilly Corporate Center, Indianapolis, IN 46285

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SUMMARY: The 20,000 dalton variant of recombinant DNA-derived methionyl human growth hormone (20K-Met-hGH) induced decreases in blood glucose and free fatty acid concentrations one hour after intraperitoneal injection into fasted, hypophysectomized rats. Similar results were obtained using the 22,000 dalton form of recombinant DNA-derived methionyl human growth hormone (22K-Met-hGH). The data reported show that 20K-Met-hGH induces early insulin-like effects similar to the responses produced by 22K-Met-hGH in fasted hypophysectomized rats.

A naturally occurring 20,000-dalton structural variant of human growth hormone (20K-hGH) differs from the major form of human growth hormone (22K-hGH) by lacking 15 amino acids comprising residues 32-46 of the primary structure (1). Purified 20K-hGH and 22K-hGH exhibit similar potency in the hypophysectomized rat weight gain and tibial cartilage bioassay (2). Lewis and coworkers reported that the administration of 20K-hGH failed to induce an early transient fall in serum glucose and free fatty acid (FFA) concentrations in fasted hypophysectomized rats (3), and did not induce glucose intolerance in fasted dogs (4). Preparations of 20K-hGH that were equipotent with 22K-hGH in the tibial cartilage bioassay were found to be less effective in rabbit liver and mammary gland membrane radioreceptor growth hormone and prolactin binding preparations (5). Both growth hormone forms are equipotent at stimulating increases in assayable serum somatomedins (6).

N-terminal methionyl human growth hormone (22K-Met-hGH) and 20,000-dalton N-terminal methionyl human growth hormone (20K-Met-hGH) were

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biosynthetically produced by recombinant DNA techniques and purified at The Lilly Research Laboratories. Experiments were undertaken to investigate the insulin-like effects of 20K-Met-hGH when administered to fasted hypophysectomized rats.

MATERIALS AND METHODS

Female, hypophysectomized, Sprague-Dawley rats (Charles River, Inc.) were fasted for eighteen hours (including overnight) prior to use in an experiment. Prior to an experiment, the rats were randomly placed into treatment groups of 6 animals each.

In the first experimental protocol each group of rats received one of the following treatments by intraperitoneal injection at time zero: vehicle 0.1M NH_4HCO_3 (0.2 ml/rat), 20K-Met-hGH (50, 100 or 200 μg/rat), or 22K-Met-hGH (25, 50, or 100 μg/rat). All rats were sacrificed by decapitation one hour after hormone administration. Serum glucose and FFA concentrations were determined using trunk blood collected at the time of sacrifice.

In the second experimental protocol, groups of 18 rats received one of the following treatments by intraperitoneal injection at time zero: 0.1M NH_4HCO_3 (0.2 ml/rat); 20K-Met-hGH (100 μg/rat) or 22K-Met-hGH (100 μg/rat). At the designated times 1.0, 2.0 and 5.0 hours after injection, 6 rats from each treatment group were sacrificed by decapitation and trunk blood was collected.

Serum glucose concentrations were determined on specimens from both experiments using the glucose oxidase-peroxidase method (7). Serum FFA concentrations were determined in the same samples using a colorimetric method previously described by Falholt and co-workers (8) and purchased in kit form (Wako Pure Chemicals, Dallas, Texas).

Oligonucleotide synthesis was used to prepare a double-stranded DNA fragment coding for the 20K-variant DNA sequence region. Similar techniques were used to prepare the 22K-Met-hGH used in these experiments. The synthesized DNA section was inserted between two convenient restriction enzyme sites located within the hGH gene, and the modified gene was cloned on a suitable plasmid, pNM1075. The gene product was expressed using the pL promoter from lambda phage under control of the temperature-sensitive CI857 gene product. These sequences are present on plasmid pNM1075, which incorporates a temperature-sensitive copy control mechanism, which also codes for tetracycline resistance. Plasmid pNM1075, inserted into *E. coli* K-12 RV308 cells, produces granules and material identified as 20K hGH by sodium dodecyl sulphate-polyacrylamide gel electrophrosis (SDS-PAGE) and radio-immunoassay.

E. coli containing the pNM1075 plasmid were grown in 150 L fermentors under conditions for optimal expression of the 20K-variant of Met-hGH. The cells were harvested and lysed, and the 20K-variant was isolated from the cell lysate by conventional procedures including DEAE-cellulose chromatography. Final purification was achieved by preparative reversed phase high performance liquid chromatography followed by size exclusion chromatography. The purified 20K-variant had the correct amino acid analysis and showed full activity compared to 22K Met-hGH by radio-immunoassay and the rat tibia assay for epiphyseal cartilage and plate width. Comparison of the SDS-PAGE patterns of two recombinant DNA-derived preparations of 20K-Met-hGH with a pituitary-derived 20K-variant, and a recombinant DNA-derived preparation of 22K-Met-hGH is shown in Figure 1.

The growth promoting activity of the growth hormone preparations used in the experiment was determined by using a 10-day body weight gain and

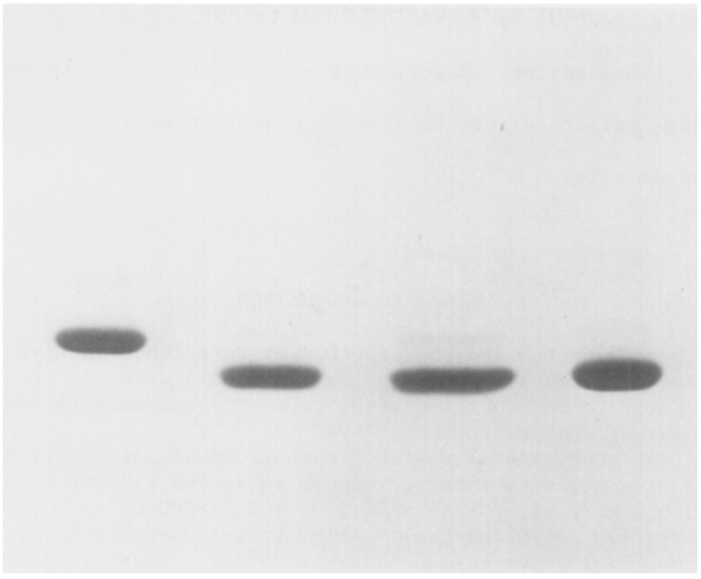

Figure 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of growth hormone preparations. Coomasie Brilliant Blue R-250 stain; load 25 µg protein. Lane 1, 22K methionyl human growth hormone; lane 2, 20K-variant of Met-hGH; lane 3, pituitary-derived 20K-hGH; lane 4, 20K-variant of Met-hGH.

tibial epiphyseal cartilage-width assay using immature hypophysectomized female rats (9-10).

The variables analyzed in the growth hormone bioassay included body weight gain during the 10-day treatment period and epiphyseal cartilage width (refers to the average of 10 individual readings across the cartilage at the termination of the bioassay). Treatment effects were evaluated by comparing the treatment means to the vehicle mean using Dunnett's test (P=0.05) on ranked data (12, 13). The potency of a test preparation relative to a standard preparation is based on the statistical methodology of the parallel line bioassay and developed by Finney (14). Confidence intervals for the computed potency are the exact fiducial limits based on Fieller's Theorem described by Finney (14).

As determined in three separate bioassays, 20K-Met-hGH had a biological potency of 2.1 IU/mg (95% C.L.=1.8-2.5 IU/mg) in the epiphyseal cartilage portion of the assays and a biological potency of 1.4 IU/mg (95% C.L.=0.8-2.2 IU/mg) in the body weight portion of the assays. The 22K-Met-hGH preparation had a biological potency in both weight gain and cartilage width portion of the assays of 2.6 IU/mg (95% C.L.=1.8-3.9 IU/mg and 2.2-3.1 IU/mg respectively). The reference preparation used in the growth hormone biological assay was a pituitary source human growth hormone AFP61 (2.2 IU/mg) purchased from Dr. A. F. Parlow (UCLA Medical Center, Torrence, CA).

RESULTS

Results of the first experiment (Table 1) demonstrate that 22K-Met-hGH at doses of 50 and 100 µg/rat, but not 25 µg/rat, induced significant

Table 1

Serum glucose and free fatty acid (FFA) concentrations in fasted hypophysectomized female rats one hour after intraperitoneal injection of 22K-Met-hGH or 20K-Met-hGH

Experimental Group (Treatment/Dose)	Number of Rats	Serum Glucose (mg/dl)	Serum FFA (μEq/ml)
I. Control (0.1M NH_4HCO_3)	12[a]	77.7 ± 3.0[b]	.572 ± .034[b]
II. 20K Met-hGH (50 μg/rat)	6	74.9 ± 4.1	.491 ± .039
(100 μg/rat)	6	65.6 ± 2.1*	.272 ± .021*
(200 μg/rat)	6	52.6 ± 1.4*	.219 ± .029*
III. 22K Met-hGH (25 μg/rat)	6	67.2 ± 4.1	.444 ± .024*
(50 μg/rat)	6	55.6 ± 5.1*	.292 ± .035*
(100 μg/rat)	6	46.4 ± 1.2*	.159 ± .009*

a. Represents two groups of six control rats. Serum glucose (79.9 ± 3.5 vs 75.4 ± 5.2 mg/dl, $p<=.43$) and FFA (.570 ± .046 vs .574 ± .053 μEq/ml, $p<=.95$) were not different than one another.

b. Mean ± S.E.M.

*Statistical significance of mean at $p<=.05$ as calculated using Dunnett's test.

reductions in serum glucose concentrations one hour after administration. All three doses of 22K-Met-hGH induced significant reductions in serum FFA concentrations one hour after administration. At the same time after administration, 20K-Met-hGH, at doses of 100 and 200 μg/rat, induced significant reductions in serum glucose and FFA concentrations. The lowest dose of 20K-Met-hGH (50μg/rat) did not effect serum glucose or FFA levels.

Table 2 shows that both 22K-Met-hGH and 20K-Met-hGH at a dose of 100 μg/rat induced significant reductions in serum glucose and FFA concentrations one hour after administration. Comparisons made with values in control rats at 2.0 and 5.0 hours after 22K-Met-hGH or 20K-Met-hGH administration revealed no significant changes in serum glucose and FFA concentrations.

DISCUSSION

Transient decreases in serum glucose and FFA concentrations characteristic of early insulin-like activity were induced in fasted

Table 2

Serum glucose and free fatty acid (FFA) concentrations in fasted hypophysectomized female rats at various times after intraperitoneal injection of 22K-Met-hGH and 20K-Met-hGH

Experimental Group (Treatment/Dose)	Dose (μg/rat)	Number of Rats	Serum Glucose (mg/dl)	Serum FFA (μEq/ml)
I. 1.0 Hour				
(0.1M NH_4HCO_3)	---	6	85.0 ± 2.3[a]	.616 ± .041
(20K Met-hGH)	100	6	67.9 ± 4.3[b]	.367 ± .048[b]
(22K Met-hGH)	100	6	59.9 ± 5.3[b]	.377 ± .033[b]
II. 2.0 Hours				
(0.1M NH_4HCO_3)	---	6	70.1 ± 3.1	.655 ± .046
(20K Met-hGH)	100	6	58.2 ± 3.6	.540 ± .046
(22K Met-hGH)	100	6	59.4 ± 4.4	.540 ± .047
III. 5.0 Hours				
(0.1M NH_4HCO_3)	---	6	66.2 ± 2.3	.494 ± .026
(20K Met-hGH)	100	6	64.3 ± 1.8	.528 ± .055
(22K Met-hGH)	100	6	65.0 ± 1.5	.599 ± .014

a. Mean ± S.E.M.

b. Statistical significance of mean at $p<=.05$ as calculated using Dunnett's test.

hypophysectomized rats one hour after administration of 22K-Met-hGH (50 and 100 μg/rat) and 20K-Met-hGH (100 and 200 μg/rat). Early insulin-like effects induced by 22K-Met-hGH in these studies were similar to those induced by 22K-hGH during experiments conducted by Frigeri and co-workers (3).

It was surprising to find that 20K-Met-hGH, at the two highest doses, induced early insulin-like effects similar to those produced by 22K-Met-hGH administration. Frigeri and co-workers (3) found that a 50 μg dose of 20K-hGH administered to fasted hypophysectomized rats failed to significantly reduce serum glucose and FFA concentrations. Likewise, 50 μg/rat doses of 20K-Met-hGH in the studies described herein failed to reduce serum glucose and FFA concentrations. However, larger doses of 20K-Met-hGH (100 and 200 μg/rat) caused significant reductions in serum glucose and FFA levels. The results reported here confirm the earlier work of Frigeri (3) which showed that a 50 μg dose of 20K-hGH did not affect serum glucose and FFA levels. However, the results found here show that higher doses do in fact alter serum glucose and FFA levels.

Several explanations may exist as to why the present results differ from earlier findings. It appears that the insulin-like activity of 20K-Met-hGH may be dose-related, so that higher doses of 20K-Met-hGH are needed to produce the same insulin-like effects as those observed with lower doses of 22K-Met-hGH. Frigeri and co-workers (3) administered 50 μg/rat, while 100 and 200 μg/rat were administered in the present studies. A second explanation may be unforeseen differences in the naturally occurring variant and synthetic methionyl forms of 20K-hGH. This explanation is unlikely because all analytical tests conducted to date indicate that the DNA-derived and human-derived hormone are identical, with the exception of the N-terminal methionine of the biosynthetic material. It is difficult to understand how a 20K-hGH variant of native human growth hormone promotes the same anabolic effects as 22K-hGH (viz. promotes body weight gain), stimulates epiphyseal cartilage width increases (2), elevates somatomedins (6), and binds to the liver and mammary gland receptors (5) and yet would be devoid of effects on carbohydrate metabolism. Thus, our results are consistant with the other physiological effects of hGH.

The results reported here demonstrate that 100 μg/rat and 200 μg/rat doses of 20K-Met-hGH produce early insulin-like effects in fasted hypophysectomized rats similar to those induced by lower doses of 22K-Met-hGH and 22K-hGH.

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