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Comparison of *in vitro* bioactivity of chicken prolactin and mammalian lactogenic hormones

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ABSTRACT

Recombinant chicken prolactin, expressed in *Escherichia coli* as an unfolded protein, was successfully refolded and purified to homogeneity as a monomeric protein. Its biological activity was evidenced by its ability to interact with rabbit prolactin receptor extracellular domain and stimulate prolactin receptor-mediated proliferation in three cell types possessing mammalian prolactin receptors. Chicken prolactin activity in those assays was 20–100-fold lower than that of mammalian lactogenic hormones, likely due to lower affinity for mammalian prolactin receptors and not to improper refolding, because in two homologous bioassays, chicken prolactin activity was equal to or higher than that of ovine prolactin and the CD spectra of chicken and human prolactin were almost identical. Our results using seven mammalian lactogenic hormones from five species in three bioassays revealed the major role of species specificity in testing biological activity *in vitro*. Heterologous bioassays may be misleading and homologous assays are strongly recommended for predicting the activity of species-specific lactogenic hormones *in vivo*.

1. Introduction

Prolactin (PRL) is a polypeptide hormone that is mainly secreted by lactotroph cells of the anterior pituitary gland (Scanes et al., 1975; Freeman et al., 2000). Its amino acid sequence is similar to that of growth hormone, placental lactogen (PL) and the newly identified PRL-like protein that shares genomic, structural and biological features and belongs to the same protein family. The gene encoding avian PRL is located on chromosome 2 (Alipanah et al., 2011), and was initially described as containing five exons and four introns (Liu et al., 2008; Yousefi et al., 2012). The mature form of the protein contains 199 residues with three disulfide bridges between six cysteines, and has a molecular weight of ~23 kDa (Watahiki et al., 1989; Kansaku et al., 2008). However, little is known about the functions of PRL modification by phosphorylation, glycosylation, deamination, sulfonation and polymerization (Bédécarrats et al., 1999; Kansaku et al., 2005). Studies in birds have indicated that the PRL-encoding gene is expressed in the hypothalamus, pituitary, oviduct and ovary, as well as in the thymus, spleen and lymphocytes (Kansaku et al., 2008). This widespread expression partially explains its involvement in various processes, such as initiation and maintenance of incubation behavior, regulation of gonadal development and functions, egg-laying, osmoregulation and immunomodulation in poultry species (Harvey et al., 1984; El Halawani et al., 1986; Skwarło-Sońta, 1990; Ben-Jonathan et al., 1996; Li et al., 2011; Chaiseha and El Halawani, 2015). In view of prolactin pleotropic action preparation of recombinant chicken prolactin in amounts suitable for *in vivo* experiments as presented in our present work is timely. The diverse actions of PRL are mediated by the PRL receptor (PRLR), a single transmembrane protein that belongs to class I of the cytokine receptor superfamily, which includes growth hormone receptor, leptin receptor, interleukins, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, leukemia inhibiting factor, oncostatin M, erythropoietin, thrombopoietin, gp130 and ciliary neurotrophic factor (Xing et al., 2011). Several isoforms of membrane-bound PRLR have been identified: few variants of short, intermediate, long, and the soluble form containing only the extracellular domain (ECD) (Tanaka et al., 1992; Ohkubo et al., 1998; Clevenger and Kline, 2001). Upon binding to PRL, the PRLR can initiate multiple intracellular signaling cascades, including activation of the Janus kinase/signal transducers and

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activators of transcription (JAK2–STAT5), the mitogen-activated protein kinase (MAPK) and pAkt signaling pathways (Jiang et al., 2005).

To better characterize the biological properties of chicken PRL (chPRL) and to provide a valuable tool for pharmacological homology studies, a procedure for large-scale production of recombinant chPRL was established and the biological activity of the purified protein was tested *in vitro* in a homologous system. An additional question addressed in this paper was whether the various *in vitro* heterologous bioassays indeed reflect the homologous biological potential of lactogenic hormones *in vivo*. We tested seven mammalian lactogenic hormones along with chPRL in three heterologous *in vitro* bioassays in cells expressing human, rabbit or rat PRL receptors.

2. Materials and methods

2.1. Recombinant proteins, cells and chemicals

Recombinant human PRL (hPRL), rabbit and human PRL ECD (rbPRL-ECD and hPRL-ECD, respectively), ovine PRL (oPRL), rbPRL, rat prolactin (rPRL), human placental lactogen (hPL), oPL and bovine placental lactogen (bPL) were prepared in our laboratory as described previously (Gertler et al., 1992, 1996, 1998; Sakal et al., 1997; Leibovich et al., 2001). Streptavidin-horseradish peroxidase (Streptavidin – HRP) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Baf3 cells stably transfected with the long form of rbPRLR were from Dr. Jean Djiane, Nb2-11C rat lymphoma cell line was from Dr. Henri Friesen, and Baf3/LP cells stably transfected with the long form of hPRLR were from Dr. Vincent Goffin. *Escherichia coli* bacterial strain A 1645 expressing chPRL was a gift from Biotechnology General Israel Inc. (Beer Tuvia, Israel). This strain constitutively produces thermostable gamma repressor at 30 °C which prevents transcription (Roberts et al., 1968). When the temperature is raised to 42 °C, repression is abolished. All other reagents were of analytical grade.

2.2. Expression, refolding, and purification of chPRL

E. coli cells (500 ml) were grown in a 2.5-L flask in LB medium (1% bacto-tryptone, 0.5% yeast extract, 1.5% NaCl, w/v) at 30 °C to an optical density at 600 nm (OD_{600}) of 1.0 and temperature was then raised to 42 °C. The cells were grown for an additional 4 h, pelleted for 6 min at 6000g, and frozen. The bacterial pellet from 5 L of culture was thawed on ice and resuspended in lysis buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8) containing 0.5 mg lysozyme/ml. Inclusion bodies (IBs) were then prepared as described previously and frozen (Gertler et al., 1998). IBs obtained from 5 L of bacterial culture were then solubilized in 50 ml of 50 mM Tris base, pH 8.5, containing 6 M guanidine-HCl and 8 mM 1.4-dithiothreitol (DTT). After 45 min of stirring at room temperature, the solution was slowly added by peristaltic pump to 1000 ml refolding buffer (50 mM Tris base, 160 mM arginine, 1 M urea, 4 mM cysteine, pH 8.5), stirred at 4 °C for 24 h, and then the clear solution was dialyzed against 3 × 10 L of 10 mM Tris-HCl, pH 8 at 4 °C. The resultant solution was applied to a Q-Sepharose column (2.5 × 6 cm) pre-equilibrated with 10 mM Tris-HCl, pH 8. Elution was carried out using a discontinuous NaCl gradient in the same buffer (50, 100, 150, 300 mM NaCl). Fractions (40 ml) were collected and protein concentration was determined at OD_{280}. Fractions containing the monomeric chPRL were identified using size-exclusion chromatography (SEC) on an analytical Superdex 75 HR column in TN buffer (25 mM Tris-HCl, 300 mM NaCl, pH 8) at room temperature. Fractions containing the monomeric chPRL were pooled (tubes 23–32 in Fig. 2), dialyzed against $NaHCO_3$ to ensure a 4:1 protein-to-salt ratio and lyophilized.

2.3. Determination of purity and monomer content

SDS-PAGE was carried out according to Laemmli (1970) in a 10% polyacrylamide gel under reducing and non-reducing conditions. The gel was stained with Coomassie Brilliant Blue R. Gel-filtration chromatography was performed on a Superdex 75 HR 10/30 column with 0.2-ml aliquots of the Q-Sepharose column-eluted fraction using TN buffer.

2.4. Circular dicroism spectra (CD)

The CD spectra in millidegrees were measured with an AVIV model 62A DS circular dichroism Spectrometer (Aviv Assoc., Lakewood, NJ) using a 0.020 cm rectangular QS Hellma cuvette. The spectrometer was calibrated with camphorsulfonic acid. The absorption spectra were measured with an AVIV model 17DS UV–vis-IR spectrophotometer (Aviv Assoc., Lakewood, NJ) using a 1.000 cm QS cuvette and corrected for light-scattering. Lyophilized chicken and human prolactins were dissolved in water, pH 8.0, and adjusted to 25 μM concentrations. The CD spectra were recorded by using a J-810 spectropolarimeter (JASCO) equipped with a Peltier thermostat using the supplied Spectra Manager software in a 0.1 cm quartz cuvette in the proteins storage buffer. Far-UV CD spectra were collected over 190–260 nm at 25 °C. For the secondary-structure determination, the CD data were expressed in degree $cm^2\ dmol^{-1}$ per mean residue, based on a respective molecular mass.

2.5. Binding assay

In-house-prepared biotinylated hPRL served as the ligand in the competitive binding experiment, chPRL and hPRL as competitors and rbPRLR-ECD as the receptor source. Polystyrene microtiter plates (96-well) were coated overnight at 4 °C with 100 μl of rbPRLR-ECD (0.8 μg/ml) in phosphate buffered saline (PBS) pH 7.4. Wells were then washed once with PBST (PBS containing 0.05% w/v Tween 20) and blocked with PBS containing 3% (w/v) skim milk for 2 h at room temperature. Wells were washed again with PBST and incubated with different concentrations of unlabeled chicken and human PRLs (50 μl/well, in triplicate) for 30 min, and then 50 μl of biotinylated hPRL (0.85 μg/ml) was added to each well for another 1.5 h. Then the wells were washed three times with PBST and incubated with 1:5000 streptavidin-HRP in PBST for 1 h. The plate was again washed three times with PBST, then developed using 100 μl/well 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (KPL, Zotal, Israel) diluted 1:4 and incubated for 30 min. The reaction was stopped with 50 μl of 2 N H_2SO_4 and the absorbance was read at 450 nm by ELISA Micro-Plate Reader ELx808 (Bio-Tek Instrument Inc., Winooski, VT, USA).

2.6. Biological activity in vitro in the Nb2-11C, Baf3/rbPRLR and Baf3/hPRLR heterologous bioassays

Cell line Nb2-11C expressing the short form of rPRLR was grown as a suspension culture in 75-cm^2 tissue-culture flasks (Nunc, Kamstrup, Roskilde, Denmark). For maximal growth and routine maintenance, cells were cultured in RPMI-1640 medium containing 5% (v/v) fetal calf serum (FCS) supplemented with antibiotic–antimycotic solution (10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml). The cells were incubated under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Stationary cultures were obtained by transferring the Nb2 cells into lactogen-free medium in which FCS was replaced with 5% (v/v) horse (gielded) serum. The experiment was performed in 96-well plates seeded with 2.5×10^4 cell/well.

Baf3/rbPRLR cells (stably transfected with rbPRLR) were grown as suspension cultures in 75-cm^2 tissue-culture flasks. For maximal