A Growth Hormone (GH) Analog that Antagonizes the Lipolytic Effect but Retains Full Insulin-Like (Antilipolytic) Activity of GH

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Abstract. **An analog of bovine growth hormone (bGH-M8: [Leu[117], Arg[119], Asp[122]]-bGH) with an idealized amphiphilic third α-helix has been proposed to be a functional antagonist of GH. In accordance with this proposition, bGH-M8 profoundly inhibited bGH-stimulated lipolysis by chicken adipose tissue *in vitro*. bGH-M8 alone was a weak agonist in the lipolytic assay (1.9% the potency of bGH). The present evidence indicates that bGH-M8 is a competitive antagonist of the lipolytic action of GH based upon the following results: (i) increasing concentrations of bGH-M8 (antagonist) produce progressively greater inhibition of GH-stimulated lipolysis; (ii) increasing concentrations of bGH (agonist) are capable of overcoming this antagonism; and (iii) Schild plot analysis (slope = −0.94) suggests a receptor antagonist with an equilibrium dissociation constant (K_B) of 4.54 n*M*. In contrast to the antagonistic effects of bGH-M8 on bGH-stimulated lipolysis, bGH-M8 retained full insulin-like ("antilipolytic") activity (i.e., inhibition of glucagon-induced lipolysis). bGH-M8 and bGH were similarly potent in eliciting antilipolytic effects *in vitro*. Moreover, the antilipolytic effects of bGH-M8 and bGH were additive. Therefore, the third α-helix (particularly residues 117, 119, and 122) of bGH contains major structural determinants for the lipolytic effects of GH. The ability of bGH-M8 to act as an antagonist for at least one action of GH (lipolysis) while being a full agonist for another (antilipolysis) suggests that different domains of GH are responsible for its various biologic activities, possibly involving different binding sites and/or signal transduction mechanisms.**

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Growth hormone (GH) is a single-chain polypeptide containing approximately 191 amino acid residues (1–3). GH may be subject to post-translational modifications, such as glycosylation, phosphorylation, proteolytic cleavage, and deamidation (4). GH exerts diverse actions including: growth promotion via insulin-like growth factor-I (5); diabetogenic effects (6, 7); immunologic effects (8, 9); "priming" preadipocyte differentiation into adipocytes (10); inhibition of lipogenesis in sheep (11), cattle (12), and pigs (13); stimulation of lipolysis in rats (14, 15) and chickens (16) but not in sheep (17) and rabbits (18); and insulin-like effects, including increased glucose uptake and oxidation (19) and decreased rate of lipolysis in the presence of potent lipolytic agents (i.e., an "antilipolytic" effect) such as norepinephrine (20) and glucagon (21). The multiplicity of GH activities, some of which occur within the same tissue site (e.g., lipolytic and antilipolytic effects in adipose tissue), may be explained by data from GH receptor characterization studies which suggest the existence of multiple receptors or receptor subunits in liver (22–25) and adipose tissue (25, 26). It has been speculated that the GH molecule has several active domains which are responsible for

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the different biologic actions observed (27–29). More recent reports provide evidence for two binding sites on both human GH (hGH) and the extracellular domain of the hGH receptor (hGH-bp: corresponding to the soluble serum hGH-binding protein) (30, 31). Specifically, two distinct, but adjacent, binding sites for hGH on the hGH-bp and two overlapping binding sites on the hGH-bp for hGH were described, such that one molecule of hGH induced hGH-bp dimerization (30, 31). hGH receptor dimerization occurs sequentially, such that a receptor first binds to site I on hGH and then a second receptor binds to site II on hGH (32).

A genetically engineered bovine GH analog (bGH-M8: [Leu^{117}, Arg^{119}, Asp^{122}]-bGH) has been generated (33) with three amino acid substitutions intended to increase the amphiphilicity of the third helix. When displayed in an Edmundson helical wheel projection (34), the hydrophilic region of helix-III (comprising residues 114, 125, 118, 111, 122, 115, 126, 119, and 112) is enhanced by hydrophobic→hydrophilic residue replacement (Ala^{122}→Asp^{122}), while the opposing hydrophobic region (comprising residues 121, 110, 117, 124, 113, 120, 109, 116, and 123) is enhanced by hydrophilic→hydrophobic residue replacement (Glu^{117}→Ala^{117}). Glycine, a neutral and helix-destabilizing residue (35), was replaced at position 119 (by a polar residue, Arg^{119}) to strengthen the helical conformation. Theoretically, these selective amino acid replacements would allow for improved ligand insertion into the amphipathic bilayer of the adipocyte membrane. Improved stability/rigidity of the α-helix by these substitutions (via increased intermolecular interactions) may increase the "tightness of fit" or affinity for the GH receptor(s).

bGH-M8 appears to be a functional GH antagonist, as it binds to liver membranes in a manner identical that of bGH, but when expressed in transgenic mice, it suppresses growth (33, 36, 37). Subsequent studies revealed that Gly^{119}, when altered to Arg, Lys, Leu, Pro, or Trp, resulted in a series of molecules that act as antagonists of GH's growth-promoting activity (38). The present study compares the ability of bGH-M8, a putative antagonist, and bGH, a known agonist, to evoke lipolytic and antilipolytic (inhibition of glucagon-induced lipolysis) responses by chicken adipose tissue *in vitro*.

Materials and Methods

Materials. Bovine serum albumin (fraction V) was obtained from Intergen Co. (Purchase, NY). Reagents for Krebs-Ringer-HEPES medium (KRH), excluding bovine serum albumin, and porcine glucagon were purchased from Sigma Chemical Co. (St. Louis, MO). Biosynthetic bGH was donated by Eli Lilly (Indianapolis, IN). Adipose tissue was incubated in 20-ml glass scintillation vials (Kimble, Vineland, NJ).

bGH-M8 and bGH Preparation. bGH or bGH-M8 was purified from serum-free culture medium derived from mouse L cells that express the respective bGH genes (33, 37). Mouse L cells were maintained in high glucose Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) with 3% Nuserum (Collaborative Research, Inc., Bedford, MA) and 10 μg/ml of gentamicin (Gibco). Following a 48-hr incubation period, the culture fluid was collected and the cell debris pelleted by centrifugation. Proteins were precipitated by addition of ammonium sulfate (50% w/v) and then separated by centrifugation (1000g × 15 min). The pellet was resuspended in 0.1 M ammonium bicarbonate (pH 7.9) and placed on a Sephacryl S-200 column (size = 2.5 × 100 cm, void volume ≈160 ml), and 3 to 4 ml eluent fractions were collected. Aliquots (100 μl) from the eluent fractions were dried and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a miniprotein II PAGE system. The 15% PAGE gel was then stained with Coomassie blue and then destained and the fractions containing bGH or bGH-M8 were pooled. The bGH or bGH-M8 pools were concentrated by ultrafiltration (Amicon YM10) to a volume of 5–10 ml and filter-sterilized and protein content was determined (39). Purity was determined by SDS-PAGE and confirmed by Western analysis.

Animals/Adipose Tissue Dissection. All studies employed abdominal adipose tissue from adult (6–11 months old) male chickens (White Leghorn strain obtained as 1-day-old chicks from Avian Services, Frenchtown, NJ). Prior to experimentation, birds were housed in individual cages with free access to water and feed (chick grower diet; Agway, Bordentown, NJ). Adipose tissues were prepared and incubated as described previously (16). Animals (three to four per trial) were sacrificed by cervical dislocation and the abdominal adipose tissue overlying the gizzard and small intestine was removed immediately. The pooled adipose tissue was maintained in warm (38°C), oxygenated KRH (pH 7.4, supplemented with 15 mM glucose, 1% bovine serum albumin, and 2.54 mM calcium chloride) and sliced into ≈5 mg explants. Explants were then distributed randomly into incubation vials (six to eight per vial).

Lipolytic Activity Assay. The *in vitro* lipolytic activity of the test compounds were determined by the method of Campbell and Scanes (16) using chicken adipose tissue explants. Adipose tissue explants, in 1 ml of KRH per vial, were preincubated for 1 hr in a shaking water bath (38°C, 60 oscillations/min) under a 95% O_2 and 5% CO_2 atmosphere. After the 1-hr preincubation period, the preincubation medium was discarded and replaced with fresh KRH medium. The adipose tissue explants were then incubated for 1 hr in the presence of the specified treatments (bGH and/or bGH-M8 or Eli Lilly bGH standard). Incubations were

terminated by removing the tissue from the vials and subsequent rapid freezing of the medium. Glycerol release into the medium (an index of lipolysis) was determined fluorometrically (11).

Insulin-like (Antilipolytic) Activity Assay. The antilipolytic activity of bGH and/or bGH-M8 was determined by their ability to reduce lipolysis (glycerol release) in the presence of a potent lipolytic agent, glucagon (1 ng/ml) (21). Adipose tissue explants were prepared and treated as described for measurement of lipolytic activity (see above).

Data Analysis/Statistics. In all studies, biosynthetic bGH (1 μg/ml), whose lipolytic and antilipolytic activities have been defined in this *in vitro* model (16, 21), was included to act as a control for tissue viability and peptide purity. bGH and bGH-M8 (0.01, 0.1, and 1.0 μg/ml), alone and in combination (4 × 4 factorial design), were compared for lipolytic and insulin-like (antilipolytic) activity. The resultant data are representative of three independent experiments, with three to five replicates per treatment. Statistical differences between means were determined by two-way analysis of variance, followed by Fisher's protected least significant differences. Biologic potencies for GH preparations were determined by a parallel line bioassay computer program (PARATL) (40) using bGH (potency = 1.00) as the standard for comparison. In all cases described, significant ($P < 0.05$) parallelism was observed with the bGH standard. Schild plots (Log[$A'/A - 1$] vs $-$Log[B] , where A' = equieffective agonist concentration in the presence of an antagonist, A = agonist concentration, and B = antagonist concentration), prepared by linear regression analysis ($r^2 \geq 0.97$), were employed to detect competitive antagonism by bGH-M8 (41, 42). By definition, if Schild regression yields a (negative) slope of 1.0, then it is assumed that the antagonism is competitive (i.e., antagonist and agonist compete for the receptor binding site or sites) (41, 42).

Results

***In Vitro* Expression of bGH and bGH-M8.** Conditioned serum-free medium from stable transformed mouse L cells was used as the source of bGH or bGH-M8 (described above). Western blot analysis (Fig. 1) was performed to ascertain the purity and concentrations of bGH and bGH-M8 prior to biologic assay (43).

Lipolytic Activity of bGH and bGH-M8. Glycerol release from adipose tissue explants was stimulated in a dose-dependent manner by bGH (0.01, 0.1, and 1.0 μg/ml) (Fig. 2). The calculated $ED_{50} \pm SE$ ($n = 3$) for this effect was 33.3 ± 5.3 ng/ml. The lipolytic response to 1 μg/ml bGH was comparable to that of the (Eli Lilly) biosynthetic bGH standard, confirming integrity of both the tissue and mouse L cell-derived bGH. The bGH-M8 analog displayed very low lipolytic activity, which resulted in stimulation of glycerol release only at the highest dose examined (1 μg/ml). The calculated potency for bGH-M8 in the lipolytic assay was 0.02 (95% confidence limits: 0.01→0.03), relative to bGH (potency = 1.00).

The ability of bGH-M8 to inhibit the lipolytic effect of bGH was also examined. Adipose tissue explants were incubated with bGH or bGH-M8 (0, 0.01, 0.1, and 1.0 μg/ml), alone or in combination, and glycerol

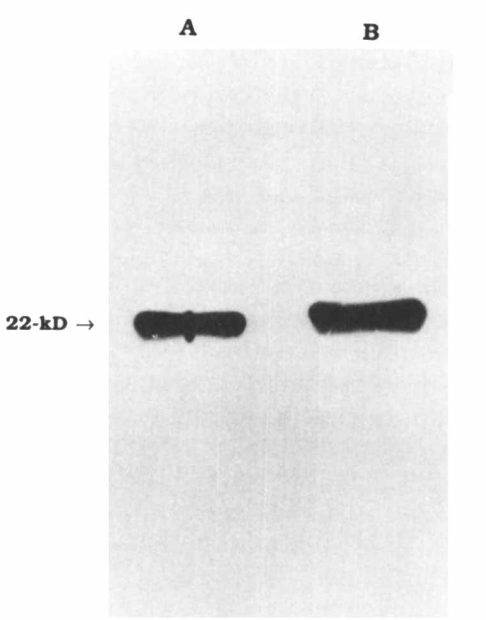

Figure 1. Western analyses of bGH (Lane A) and bGH-M8 (Lane B) expressed by mouse L cells after sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) (bands correspond to ≈22,000–23,000 daltons). Fifty nanograms each of the respective purified protein preparations were applied to the gel. Conditions for SDS-PAGE and Western blotting were as described (38).

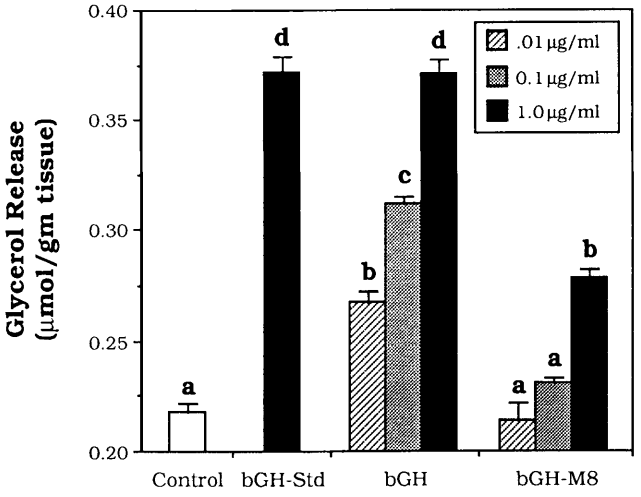

Figure 2. Effects of bGH, biosynthetic bGH standard (bGH-Std), and the putative functional antagonist, bGH-M8, on basal lipolysis by adipose tissue explants *in vitro*. Columns not sharing common letters (a, b, c, d) differ ($P < 0.001$) by two-way analysis of variance and Fisher's protected least significant differences.

release was determined. The lipolytic potency of bGH was reduced ($P < 0.05$) by 91.2% and 72%, respectively, in the presence of 1 μg/ml and 0.1 μg/ml of bGH-M8 (Fig. 3a). Increasing concentrations of bGH were capable of overcoming the antagonistic effects of bGH-M8 on lipolysis (Fig. 3b).

In order to evaluate whether the observed antagonism between bGH-M8 and bGH was competitive in nature, Schild plot analysis was performed. Utilizing the three concentrations of bGH-M8 employed, a slope of 0.94 ± 0.10 (± SE, $n = 3$) was calculated. The x-intercept of the Schild plot, yielding the equilibrium dissociation constant (K_B) of the antagonist, was determined to be 4.54 ± 0.47 nM. Taken together, these data are consistent with bGH-M8 being a high affinity, competitive antagonist of bGH for lipolytic activity.

Insulin-like (Antilipolytic) Activity of bGH and bGH-M8. bGH was observed to inhibit glucagon-induced lipolysis by adipose tissue explants, with the effect being progressively greater with increasing doses (0.01, 0.1, and 1.0 μg/ml) (Fig. 4). The antilipolytic effect of 1 μg/ml of bGH was equivalent to that of the internal bGH standard, again confirming polypeptide and tissue integrity. The ED_{50} ± SE for the antilipolytic effect of bGH was estimated to be 5.03 ± 0.87 ng/ml ($n = 3$). Unexpectedly, bGH-M8 exerted an antilipolytic effect comparable to that of bGH (potency = 0.81 [95% confidence limits: 0.48→1.37], relative to bGH). The antilipolytic effects of bGH and bGH-M8 were approximately additive (i.e., where bGH:bGH-M8 dose ratios exceed 1:2). The maximal antilipolytic effect of GH occurred at 1 μg/ml. This conclusion is based upon the lack of additivity of bGH and bGH-M8 at that dose.

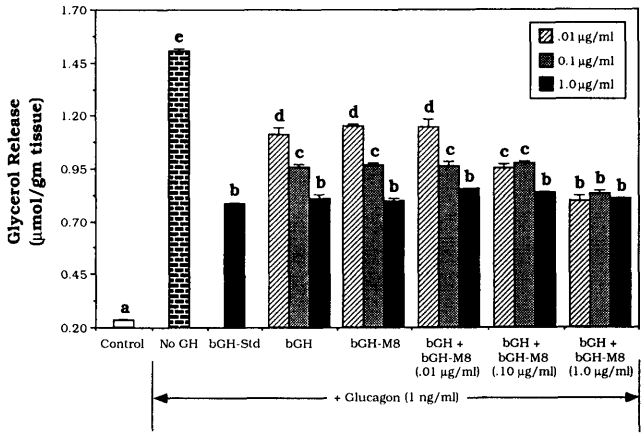

Figure 4. Antilipolytic effects of bGH, bGH internal standard (bGH-Std), bGH-M8, and bGH + bGH-M8 on glucagon-induced glycerol release by adipose tissue explants *in vitro*. Columns not sharing common letters (a, b, c, d) differ ($P < 0.01$) by two-way analysis of variance and Fisher's protected least significant differences.

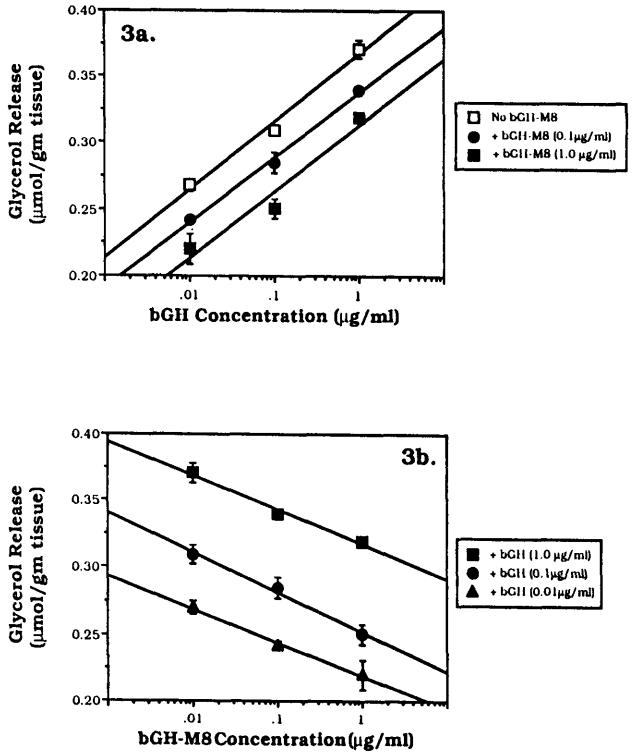

Figure 3. (a) Effect of GH analog, bGH-M8, on GH-stimulated lipolysis by adipose tissue explants. bGH-M8 (0.01 μg/ml) did not affect bGH-stimulated lipolysis and is not shown; (b) Effect of varying concentrations of bGH-M8 with constant bGH concentration.

Discussion

In the present studies, bGH-M8 has been demonstrated to be: (i) a competitive antagonist for the lipolytic effect of GH; (ii) a weak agonist in the lipolytic assay; and (iii) a full agonist in the insulin-like (antilipolytic) assay.

The bGH analog, bGH-M8, contains three amino acid substitutions (Glu^{117}→Leu^{117}, Gly^{119}→Arg^{119}, and Ala^{122}→Asp^{122}) within the putative third α-helix (44) selected to optimize amphiphilic, helical character (33). The competitive antagonist activity of bGH-M8, observed on bGH-stimulated lipolysis in the present study, is complementary to previous studies employing other model systems. For example, bGH-M8 inhibited growth and insulin-like growth factor-I release when expressed in transgenic mice (33, 36). Moreover, bGH-M8 functions as a competitive GH antagonist *in vitro* in stimulating preadipocyte differentiation and insulin-like activity (glucose oxidation) (45). bGH-M8 is fully competitive with bGH (i.e., similar binding affinity) in receptor binding assays, displacing ^{125}I-GH from liver (33, 36) and preadipocyte and adipocyte (45) membrane preparations. Considering these and the present observations (indicating that bGH-M8 acts as a partial agonist alone and as a competitive antagonist in the presence of bGH), it would appear that these amino acid substitutions occur in a region critical to eliciting a lipolytic effect, but not to binding *per se*. However,

these binding assays may not fully elucidate the nature of the binding complex (i.e., multiple binding domains), as has been demonstrated for hGH binding to hGH-bp (30, 31). It is conceivable that bGH-M8 may bind to the GH receptor via site I of the ligand, but not bind to a second receptor via site II of the ligand, since this site has been altered. The alteration of binding site II could then result in a failure to initiate signal transduction.

In view of the antagonist activity observed in the lipolytic assay, it was indeed surprising that bGH-M8 retained full antilipolytic activity. According to x-ray crystallographic data describing hGH-hGH-bp binding, the substituted residues contained within bGH-M8 (residues 117, 119, and 122) are not part of binding site I on GH, but correspond to site II (30, 31). As bGH-M8 displays full antilipolytic potency (relative to intact bGH), it is unlikely that these particular third-helix residues are critical to the antilipolytic action of GH.

The differential blockade of the lipolytic, but not insulin-like (antilipolytic), response suggests that there are functionally at least two receptors/receptor domains for GH on adipose tissue. This idea is further supported by studies on the ability of GH from different species to evoke lipolytic and antilipolytic responses by chicken adipose tissue *in vitro*. Both lipolytic and antilipolytic effects are consistently observed with bovine, human, and chicken GH (irrespective of whether native or recombinant preparations are employed) (16, 21, 46). However, GH preparations from lower vertebrates (turtle, frog, and six species of fish) exhibit very little or no lipolytic activity, yet retain substantial antilipolytic activity (29). It might also be noted that pharmacologic agents can differentially inhibit either lipolytic (cycloheximide, actinomycin D, puromycin, or verapamil) or antilipolytic (difluoromethylornithine) effects of GH (47). This data strongly suggest that the lipolytic and insulin-like (antilipolytic) effects of GH are elicited by different receptors and/or signal transduction mechanisms. An alternative explanation of the phenomenon is that different domains of GH may be responsible for different physiologic actions utilizing the same receptor(s) system, i.e., a ligand-dependent, receptor-mediated event whereby mutations in helix-III abolished the lipolytic, but not the antilipolytic, action.

The identification of a peptide or protein that acts as an antagonist for one (or more) biologic activity while retaining full agonist activity on a separate biologic action is novel. It is particularly unique that these differential effects are observed in the same dose range and time span. Moreover, the availability of an analog (bGH-M8) with antagonist activities in growth and lipolytic assays and full agonist activity in an antilipolytic assay offers considerable potential as a tool to further investigate the role and mechanism(s) of GH action(s).

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