Effects of growth hormone antagonists on 3T3-F442A preadipocyte differentiation

B C Xu, W Y Chen, T Gu, D Ridgway, P Wiehl, S Okada and J J Kopchick

Department of Biological Sciences, Molecular and Cellular Biology Program and Edison Biotechnology Institute, Ohio University, Athens, Ohio 45701, USA

Abstract

We have previously shown that a bovine (b) GH antagonist, bGH-M8, which possesses three amino acid substitutions in its third α-helix, inhibits mouse 3T3-F442A preadipocyte differentiation. In the current studies, we used the bGH and human (h) GH analogs with single amino acid substitution, bGH-G119R and hGH-G120R, for determining their biological activity using the preadipocyte differentiation assay. Short-term and long-term GH-inducible events were studied during adipose differentiation, including late marker gene expression (adipocyte protein 2), immediate early gene induction (c-fos), and tyrosine phosphorylation of intracellular proteins. The results demonstrated that these GH analogs not only failed to induce these three events, but also antagonized GH induction of c-fos expression and phosphorylation of proteins of apparent molecular mass of 95 kDa. Our present study agrees with the notion that GH must bind to the GH receptor via site one and with a second GH receptor molecule (or with some yet unidentified 'second target') through GH binding site two. This interaction is important for subsequent GH-dependent biological events.

Introduction


During this adipocyte differentiation process, GH induces an antimitotic state and a differentiation permissive condition (Corin et al. 1990). Cells in this GH-primed condition are sensitive to the differentiation activity of insulin and undergo terminal differentiation into mature adipose cells. Many differentiation-related changes in proteins and corresponding mRNA have been characterized. These include many of the enzymes involved in fatty acid and triglyceride metabolism, such as glycerol phosphate dehydrogenase (GPDH), fatty acid synthetase, lipoprotein lipase, etc. (Spiegelman 1988). These proteins, along with some novel adipocyte gene products such as adipocyte protein 2 (aP2) and adipin, are considered to be specific markers of cellular differentiation for 3T3-F442A preadipocytes (Spiegelman 1988).

Previously we have demonstrated that the third α-helix of bovine (b) GH (residues 109-126) is important for growth-promoting activity in transgenic mice (Chen et al. 1990, 1991a,b). A bGH analog, bGH-M8, which contains three amino acid substitutions (Glu-117, Gly-119 and Ala-122 to Leu, Arg, and Asp respectively) was generated. This bGH analog possesses an idealized amphiphilic structure in the third α-helix of the molecule (Chen et al. 1990). Ligand binding assay has shown that bGH-M8 has the same apparent binding affinity to the GHRs as bGH. However, the mutations destroyed the growth-promoting activity of the molecule in transgenic mice and it was found to act as a GH antagonist. A study on...
GH-dependent in vitro adipose differentiation of 3T3-F442A cells indicates that bGH-M8 could not trigger the terminal differentiation of the cells in a serum-free defined condition and it was found to act as a GH antagonist (Okada et al. 1992). We later generated a bGH analog containing a single amino acid substitution at Gly-119 to Arg which lacks growth-promoting activity in transgenic mice (bGH-G119R) (Chen et al. 1991b). A human (h) GH analog with a single amino acid substitution at Gly-120 (which corresponds to Gly-119 of bGH) to Arg (hGH-G120R) also acts as a GH antagonist in vivo and in vitro (Cunningham et al. 1991, Chen et al. 1994).

One of the most striking discoveries in the study of GH binding to GHR was that of the formation of the hGH:bGHbp2 complex (Cunningham et al. 1991, Fuh et al. 1992, de Vos et al. 1992). These findings revealed a novel manner of ligand–receptor interaction, one in which a monomeric protein ligand binds and brings together two receptor molecules. The two GHR–binding sites on the hGH molecule are located on opposite sides of the four-helical bundle and the binding of GH to the two hGHbps is a sequential event (Cunningham et al. 1991). It has been shown that Gly-120 of hGH is located in binding site two of hGH. Studies using a chimeric hGH receptor and granulocyte colony-stimulation factor receptor suggest that the association of the hGH ligand with the second receptor molecule through binding site two may be important for receptor dimerization and biological effects (Fuh et al. 1992). However, there is no direct evidence showing that hGH can form a one ligand–two receptors complex in intact cells.

Although bGH-M8 and hGH-G120R have been shown to act as antagonists in vivo, various results have been seen while using different cell lines for in vitro studies. For example, it has been shown that bGH-M8 can antagonize the lipolytic effect but retain the full insulin-like action of GH when assayed in vitro using chicken adipose tissue (Campbell et al. 1993). Also, hGH-G120R, which acts as an antagonist in transgenic animals (Chen et al. 1994), has been found to be a partial agonist in Nb2 cell mitogenesis assays (J Kopchick, unpublished data). In this study we tested the ability of two GH analogs, bGH-G119R and hGH-G120R, to induce early 'events' and terminal adipose differentiation of 3T3-F442A preadipocytes.

Materials and Methods

Materials

Mouse 3T3-F442A cells were generously provided by Dr Howard Green (Harvard University, Boston, MA, USA). Media and bovine calf serum were obtained from Gibco (Grand Island, NY, USA). Nu–Serum was obtained from Collaborative Research (Bedford, MA, USA). Cat serum was purchased from RJO Biochemicals (Kansas City, MO, USA). Bovine transferrin, bovine insulin, bovine fetuin, mouse epidermal growth factor (EGF) and 3,3',5-triiodothyronine (T3) were purchased from Sigma (St Louis, MO, USA). Rainbow protein markers, horseradish peroxidase (HRP)-conjugated phosphotyrosine antibody PY-20, Hybond-enhanced chemiluminescent (ECL) membrane and ECL developing reagents were purchased from Amersham (Arlington Heights, IL, USA). RNAzol was purchased from Cinna/Biotex Laboratories (Houston, TX, USA). GeneScreenPlus membranes were purchased from New England Biolabs (Boston, MA, USA). Random–primed DNA labeling kits were purchased from Boehringer Mannheim (Indianapolis, IN, USA). Other reagents for Northern analyses were purchased from Sigma. The mouse c-fos cDNA was a gift from Dr HJ Kung (Case Western Reserve University, Cleveland, OH, USA).

Purification of GH and GH analogs

Stably transformed mouse L cells which express bGH, bGH-G119R, hGH and hGH-G120R were maintained in roller bottles (850 cm²) in Dulbecco’s modified Eagle’s medium (DMEM) containing 4:5 g glucose/l supplemented with 10 μg gentamycin/ml and 3% Nu-Serum in an atmosphere of 10% CO₂:90% air. Cells were attached to Cytodex-2 microcarrier beads and grown to confluence. Then the cells were washed with PBS and placed on DMEM minus serum for 48 h. Cell culture media were collected and debris removed by centrifugation at 3000 g for 10 min. Proteins in the culture media were precipitated by 50% ammonium sulfate and further purified by membrane ultrafiltration HPLC (Gu et al. 1995). The resulting protein samples were assayed for purity by HPLC. GH concentrations were determined by RIA.

Cell culture and treatment

Mouse 3T3-F442A cells were plated at 200 cells/cm² and grown to confluence on six-well plates, using DMEM containing 4:5 g glucose/l supplemented with 10 μg gentamycin/ml and 10% bovine calf serum in an atmosphere of 10% CO₂:90% air. For acute GH and GH antagonist treatments, confluent 3T3-F442A cells were rendered quiescent by incubating in DMEM minus serum for 18 h. The purified and concentrated GHs or GH analogs in sodium bicarbonate buffer were added directly to the serum-free media. Cells were then incubated at 37 °C for 30 min before total cellular proteins or RNAs were isolated. 3T3-F442A cell differentiation was performed as described by Guller et al. (1988, 1989). Briefly, cells were plated at 600 cells/cm² in calf serum and switched to DMEM supplemented with 10% cat serum 24 h later. After becoming confluent, the cells were washed extensively with PBS and the medium was replaced by serum-free defined medium (DM), consisting
of a basal medium (Ham's F-12/F12 medium 2:1) supplemented with bovine fetuin (50 ng/ml), EGF (50 ng/ml), insulin (10 µg/ml), T3 (100 µg/ml) and transferrin (10 ng/ml). Purified GHs or GH analogs were added to the DM to induce differentiation.

**Western blot analyses using a phosphotyrosine antibody**

Western blot analyses using phosphotyrosine antibody were performed as previously described (Wang et al. 1994). Briefly, cells growing in six-well plates were quickly washed with PBS after GH treatment. Cells were then lysed by adding 350 µl hot lysis buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 1% β-mercaptoethanol, 0.1 M dithiothreitol, 5% sucrose, 100 µM sodium orthovanadate) directly to the cell monolayer. Cell lysates were transferred to Eppendorf tubes and boiled for 5 min before being placed on ice. Genomic DNA in the cell lysate was sheared by passing the lysate through a 26 gauge needle. Protein samples were separated by SDS-PAGE using the Bio-Rad Protein system. After electrophoresis, gels were rinsed twice with a transfer buffer and transferred to a Hybond-ECL membrane for 3 h at 10 W constant power. The membrane blots were rinsed twice with rinsing buffer (10 mM Tris-HCl, pH 7.5, 75 mM NaCl, 0.1% Tween 20, 1 mM EDTA). Blots were incubated in a blocking solution of 4% BSA in PBS for 1-2 h. After incubation, blots were washed extensively with PBST and then re-incubated with the specific antibody at a concentration of 0.1 µg/ml in the blocking solution for 1-2 h. After incubation, blots were washed extensively with PBST, developed with ECL developing reagent and exposed to X-ray films according to the manufacturer's protocol.

**Northern blot analyses**

Cells in six-well plates were washed with PBS and lysed in 1 ml RNAzol. Total cellular RNAs were isolated according to the manufacturer's instructions and separated by 1% agarose-formaldehyde gel electrophoresis. The separated RNAs were transferred to a GeneScreenPlus membrane and hybridized with the indicated DNA probes, each labeled using the random priming kit. Hybridization was carried out overnight at 42 °C. The membrane was then washed in 2 x SSC/1% SDS and exposed to Kodak film at −70 °C using an intensifying screen. For determining the levels of β-actin RNA, the membrane was stripped and probed again with labeled β-actin cDNA. To control for possible variations in RNA loading, the denaturing gel containing the separated RNAs was stained with ethidium bromide and the stained RNA bands were photographed and analyzed.

**Results**

**bGH-G119R and hGH-G120R failed to stimulate aP2 expression**

A serum-free differentiation procedure was used to examine the adipogenic effect of GH and GH analogs on 3T3-F442A preadipocytes. Confluent 3T3-F442A cells, propagated in serum-supplemented media, were switched to serum-free DM supplemented with 2.2 nM bGH. Serum-free DM was used as control. Cell morphological changes were observed at various times during the differentiation program began. As reported previously (Guller et al. 1989a), there was a striking change in cell shape from a flat fibroblastic morphology to a rounded one after 7 days of incubation in DM supplemented with GH. Fat droplets were observable in these cells, indicating that differentiation to mature adipocytes had occurred. Cells incubated with DM did not show such morphological changes. Total cellular RNAs were isolated from post-confluent cell cultures at various times after treatment and subjected to Northern blot analyses using a mouse aP2 gene-specific probe (Fig. 1). The resulting autoradiogram indicated that aP2 mRNA was increased in cells incubated with bGH (2.2 nM) up to 7 days after the addition of GH (Fig. 1A, lanes 1-9). The size of the aP2 RNA observed was 0.6 kb. No elevated aP2 RNA was observed in the cells incubated in serum-free DM without bGH (Fig. 1A, lanes 1-5). These data correlated very well with the morphological changes observed during adipose differentiation (data not shown). The aP2 probe was then stripped from the membrane and a DNA probe specific for β-actin gene was used to reprobe the blot. It was observed that the β-actin RNA decreased after 7 days of incubation in the serum-free DM with bGH (Fig. 1B, lane 9) but did not change in control cells, indicating down-regulation of actin RNA during the adipogenesis process as reported before (Guller et al. 1989a). To test whether the bGH and hGH analogs can induce expression of aP2, cells were treated with different concentrations of GHs, bGH-G119R, or hGH-G120R. Total cellular RNA was isolated 7 days after commencement of the differentiation program. The resulting Northern blot is shown in Fig. 2. The upper panel reveals that aP2 RNA was elevated after bGH (Fig. 2, lanes 2 and 3) and hGH (Fig. 2, lane 5) treatment. The GH analogs, bGH-G119R (Fig. 2, lanes 3 and 4) and hGH-G120R (Fig. 2, lanes 6 and 7) at 2.2 nM and 220 nM did not induce aP2 gene expression.

**bGH-G119R and hGH-G120R failed to induce c-fos expression and antagonized the ability of GHs to induce c-fos expression**

It has been shown that acute treatment of 3T3-F442A preadipocytes with a physiological concentration (2.2 nM)
of GH could rapidly and transiently induce the expression of the proto-oncogene c-fos RNA (Gurland et al. 1990). This effect of GH on c-fos induction was protein kinase C-dependent and occurred at the level of gene transcription. Since both GH antagonists could not induce aP2 expression, we tested whether they could trigger induction of the immediate early gene, c-fos. Serum-depleted preadipocytes were treated with different concentrations of GHs or GH analogs (Fig. 3, lanes 2–4 and 7–9) for 30 min at 37 °C. Total RNAs were isolated and subjected
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**FIGURE 2.** bGH-G119R and hGH-G120R failed to induce aP2 expression during 3T3-F442A cell differentiation. Confluent 3T3-F442A cells were incubated with differentiation concentration of wild-type bGH, hGH, bGH-G119R or hGH-G120R as indicated. Total cellular RNAs were isolated on day 7 and subjected to Northern blot analyses. The arrow shows the 0.6 kb aP2 RNA signal. The lower panel shows the stained RNA resolved in denaturing gel electrophoresis. The arrows show the major 28S and 18S ribosomal RNA bands.

FIGURE 2. bGH-G119R and hGH-G120R failed to induce aP2 expression during 3T3-F442A cell differentiation. Confluent 3T3-F442A cells were incubated with differentiation concentration of wild-type bGH, hGH, bGH-G119R or hGH-G120R as indicated. Total cellular RNAs were isolated on day 7 and subjected to Northern blot analyses. The arrow shows the 0.6 kb aP2 RNA signal. The lower panel shows the stained RNA resolved in denaturing gel electrophoresis. The arrows show the major 28S and 18S ribosomal RNA bands.

bGH-G119R and hGH-G120R failed to induce tyrosine phosphorylation of pp95/96 and antagonized the ability of GHs to induce this phosphorylation event

GHs and the GH analogs were used to treat serum-deprived 3T3-F442A cells as observed above. Total cellular proteins were isolated 10 min after GH treatment and subjected to Western blot analyses. Figure 4 showed that bGH and hGH could trigger tyrosine phosphorylation of proteins of apparent molecular mass of 95 kDa (pp95/96) (Fig. 4, lanes 2 and 5) and agree with our previous results (Wang et al. 1994). bGH-G119R and hGH-G120R at increasing concentrations could not induce tyrosine phosphorylation of pp95/96 (Fig. 4, lanes 3–4 and 6–7). Serum-depleted 3T3-F442A cells were treated with
bGH and bGH-G119R  hGH and hGH-G120R
(-)  

GH      L   L   L   L   L   L   L
GH antagonist  L   H   L   H   L   H   L   H

FIGURE 3. GH antagonists do not induce c-fos expression and antagonize the ability of GH to induce c-fos. Confluent serum-depleted 3T3-F442A cells were treated with different concentrations of GH or GH antagonists for 30 min before total RNAs were isolated and subjected to Northern blot analyses (lanes 2-4 and 7-9). The type of GH ligand and the concentration applied are indicated at the top of the panel (L: 2 nM; H: 0.2 µM). For examining the interference of GH analogs to the c-fos induction by GH (lanes 6-7 and 10-11), cells were treated with different concentrations of GH antagonist along with 2.2 nM GH. Total RNA was isolated after 30 min of treatment and subjected to Northern blot analyses. The arrow shows the 2.2 kb c-fos RNA signal. The lower panel shows the stained RNA on the denaturing gel. The arrows show the major 28S and 18S ribosomal RNA bands.

different concentrations of GH antagonists and 2.2 nm GHs for 10 min. The resulting Western blots showed that incubation of cells with media containing bGH-G119R or hGH-G120R at 220 nm totally abolished tyrosine phosphorylation of pp95/96 triggered by 2.2 nm bGH or hGH (Fig. 5, lanes 5 and 9).

Discussion

GH-dependent conversion of 3T3-F442A preadipocytes to mature adipocytes provides a reliable model for studying short-term and long-term effects of GH on cultured cells (Gurland et al. 1990). In this process, GH accounts for 60% of the adipogenic activity of the serum (Nixon & Green 1984). It has been reported that GH acts on serum-starved preadipocytes to induce a GH-primed antimitotic state (Corin et al. 1990). Cells in this state are sensitive to the adipogenic activity of insulin and terminally differentiate to adipose cells.

To examine the effect of different GH antagonists, bGH-G119R and hGH-G120R, on 3T3-F442A cell differentiation, a serum-free differentiation procedure was used as described by Guller et al. (1988, 1989b). In this procedure, the effect of GH can be studied without the influence of low amounts of GH in the serum media. Previously we examined the adipogenic effect of bGH on these cells. Cells incubated in DM supplemented with bGH showed adipocyte morphology after 7 days of incubation (Okada et al. 1992). In the present study, Northern blot analyses revealed that the expression of the differentiation specific marker gene, aP2, was elevated and β-actin gene was down-regulated. Cells incubated in the control DM did not show such changes. These findings agree with previous reports about adipose conversion of 3T3-F442A cells (Spiegelman 1982, 1988), indicating that the serum-free differentiation was successful. hGH also showed the ability to trigger the terminal differentiation of 3T3-F442A preadipocytes (data not shown).
We have previously shown that a bGH analog, bGH-M8 in which three amino acid residues were altered, acted as a functional antagonist of GH (Okada et al. 1992). However, this analog has been found to antagonize the lipolytic effect but retain full insulin-like activity in chicken adipose tissue (Campbell et al. 1993). In this study...
we wanted to determine whether single amino acid substitution GH analogs, i.e. bGH-G119R and hGH-G120R, could affect adipose conversion of 3T3-F442A cells as judged by aP2 expression and whether these GH analogs acted as antagonists for the GH differentiation-promoting activity. The morphological changes of the preadipocyte could not be observed when using these GH antagonists. These data suggest that mutation of the conserved Gly residue in the third α-helix of the GH molecule to Arg abolished the adipogenic activity of the molecule.

It has been demonstrated that one of the acute effects of GH on 3T3-F442A cells is the induction of c-fos and c-jun gene expression (Gurland et al. 1990). GH treatment also elevates the production of c-fos proteins (Barcellini-Couget et al. 1993). The association of Fos and Jun proteins results in the Fos/Jun heterodimers which serve as transcription factors that regulate the transcription of differentiation specific genes, including aP2 (Distel et al. 1987, Rauscher et al. 1988). This has been suggested to be one means by which GH exerts its long-term effect on cells (Gurland et al. 1990). Since neither of the GH analogs tested could induce aP2 gene expression, it was of interest to examine the effect of these GH analogs on c-fos induction. In this study, both GH analogs failed to induce c-fos gene expression. However, hGH-G119R and hGH-G120R did prevent GH from inducing c-fos, suggesting that they acted as functional antagonists.

To elucidate the defect of bGH-G119R and hGH-G120R in adipose conversion of 3T3-F442A cells further, another GH-dependent event was examined. The rapid and transient tyrosine phosphorylation of intracellular protein pp95/96 has been observed in GH-treated 3T3-F442A cells (Wang et al. 1994). This is a rapid event which is observable as early as 1 min after GH treatment. Similar to the effects observed in the c-fos expression, the GH analogs failed to induce this GH-dependent phosphorylation event and antagonize the ability of GHS to induce the phosphorylation of pp95/96. These data suggest that the defect of bGH-G119R and hGH-G120R in inducing GH-dependent conversion of adipose cells occurs after GH binding but before the induction of pp95/96.

Studies using hGH-binding protein revealed that the formation of the hGH:GHR complex is important for the effect of GH (Cunningham et al. 1991, de Vos et al. 1992, Silva et al. 1993). Since Gly-119 in bGH and Gly-120 in hGH are considered as a GHR binding site two amino acid, it is possible that these GH analogs exert their antagonistic effect by association with GHR through binding site one and subsequently fail to associate with a second GHR ultimately to form a functional ternary complex. If this is the case, the defect of the analogs to trigger biological effects should be at the GH/GHR interaction level. Our data in this study, showing that bGH-G119R and hGH-G120R could not trigger any of the GH-inducible biological events, support the hypothesis that the defect of bGH-G119R and hGH-G120R may be due to their inability to associate with a second GHR molecule through binding site two. However, one cannot rule out the possibility that an as yet unidentified site two specific molecule may be important for the effects of GH.

Different antagonist:agonist ratios have been reported in other studies to obtain inhibition of GH agonist effects. In hGH-treated human IM-9 cells, a tyrosine phosphorylated 97 kDa protein could be observed. IM-9 cells treated with hGH-G120R and hGH at a 10:1 ratio showed obvious GH antagonist effects (Silva et al. 1993, Chen et al. 1994), while a 1:1 ratio of hGH-G120R to hGH had no effect. Tyrosine phosphorylation of a pp95 can be observed in GH-treated mouse L cells expressing mouse GHRs (Zhou et al. 1994). Inhibition of GH-induced pp95 can be observed at a hGH-G120R:hGH ratio of 1:1 with a maximum effect at 10:1. Additionally, GH-induced GPDH enzyme activity in 3T3-F442A cells can be inhibited 50% at equimolar concentration of bGH and bGH-M8 of 0.03 nM (Okada et al. 1992). In the studies reported here, a 100:1 antagonist:agonist ratio was used to ensure full inhibition of GH-induced c-fos expression and tyrosine phosphorylation of intracellular proteins.

Our data in the present study suggest that (1) mutation of the conserved Gly residue in the third α-helix of bGH and hGH molecule abolished its ability to trigger the adipose conversion of 3T3-F442A cells and (2) the defect of bGH-G119R and hGH-G120R to the induction of terminal differentiation of 3T3-F442A cells is upstream of the GH-induced pp95/96 phosphorylation, and likely to be due to their inability to associate with the second GHR through binding site two.

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