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Human Growth Hormone Fragment (hGH_{44-191}) Produces Insulin Resistance and Hyperinsulinemia but Is Less Potent than the 22 kDa hGH in the Rat

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A 17 kDa fragment of human growth hormone (22 kDa hGH), identified as hGH_{44-191}, has lower binding affinity for growth hormone receptors (GHRs), but has been reported to be more potent in producing glucose intolerance in yellow obese mice. Our aim was to investigate this anomaly by comparing acute development of hyperinsulinemia and insulin resistance ("diabetogenic activity") during hGH_{44-191} or 22 kDa hGH infusion in normal rats. Fasted awake male rats (350–370 g) were infused via a carotid cannula with saline (CON), 22 kDa hGH (at 0.125 μg/min), or hGH_{44-191} (at 0.64 or 0.32 μg/min) for 5.75 h. Over the last 2 h, a euglycemic hyperinsulinemic clamp (insulin infusion rate 0.25 U/kg/h) was performed. After 3.75 h infusion, 22 kDa hGH at 0.125 and hGH_{44-191} at 0.64 μg/min produced basal (preclamp) hyperinsulinemia compared to CON. During the clamp, insulin resistance was consistently produced by 22 kDa hGH at 0.125 and hGH_{44-191} at 0.64 μg/min compared to CON. Using specific radioimmunoassays for 22 kDa hGH and hGH_{44-191}, we determined that under conditions of equivalent diabetogenic activity, molar circulating levels of hGH_{44-191} were 50–60-fold higher than 22 kDa hGH. It was concluded that whereas 22 kDa hGH and hGH_{44-191} are both capable of generating acute hyperinsulinemia and insulin resistance in the normal rat, the diabetogenic potency of hGH_{44-191} is not enhanced compared to 22 kDa hGH, and that diabetogenic potency is in accord with the reported lower binding affinity of hGH_{44-191} to the GHR.

Key Words: Hyperinsulinemia; glucose clamp; insulin resistance; glucose infusion rate (GIR).

Introduction

As well as its growth promoting activity, human growth hormone (22 kDa hGH) possesses diabetogenic activity *(1–4)*. Acute and chronic elevation of 22 kDa hGH have been shown to cause insulin resistance and hyperinsulinemia in humans and animals *(1–4)*. It has also been demonstrated that different fragment forms of hGH exist in humans in addition to the 22 kDa form *(5)*, and that these exhibit varying degrees of diabetogenic activity *(5–6)*.

One particular fragment of hGH, hGH_{44-191}, which is devoid of 43 amino acid residues from the amino terminal end, has recently been demonstrated not only to exist in the human pituitary gland and serum *(7)*, but also to be 10 times more potent in causing glucose intolerance in yellow obese mice compared to the 22 kDa form *(8)*. Paradoxically, in a recent study by Rowlinson et al. *(9)*, comparing the in vitro binding affinities of hGH_{44-191} with 22 kDa hGH to the growth hormone receptor (GHR), it was found that hGH_{44-191} had a much lower binding affinity for GHRs. Furthermore, the lower binding affinity was in accord with lower growth promoting activity of hGH_{44-191} in vitro as well as in vivo *(8)* compared to 22 kDa hGH. The authors, therefore, thought that the apparent anomaly of greater diabetogenic activity, but lower GHR binding of hGH_{44-191} compared with 22 kDa hGH should be investigated. This would provide information as to the likelihood or otherwise that the diabetogenic action of hGH_{44-191} was mediated via GHR binding.

Therefore, in this study the aim was to compare the diabetogenic activity of infusions of hGH_{44-191} and 22 kDa hGH in chow-fed rats by examining effects on the basal insulin level and also on insulin sensitivity with the euglycemic hyperinsulinemic clamp.

Materials and Methods

Experimental Animals

All surgical and experimental procedures performed were approved by the Garvan Institute of Medical Research, St. Vincent's Hospital Animal Experimentation Ethics

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Committee, and were in accord with the National Health and Medical Research Council of Australia Guidelines for the use of animals in research. Adult male Wistar rats (350 ± 7 g), were fed standard laboratory chow (Allied Feeds, Rhodes, NSW) with water *ad libitum*. They were housed individually after surgery in enclosed, well-ventilated metabolic cages in a temperature-controlled (22 ± 1°C) environment with 12/12 h light/dark cycle (lights on at 0600 h). Rats were accustomed to human contact in order to minimize stress associated with handling during studies.

Surgery

One week prior to the study, rats were chronically cannulated via the right jugular vein and left carotid artery under ketamine hydrochloride (90 mg/kg)/xylazine (10 mg/kg) anesthesia. Cannulae were exteriorized and patency was maintained as previously described *(10)*. Recovery following surgery during the 7-d period prior to the study was closely monitored with measurement of food intake and body weight gain.

Study Protocols

Materials

Authentic human recombinant growth hormone (potency of 2.6 U/mg) produced from a mammalian cell line was used *(11)*. hGH_{44-191} was produced by recombinant DNA technology and was a gift from the laboratory of U. James Lewis, La Jolla, CA.

Continuous Infusion Protocol

Acute studies were performed 7 d after surgical implantation of jugular and carotid cannulae. On the morning of the study, food was withdrawn 5–7 h before study commencement, and the carotid cannula was flushed and connected to a continuous infusion pump (Harvard Instruments). Infusions and the subsequent clamp studies were performed with animals in the awake, unrestrained state. The cannulated rats were randomly assigned to one of four groups—saline control, 22 kDa hGH, low and high dose hGH_{44-191}—and were continuously infused over a 5.75 h period via the carotid cannula. 22 kDa hGH was infused at the rate of 0.125 μg/min whereas hGH_{44-191} was infused at 0.32 μg/min (low dose) or 0.64 μg/min (high dose), respectively in 0.9% saline. Blood samples were collected before the start of the infusion (time zero) and before the start of the clamp (designated basal) for the measurement of plasma glucose, insulin, and triglycerides.

Euglycemic Clamp Studies

During the last 2 h of the 5.75 h infusion, insulin action was assessed using the euglycemic hyperinsulinemic clamp as previously described *(10)*. In brief, porcine insulin (Actrapid, Novo-Nordisk, Copenhagen) was infused at a constant rate of 0.25 U/kg/h for 120 min. Blood glucose concentration was maintained at a euglycemic level (4.5 mmol/L) by a variable infusion of 30% (w/v) glucose. At the end of the clamp period the rats were sacrificed with a lethal dose of pentobarbitone sodium (Nembutal, Abbott Laboratories, Sydney).

Analytical Methods

Blood and plasma glucose concentrations were measured using a Yellow Springs Glucose Analyser (Yellow Springs Instruments, Yellow Springs, OH). Plasma triglycerides were estimated using a colorimetric assay (Triglyceride [INT], Procedure # 336, Sigma, St. Louis, MO). Basal and clamp plasma insulin was measured using an in-house double antibody radioimmunoassay (RIA) with guinea pig antiserum as described previously *(10)*. Estimation of plasma 22 kDa hGH levels were achieved by an in-house RIA using ^{125}I-labeled hGH, an antiserum against pituitary derived hGH raised in rabbits and a double antibody–polyethylene glycol (PEG)-facilitated precipitation technique as previously described *(12)*. This antiserum had cross-reactivity of about 3% to hGH_{44-191}. The ID_{50} of the RIA for 22 kDa hGH was 3.1 ng/mL.

Radioimmunoassay for hGH_{44-191}

A RIA specific for hGH_{44-191} was developed using a rabbit anti-hGH_{44-191} antiserum raised in the laboratory. The assay comprised of a competitive immunoassay using a double antibody–polyethylene glycol-facilitated precipitation technique, with ^{125}I-hGH_{44-191} as a tracer. hGH_{44-191} was iodinated by the Iodogen method to a specific activity of 20–30 μCi/μg. For iodination, 20–30 μg of the fragment was dissolved in 25 μL of 10 m*M* NaOH and neutralized with 65 μL of 0.5*M* sodium phosphate, pH 7.4. The iodination was performed in a tube coated with 1 μg of Iodogen (Pierce, Rockford, IL) and commenced by addition of 1 μCi of Na ^{125}I (ARI, Sydney, Australia). After incubation at 22°C for 10 min, the reaction was terminated by adding 200 μL of phosphate-buffered saline (PBS) with 2 mg/mL bovine serum albumin (BSA). The radiolabeled fragment was separated from free iodide by an Excellulose GF5 column (Pierce) and stored at –20°C. Before use, the monomeric ^{125}I-hGH_{44-191} was purified on an AcA54 Ultragel column (1 × 22 cm).

A rabbit polyclonal antiserum to hGH_{44-191} produced in the laboratory was used for immunoprecipitation. The antiserum was highly specific to the fragment; it had crossreactivity <0.2% to 22 kDa hGH and did not react with 20 kDa hGH or human prolactin. Rat plasma samples were routinely diluted 1/40 in PBS with 10 mg/mL BSA (PBS/1% BSA), and hGH_{44-191} standard of concentration 1–100 ng/mL was made up in PBS/1%BSA containing 1/40 diluted normal rat serum. To set up the assay, 50 μL of sample or standard was added in duplicate to 350 μL of PBS/1%BSA containing 1/52,500 diluted antiserum. After incubation at 4°C for 4 d, 100 μL of ^{125}I-hGH_{44-191} ($1.5–2.0 \times 10^4$ cpm/tube) was added. The incubation was continued at 4°C for 1 d. Then, 100 μL of 1/25 diluted sheep antirabbit Ig anti-

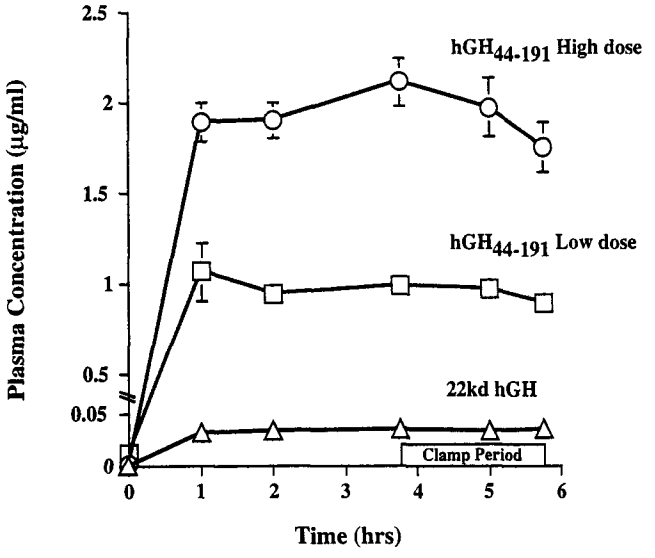

Fig. 1. Time course of basal and clamp plasma levels of 22 kDa hGH, hGH_{44-191} (32 μg/min; low dose), hGH_{44-191} (64 μg/min; high dose) are shown. Results are expressed as mean ± SE for $n = 6–7$ rats. Plasma hGH_{44-191} concentration reached a plateau level 1 h after the infusion began. hGH_{44-191} levels were below the assay minimum detection level (1 ng/mL) in control rats (saline infusion).

serum (Antibodies, Davis, CA) and 100 μL of 1:80 diluted normal rabbit serum were added. After incubation at 22°C for 10 min, the complex was precipitated with 1 mL of ice-cold 10% polyethylene glycol 6000 in PBS. Radioactivity of the pellet was counted on a γ counter (Packard Instrument Co., Meriden, CT). The standard curve was constructed with a four-parameter weighted logistic curve fitting program (AssayZap, Biosoft, Cambridge, UK) and hGH_{44-191} concentrations of samples were extrapolated from the standard curve. The minimum detectable level of hGH_{44-191} by this RIA was 1 ng/mL. The ID_{50} of the RIA for hGH_{44-191} was 14.0 ng/mL.

Statistical Analysis

All results are expressed as mean ±SE. Overall group effects were analyzed by ANOVA. The differences between individual groups were examined using a post hoc test (Scheffes *F*-test) (Macintosh Statview SE + Graphics program, Abacus Concepts, Brain Power, Berkeley, CA).

Results

Plasma levels of 22 kDa hGH and hGH_{44-191}

hGH_{44-191} was undetectable in normal rat plasma. Continuous infusion of hGH_{44-191} at low (0.32 μg/min) and high (0.64 μg/min) doses resulted in maximum plasma levels of 1.06 ± 0.2 and 1.9 ± 0.1 μg/mL, respectively by 1 h. These levels did not change significantly for the rest of the infusion (average plateau levels were 0.97 ± 0.03 and 1.92 ± 0.06 μg/mL, respectively) (Fig. 1). 22 kDa hGH also reached the maximum level by 1 h and remained unchanged at an average plateau concentration of 0.033 ± 0.001 μg/mL (Fig. 1).

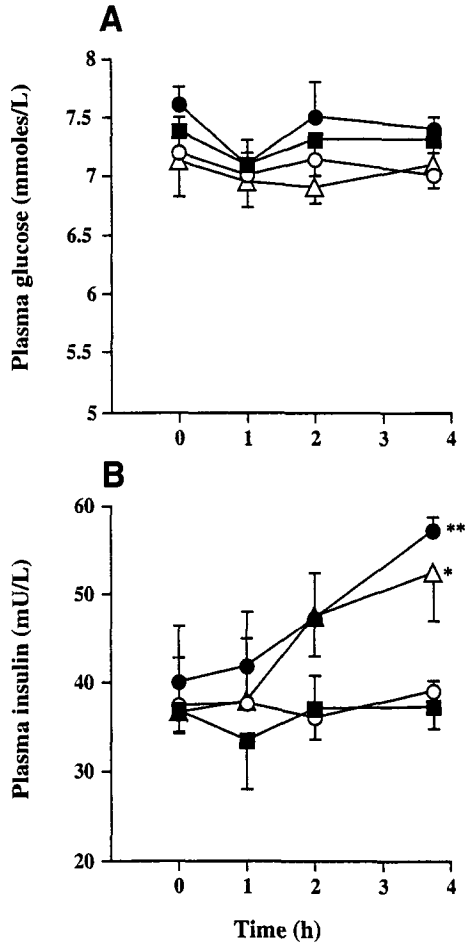

Fig. 2. Time course of preclamp plasma glucose **(A)** and insulin **(B)** levels during infusions of ■, saline; ○, hGH_{44-191} (32 μg/min; low dose), ●, hGH_{44-191} (64 μg/min; high dose); △, 22 kDa hGH. Results are expressed as mean ± SE, $n = 5–12$ rats. $*p < 0.05$, $**p < 0.02$ vs control.

The metabolic clearance rates (MCRs) calculated from the infusion data and the steady state plasma level indicated that hGH_{44-191} was cleared at approx one-tenth the rate of 22 kDa hGH (1.12 ± 0.14 vs 10.4 ± 0.5 mL/kg/min; $p < 0.01$).

Preclamp Data

Fasting preclamp plasma glucose levels during the fragment or 22 kDa hGH infusion were not significantly different among the four groups (Fig. 2A). Similarly, plasma triglycerides also remained unchanged (at approx 100 mg/dL) during the preclamp infusion period (Table 1). Fasting preclamp plasma insulin levels during the early part of the infusion (0–2 h) of the hGH_{44-191} (at low and high doses) or 22 kDa hGH were not different from that of the saline control (Fig. 2B). However, plasma insulin levels were significantly elevated compared to control after 3.75 h of hGH_{44-191} infusion at the higher dose (0.64 μg/min) (CON vs hGH_{44-191}; $p < 0.02$), but not at the lower dose (0.32 μg/min) (Fig. 2B). Significant hyperinsulinemia was also evident in response to 3.75 h of 22 kDa hGH infusion (i.e., CON vs 22 kDa hGH; $p < 0.05$) (Fig. 2B).

Table 1
Basal and Clamp Plasma Responses to Infusions of Saline, 22 kDa hGH and Two Dose Levels of hGH_{44-191}

Measurement	Saline	22 kDa hGH	hGH_{44-191} (Low dose)	hGH_{44-191} (High dose)
Basal data				
Plasma glucose, mmol/L	7.3 ± 0.2	7.1 ± 0.2	7.0 ± 0.2	7.4 ± 0.1
Plasma triglycerides, mg/dL	108 ± 10	100 ± 12	95 ± 11	111 ± 21
Clamp data				
Plasma glucose, mmol/L	7.3 ± 0.2	7.3 ± 0.2	7.3 ± 0.2	7.3 ± 0.2
Plasma triglycerides, mg/dL	53 ± 5	47 ± 7	49 ± 6	66 ± 8
Plasma insulin, mU/L	101 ± 5	103 ± 6	106 ± 17	111 ± 15

Results are mean ± SE, n = 6–12. Basal corresponds to measurements made immediately before the clamp (after 3.75 h of infusion) whereas clamp corresponds to measurements made after the clamp.

Clamp Data

Glucose clamps were performed at similar plasma glucose levels in all four groups (Table 1). Plasma triglyceride levels were suppressed during the clamp compared to basal values as expected throughout all four groups (Table 1). Plasma insulin levels obtained during the clamp were also maintained at similar levels among all four groups (Table 1).

In comparison to controls, glucose infusion rate (GIR), the measure of whole body insulin sensitivity, was suppressed by 28 ± 6% with high dose hGH_{44-191} (i.e., CON vs hGH_{44-191}; $p < 0.05$) and by 36 ± 6% with 22 kDa hGH (i.e., CON vs 22 kDa hGH ; $p < 0.01$) (Fig. 3). The GIR for low dose (0.32 μg/min) hGH_{44-191} was similar to control and was significantly higher than that of 22 kDa hGH (i.e., low dose hGH_{44-191} vs 22 kDa hGH; $p < 0.01$) (Fig. 3). Furthermore, the GIR was significantly reduced with the higher dose (0.64 μg/min) of hGH_{44-191} ($p < 0.05$) (Fig. 3). Thus the results indicate reduced whole body insulin sensitivity following 22 kDa hGH and high dose hGH_{44-191} infusion, but no change in insulin sensitivity following low dose hGH_{44-191} infusion.

To examine the relation between altered basal plasma insulin level (obtained after 3.75 h of infusion) and insulin sensitivity as determined by the euglycemic clamp, basal (preclamp) insulin and clamp glucose infusion rate in all individual infused rats were compared (Fig. 4). This showed a significant negative relation ($r = -0.55$, $p < 0.002$).

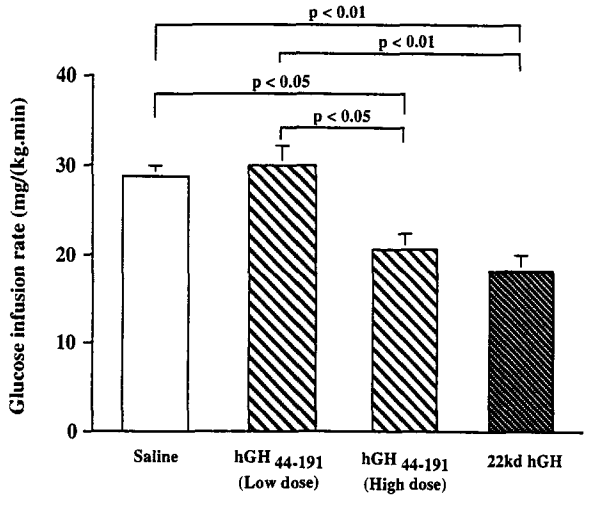

Fig. 3. Plateau GIR during a hyperinsulinemic euglycemic clamp performed from 3.75–5.75 h. Results are expressed as mean ± SE, n = 7–12 rats.

Discussion

These results, in contrast to the previous published data *(8)*, demonstrate that whereas hGH_{44-191} was capable of causing insulin resistance and hyperinsulinemia in normal rats, its diabetogenic potency was much lower than that of 22 kDa hGH. In rats, the degree of insulin resistance achieved with high dose hGH_{44-191} was similar to that achieved with a lower dose (on a molar basis) of 22 kDa hGH.

To investigate in vivo plasma kinetics in more detail, a new specific radioimmunoassay for hGH_{44-191} was set up. This revealed that similar degrees of insulin resistance are produced when hGH_{44-191} levels are 50–60 times higher than that of 22 kDa hGH. The plasma concentration data of hGH_{44-191} also indicate a much lower clearance rate compared to that of 22 kDa hGH. Both the lower diabetogenic activity and lower clearance rates are consistent with lower affinity to GHRs. In addition, the degree of basal hyperinsulinemia generated by the hGH_{44-191} (high dose) infusion, despite its higher molar concentration, was the same as for 22 kDa hGH infusion.

Our results are consistent with the in vitro findings recently reported by Rowlinson et al. *(9)*, that hGH_{44-191} was 3300–5000-fold less potent than hGH in stimulating proliferation of myeloid cells transfected with human GHRs. The effects of hGH_{44-191} were species specific because it did not affect the proliferation of myeloid

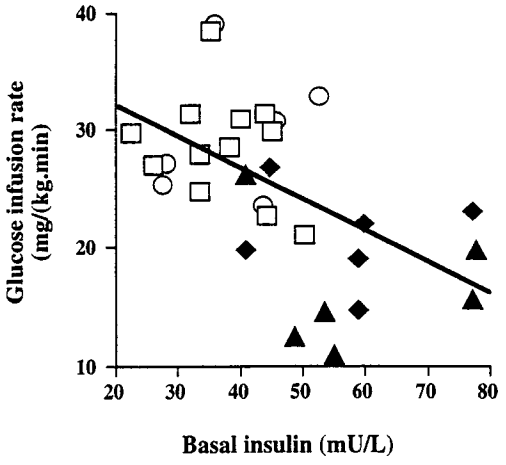

Fig. 4. Correlation between GIR and basal (preclamp) plasma insulin levels. All four groups are included in the plot. Each group is identified by an individual symbol. □, saline; ◆, hGH_{44-191} (high dose); ○, hGH_{44-191} (low dose); ▲, 22 kDa hGH. Points represent individual animals. $r = -0.55$, $p < 0.002$.

transfectants with mouse or rabbit GHRs *(9)*. In contrast, hGH had similar proliferative effects on all three transfectants. Taken together with the findings of the diabetogenic properties of the two forms of hGH, the collective data reveals that hGH_{44-191} has a much lower biological potency than hGH.

The authors' physiological findings are different to those of Lewis et al. *(8)*. However, these studies are difficult to compare directly. Lewis et al. *(8)* reported greater glucose intolerance following subcutaneously administered hGH_{44-191}. As a result, it is not possible to establish whether the measurements made by Lewis et al. *(8)* explicitly indicate insulin resistance. In contrast, insulin resistance under the controlled conditions of the euglycemic hyperinsulinemic clamp was measured *(10)*. The authors have clearly demonstrated that hGH_{44-191} infusion causes a significant reduction in net whole body glucose disposal or insulin resistance as measured by the glucose infusion rate under euglycemic clamp conditions.

The mechanism whereby 22 kDa hGH or hGH_{44-191} causes insulin resistance is not fully understood. Recent studies *(13,14)* have suggested 22 kDa hGH may be capable of directly affecting the insulin signaling pathway. Accordingly, other studies have suggested that hGH is capable of causing postreceptor defects without alterations in insulin binding to its receptors *(15)*. The cellular mechanism of the actions of hGH_{44-191} is largely unknown. There is ample evidence that the biological actions of hGH are mediated by the hGH and prolactin receptors *(16)*, and that Helix 1 of GH, which is comprised of amino acids 9–34 at the N-terminus, is responsible for binding to these receptors *(17,18)*. The absence of 43 amino acids at the N-terminus in hGH_{44-191}, and thus absence of Helix 1, would disrupt the intactness of these binding sites and the binding to the receptors. Indeed, Rowlinson et al. *(9)* have shown that hGH_{44-191} bound to the GH and prolactin receptors at affinities 10–30-fold lower than their corresponding ligands. How the reduction in receptor binding of hGH_{44-191} affects its biological actions remains to be determined.

The presence of basal hyperinsulinemia has been suggested as a major inducer of insulin resistance in some studies *(19–21)*. The significant negative correlation between basal plasma insulin levels and GIR provides support for a possible role of hyperinsulinemia in hGH_{44-191} (and 22 kDa hGH) induced insulin resistance. hGH-induced hyperinsulinemia may result from two possible mechanisms. One possibility is that hGH directly stimulates insulin release; this has been demonstrated in vitro in cultured rat fetal and adult pancreatic islets *(22,23)* and the other possibility is that it occurs as a compensatory mechanism to control the hyperglycemia that might result from insulin resistance *(24)*. So far, little is known about whether hyperinsulinemia follows or precedes insulin resistance in this or other states of insulin resistance *(19,25,26)*. Therefore, hyperinsulinemia *per se* needs to be considered as a possible important contributor to hGH insulin resistance. However, it is not clear how short-term hyperinsulinemia, as in this study, affects the development of insulin resistance *(27)*.

The authors conclude that 22 kDa hGH and hGH_{44-191} are both capable of generating acute hyperinsulinemia and insulin resistance in the normal rat; however, hGH_{44-191} has much lower diabetogenic potency compared to that of 22 kDa hGH in this model. Relative diabetogenic potencies and in vivo metabolic clearance rates, are in accord with recently reported relative affinities for binding to the GHR. Our data do not, therefore, support a diabetogenic action of GH independent of GHR binding.

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