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Human growth hormone: 45-kDa isoform with extraordinarily stable interchain disulfide links has attenuated receptor-binding and cell-proliferative activities

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ABSTRACT

Background: Human growth hormone (hGH) is a complex mixture of molecular isoforms. Gaps in our knowledge exist regarding the structures and biological significances of the uncharacterized hGH molecular variants. Mercaptoethanol-resistant 45-kDa human growth hormone (MER-45 kDa hGH) is an extraordinarily stable disulfide-linked hGH homodimer whose biological significance is unknown.
Objectives: To elucidate the pharmacokinetic abilities of dimeric MER-45-kDa hGH to bind to GH and prolactin (PRL) receptors and to elucidate its abilities to stimulate cell proliferation in lactogen-induced and somatogen-induced *in vitro* cell proliferation bioassays.
Design: The binding of MER-45-kDa hGH to GH and PRL receptors was tested in radioreceptor assays (RRAs). Competitive displacements of [^{125}I]-bovine GH from bovine liver membranes, [^{125}I]-ovine PRL from lactating rabbit mammary gland membranes and [^{125}I]-hGH from human IM-9 lymphocytes by unlabelled GHs, PRLs or dimeric MER-45-kDa hGH were evaluated. The abilities of dimeric MER-45-kDa hGH to stimulate proliferation of lactogen-responsive Nb2 lymphoma cells and to stimulate proliferation of somatogen-responsive T47-D human breast cancer cells were assessed by incubation of cells with GHs or PRLs and subsequently measuring growth using the MTS cell proliferation assay.
Results: Dimeric MER-45-kDa hGH, compared to monomeric hGH, had reduced binding affinities to both GH and prolactin receptors. In a bovine liver GH radioreceptor assay its ED_{50} (197.5 pM) was 40.8% that of monomeric hGH. In a human IM-9 lymphocyte hGH RRA its ED_{50} (2.96 nM) was 26.2% that of monomeric hGH. In a lactating rabbit mammary gland prolactin RRA its ED_{50} (3.56 nM) was 16.8% that of a monomeric hGH. Dimeric MER-45-kDa hGH, compared to monomeric hGH, had a diminished capacity to stimulate proliferation of cells *in vitro*. In a dose–response relationship assessing proliferation of Nb2 lymphoma cells its ED_{50} (191 pM) was 18.0% that of monomeric hGH. While monomeric hGH stimulated a 2.2-fold proliferation of T47-D human breast cancer cells above vehicle control, dimeric MER-45-kDa hGH was unable to stimulate the cells to proliferate and slightly inhibited their proliferation to 77.6% that of control.
Conclusions: The topological arrangement of monomeric hGHs to form an unusually stable disulfide-linked dimer markedly diminishes hGH's binding affinities to both GH and PRL receptors and also drastically attenuates its ability to stimulate proliferation of cells *in vitro*.

Abbreviations: BSA, Bovine serum albumin; DMS, Dimethylsuberimidate; EDC, 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide; EDTA, Ethylenediaminetetraacetic acid; EGTA, Ethylene glycol-bis(β-aminoethylether)-N, N, N′, N′-tetraacetic acid; GH, Growth hormone; bGH, Bovine growth hormone; hGH, Human growth hormone; hPRL, Human prolactin; MER-45-kDa hGH, Mercaptoethanol-resistant 45-kDa human growth hormone; NHPP, National Hormone and Pituitary Program; NIDDK, National Institute of Diabetes, Digestive and Kidney Diseases; NIH, National Institutes of Health; oPRL, Ovine prolactin; PRL, Prolactin; RRA, Radioreceptor assay; Tris, 2-Amino-2-(hydroxymethyl)-1,3-propanediol; hGH-N, Normal hGH gene.

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1. Introduction

Pituitary GHs are important regulators of growth, metabolism and development [1–3]. The hGHs are a complex mixture of molecular isoforms that constitute approximately 10% of the dry weight of the pituitary gland [4–10]. Although a 22-kDa hGH is the predicted protein product of the hGH-N gene, posttranscriptional and post-translational processing produce different hGH isoforms, as shown in a western blot of human pituitary extract separated by 2-D IEF/SDS-PAGE [7]. Small hGH isoforms (5-kDa and 17-kDa) are produced by fragmentation of the 22-kDa hGH and a slightly smaller alternatively spliced 20-kDa hGH is also produced [4–8,10]. Other molecular variants of hGH include the 24-kDa and 12-kDa glycosylated hGHs [11–13], deamidated hGHs [14] and phosphorylated hGHs [15,16]. In addition, large hGH isoforms such as a 35-kDa hGH, 45-kDa hGH dimers and higher oligomers of hGH have been reported [4–8,10]. The molecular variants are present in serum to varying degrees [8,17]. A few isoforms of hGH have enhanced biological activities when compared to the 22-kDa hGH, others have reduced bioactivity, several are completely bioinactive and some can only regulate a limited number of metabolic and physiological processes [4–8,10]. The hGH isoforms can thus act as full or partial agonists and antagonists of monomeric 22-kDa hGH. Many of the hGH isoforms have not been isolated, hence, their biological significances remain unknown.

Nevertheless, certain progress is being made, as in a recent study in which an extraordinarily stable dimeric 45-kDa hGH isoform was isolated and structurally characterized [18]. The mercaptoethanol-resistant (MER) 45-kDa hGH was shown to be an interchain disulfide-linked 22-kDa hGH homodimer which does not readily dissociate into monomers even when incubated in 10% 2-mercaptoethanol at 100 °C for extended periods of time. Development of a method for preparation of dimeric MER-45-kDa hGH suitable for biological testing [19] has led us to the current work assessing its capacity to interact with lactogenic and somatogenic receptors and its ability to stimulate proliferation of rat Nb2 lymphoma cells and T47-D human breast cancer cells. Our findings demonstrate that dimerization of hGH through unusually stable disulfide bonds attenuates hGH's receptor-binding and cell-proliferative functions.

2. Materials and methods

2.1. Chemicals

EGTA, bromocriptine (Lot 107H1244), sodium azide, sodium phosphate, sodium chloride, Fischer's medium, BSA, penicillin and streptomycin were purchased from Sigma (St. Louis, MO, USA). EDTA, ammonium bicarbonate and sodium bicarbonate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Tris, was purchased from EM Science (Gibbstown, NJ, USA). Gelding horse serum was purchased from Animal Technologies, Inc. (Tyler, TX, USA). Fetal calf serum, Dulbecco's modified Eagle's medium and Ham nutrient mixture F-12 medium 50:50 (DMEM/Ham's F-12), and RPMI-1640 medium were purchased from Gibco (Gibco BRL Div. of Invitrogen, Gaithersburg, MD, USA). Trypsin–EDTA was purchased from HyClone (Logan, UT, USA).

2.2. Hormone preparations

Bovine GH (bGH; NHPP AFP10325C), monomeric hGH (monomeric NIH-hGH; NIDDK-NIH AFP 9755A), radioiodination grade hGH (radioiodination grade monomeric NIH-hGH, hGH AFP11019B), ovine PRL (oPRL; NIDDK-NIH AFP10692C), and human PRL (NHPP-hPRL; recombinant human prolactin, NHPP AFP 795) were obtained from Dr. A.F. Parlow of the National Hormone and Pituitary Program (Harbor-UCLA Medical Center, Torrance, CA, USA). MER-45-kDa hGH was previously isolated [19].

2.3. Protein determination

Quantification of protein [20] was carried out using the Microassay Procedure for microtiter plates (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as a standard.

2.4. Radioiodination of hormones

Bovine GH, radioiodination grade monomeric NIH-hGH, and oPRL were each radiolabeled using an established procedure [21] with modifications [22]. The specific activities of the bGH, radioiodination grade monomeric NIH-hGH, and oPRL preparations were 62 μCi/μg, 63 μCi/μg, and 44 μCi/μg, respectively.

2.5. Experimental animals

All animal experimentation was conducted in accordance with the accepted standards of humane animal care. All research animals were acquired and used in compliance with federal, state, and local laws and institutional regulations. Animals were maintained in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

2.6. Membrane preparations

Bovine liver membranes were prepared as described [23]. Lactating rabbit mammary gland membranes were prepared as described [22]. The lactating New Zealand White Rabbit, obtained from Harlan (Indianapolis, IN, USA), was subcutaneously injected at times 0, 12, and 24 h with 1.0 mL/injection of a 1.0 mg bromocriptine/mL solution then sacrificed at 36 h. The mammary glands were subsequently dissected, frozen on dry ice then stored at −20 °C until used for preparation of microsomal membranes.

2.7. Non-linear curve fitting and statistical analyses

Simultaneous non-linear curve fitting of families of sigmoidal dose–response curves of radiolabelled hormone displacement data obtained in the RRAs and of rat Nb2 lymphoma cell number data obtained in the hormone-induced cell proliferation bioassay was carried out using the ALLFIT [24]. The ED_{50} values for curves within an assay were tested for significant differences using One-Way ANOVA. If the ANOVA *F*-test indicated that ED_{50} values were significantly different from each other ($P<0.05$), a *post hoc* Bonferroni Multiple Comparison Test was used to determine differences among the ED_{50}s ($P<0.05$). An unpaired two-tailed Student's *t*-test was used when comparing ED_{50} values for significant differences ($P<0.05$) in assays that had only two displacement curves.

2.8. Bovine liver GH radioreceptor assay

The bovine liver GH RRA was carried out as described [23] using [^{125}I]-bGH (20,000 cpm) with each dose point assayed in duplicate ($n=2$).

2.9. Human IM-9 lymphocyte hGH radioreceptor assay

The human IM-9 lymphocyte hGH RRA was carried out as reported [25,26] with some modifications. Human IM-9 lymphoblastoid cells were grown in suspension culture in RPMI-1640 medium supplemented with 2 g/L sodium bicarbonate, 10% (v/v) fetal calf serum, 50 U/mL penicillin, 50 μg/mL streptomycin at pH 7.5 in a humidified 5% CO_2 atmosphere at 37 °C. Before initiating the RRA, the RPMI-1640 medium was replaced with phosphate assay buffer [10 mM sodium phosphate, 150 mM sodium chloride, 10 mM EDTA, 10 mM EGTA, 0.1% (w/v) BSA, pH 7.6]. Cells were aliquoted into 12×75 mm polypropylene tubes (2×10^6 cells/tube) in phosphate assay buffer