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Biological activity of bovine placental lactogen in 3T3-F442A preadipocytes is mediated through a somatogenic receptor

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Bovine placental lactogen (bPL) exhibited antimitogenic differentiation-promoting biological activity in 3T3-F442A preadipocytes. Competitive binding studies and affinity labelling revealed bPL activity to be mediated through a somatogenic type of receptor that recognizes human growth hormone (hGH) and bovine GH, but not ovine prolactin or human PL. The bioactivity of bPL was sixfold lower than that of hGH despite that bPL is binding to the somatogenic receptors with fivefold higher affinity. This discrepancy may result from the relatively low ability of bPL to induce post-receptoral effects such as receptor dimerization.

Placental lactogen; Somatogenic receptor; Affinity labelling; 3T3-F442A preadipocyte

1. INTRODUCTION

Placental lactogens (PLs) are polypeptide hormones produced and secreted by mammalian placentas throughout pregnancy. Human, rodent, ovine and bovine PLs have been isolated and sequenced [1]. Their structure is similar to that of related pituitary hormones, such as prolactins (PRLs) and growth hormones (GHs). Bovine PL (bPL) has been purified from term placental homogenates [2] and from isolated secretory granules obtained from binucleate cells of the fetal cotyledon [3]. The native 31–33 kDa protein has at least five isoelectric variants which are the result of heterogeneity of the attached oligosaccharides as well as other, as yet unidentified modifications. The gene for bPL has been cloned and expressed with high efficiency in *E. coli*, and the recombinant bPL was purified to homogeneity [4]. The predicted mature bPL has 200 residues and the primary sequence has 50% and 23% homology to bPRL and bGH, respectively [4]. Deglycosylation of native bPL has no effect on PRL-like mitogenic activity in the Nb_2 lymphoma cell proliferation assay and only slightly reduces its binding to liver bGH receptors [5]. It has been suggested that bPL or ovine PL (oPL) may mimic the action of either GH [6] or PRL [7], though evidence that the resultant activity is transduced through respective GH or PRL receptors was not provided. Other studies have suggested a distinct and unique PL receptor in fetal and maternal sheep livers [8,9]. We recently reported [10] this unique receptor for bPL in endometrium from mid-pregnant heifers. Thus, the question of whether the biological activity of bPL is mediated through several distinct receptors has yet to be resolved. In the present study we attempted to answer this question, using 3T3-F442A rat preadipocytes as a model. These cells possess a well-defined somatogenic receptor that recognizes not only hGH, but other GHs as well [11,12]. It has been reported that hGH is required for differentiation of these cells to adipocytes [13] and that under defined conditions, hGH exhibits antimitogenic activity [14].

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2. MATERIALS AND METHODS

2.1. *Materials*

Recombinant bPL was obtained from Monsanto Co. (St. Louis, MO, USA) prepared as described previously [15]. Recombinant bovine and human growth hormones were obtained from Biotechnology General Inc. (Rehovot, Israel), carrier-free Na[^{125}I] was purchased from the New England Nuclear Corp. (Boston, MA, USA) and a 20K hGH variant, hPL and bPRL from the National Institute of Health (Bethesda, MD, USA). Molecular weight (M_r) markers for gel electrophoresis, Fischer's, RPMI-1640 and DME media and bovine serum albumin (BSA, RIA grade) were obtained from Sigma Chemical Corp. (St. Louis, MO, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents and Protein Assay Kit were purchased from Bio-Rad Laboratories (Richmond, CA, USA) and disuccinimidyl suberate (DSS) from Pierce Chemical Co. (Rockford, MA, USA). Lactogen-free horse serum, fetal calf serum (FCS) and calf serum (CS) were obtained from Biolab Co. (Jerusalem, Israel) and Bet-Haemek Co. (Bet-Haemek, Israel). 3T3-F442A preadipocytes developed in the laboratory of Dr. H. Green [16] (Boston, MA, USA), were obtained from Dr. J. Schwartz (Ann Arbor, MI, USA). All other chemicals were of analytical grade.

2.2. *Binding assays*

3T3-F442A preadipocytes were seeded in six-well plates at 80,000–100,000 cells/well and cultured in DME medium supplemented with

10% CS for 12–14 days until full confluence was achieved. The wells were then washed with 1 ml phosphate-buffered saline (PBS) followed by 0.5 ml PBS containing 1% BSA. One ml PBS containing 1% BSA was then added and the plates were transferred to 4°C. After 30 min, the tracer ([^{125}I]hGH or [^{125}I]bPL) with or without unlabelled hormone was added and the incubation was continued for 16 h. Wells were then washed (3 × 1 ml) with ice-cold PBS, and 1 ml of 10 mM NaOH was added. Four hours later, the well contents were transferred to Eppendorf tubes and counted in a gamma counter. Iodination of hGH and bPL was carried out as described previously [17].

2.3. *Affinity labelling*

3T3-F442A preadipocytes were seeded in 15-cm diameter dishes and cultured as described above. After washing, 10 ml of cold PBS containing 1% BSA and radiolabelled bPL or hGH (~2 × 10^6 cpm, ~10 ng) was added and the cells were incubated at 4°C. For a determination of non-specific binding, 40 μg of either bPL or hGH were added. After 16 h of incubation, DSS was added to a final concentration of 0.5 mM and the incubation was continued for 25 min. The reaction was then quenched by addition of 0.1 ml 5 M Tris-HCl, pH 7.5. The dishes were washed thoroughly with cold PBS (3 × 5 ml) and cells were removed with a rubber policeman and collected to Eppendorf tubes. Following addition of 250 μl of sample buffer containing 50 mM DTT, the cells were briefly sonicated, boiled for 5 min and subjected to SDS-PAGE [18]. The gels were dried and autoradiographed at −70°C using Agfa RP2 film.

2.4. *3T3-F442A preadipocyte bioassay*

3T3-F442A preadipocytes were seeded at high confluence in 24-well plates in medium containing 10% CS. After 24 h the cells were transferred to a serum-free medium containing supplemental proteins [14] and increasing concentrations of various tested hormones (0.09–25.0 ng/ml) and cultured for five days. Following incubation, the medium was changed to 4% FCS and DNA synthesis was determined 20 h later by a 2-h pulse of 2 μCi/ml [^{3}H]thymidine.

2.5. *Nb2 lymphoma cell culture*

Culture of the Nb_2-11C lymphoma cell line, synchronization of Nb_2-11C cells in the G_0/G_1 phase and monitoring of cell proliferation were carried out as described previously [19].

3. RESULTS

3.1. *Binding studies*

Results of the competitive binding studies to intact 3T3-F442A preadipocytes indicated that binding of either [^{125}I]hGH or [^{125}I]bPL to be competed away by either of the respective unlabelled hormones (Fig. 1). Bovine GH competed with both tracers similarly, while oPRL did not compete at all. However, the affinities of the bPL, hGH and bGH were not identical. Using, respectively, the radiolabelled bPL or hGH as tracers, the corresponding IC_{50} values for bPL, hGH and bGH (in ng/tube) were: 6, 32, 18 and 1.2, 7, 15. These results were confirmed using homologous binding assays with [^{125}I]bPL or [^{125}I]hGH as tracers. Scatchard analyses revealed a single receptor population for both cases. The respective K_d values for bPL and hGH were 64 and 328 pM while the B_{max} values (in fmol/well) were 17.8 and 17.7. Although the precise cell number could not be determined for each well, an average number observed in other experiments for confluent wells was 1.34 × 10^6. Based on this, the average number of receptors/well was ~8,000, in agreement with others [16].

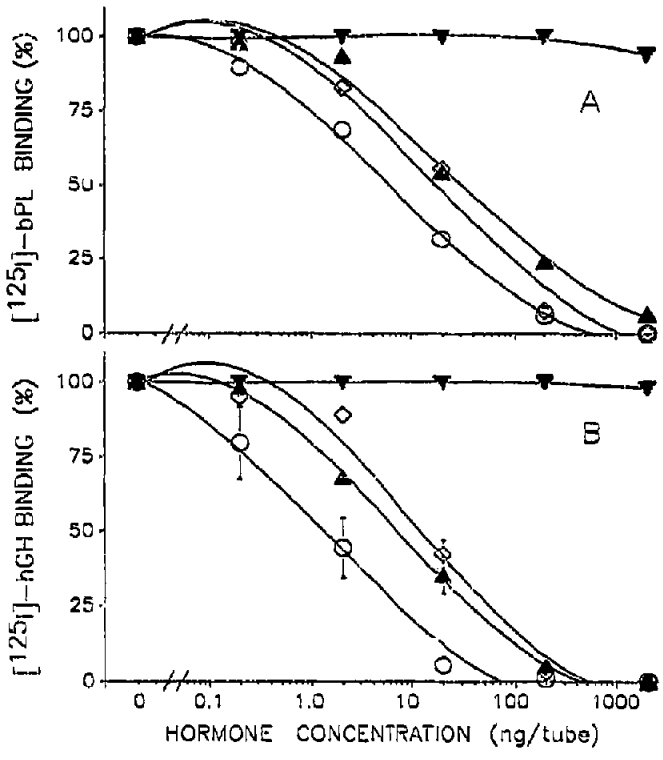

Fig. 1. Competition of unlabelled hGH (▲), bPL (○), bGH (◇) and oPRL (▼) with the binding of [^{125}I]bPL (A) and [^{125}I]hGH (B, average of two experiments). The full lines were calculated using the Sigma plot curve fitting program and the symbols represent actual results.

3.2. *Affinity labelling*

Affinity labelling of the receptors in intact 3T3-F442A cells was carried out in the presence of [^{125}I]hGH and [^{125}I]bPL. Both tracers gave similar electrophoretic profiles and labelling was prevented by both excess unlabelled hGH or bPL (Fig. 2). The main labelled band had an M_r of 130 kDa, indicating the M_r of the receptor to be ~108 kDa. Other bands having the respective M_r values of 109, 89, 83 and 43 were observed in both tracers, hinting that they may have resulted from partial proteolysis, which is independent of tracer type.

3.3. *Biological activity*

The antimitogenic activities of bPL, hGH, bGH and other related hormones determined in four experiments were compiled and averaged and are presented in Fig. 3. Since the absolute values of [^{3}H]TdR incorporation and the maximal inhibition varied in different experiments, the final results were normalized, by assuming that maximal inhibition (residual activity = 0) was achieved in the presence of 25 ng/ml of hGH. The values of treatments obtained in the absence of the assayed hormones were taken as 100. Each experimental result obtained in the presence of a different dose of each hormone was normalized and these values were then averaged and presented as mean ± S.E.M.

The IC_{50} values calculated from the results shown in

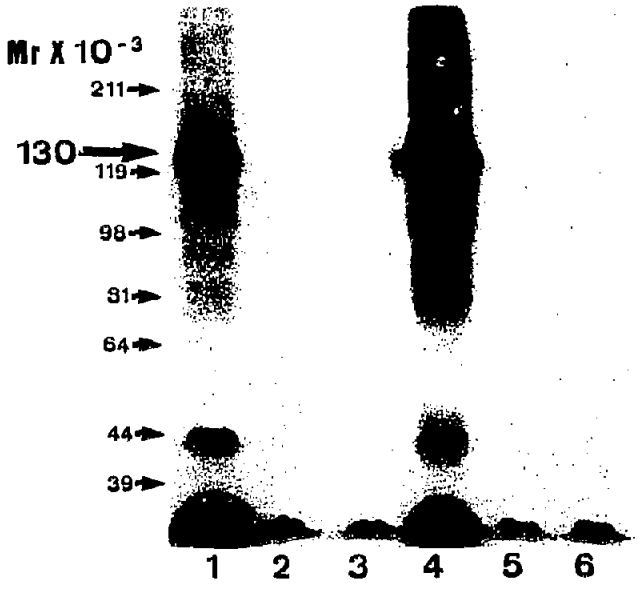

Fig. 2. Affinity labelling of lactogen receptors in intact 3T3-F442A preadipocytes. Intact cells were incubated with [^{125}I]hGH (lanes 1–3) or [^{125}I]bPL (lanes 4–6) in the absence (lanes 1 and 4) or presence (lanes 2 and 6) of 40 μg unlabelled hGH or unlabelled bPL (lanes 3 and 5). After cross-linking with DSS, the cells were washed, subjected to SDS-PAGE (7.5%) and autoradiographed.

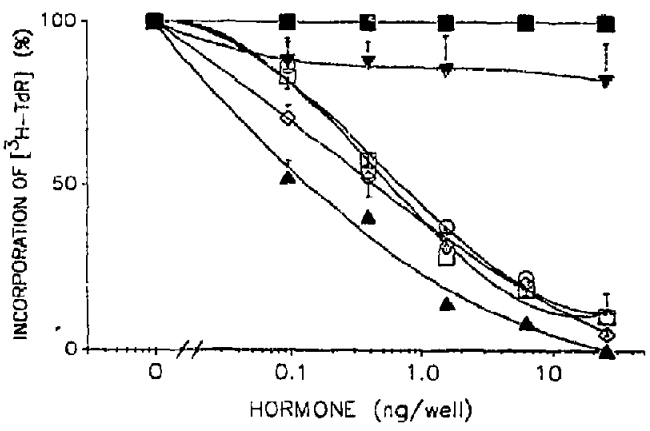

Fig. 3. The antimitogenic effect of bPL (○), hGH (▲), bGH (◇), oPRL (▼), 20K hGH (□) and hPL (■) in 3T3-F442A preadipocytes. After seeding the cells were transferred to a serum-free medium containing supplemental proteins (14) and increasing concentrations of bPL or other hormones and cultured for five days. Then the medium was changed to 4% FCS and DNA synthesis was determined 20 h later by a 2 h pulse of 2 μCi/ml [^{3}H]thymidine. Results represent the normalized mean ± S.E.M. of four experiments.

Fig. 3 indicated the strongest antimitogenic activity to be exhibited by hGH (IC_{50} = 0.13 ng/well), while bGH, bPL and the 20K variant of hGH were three- to sixfold less potent. Their respective IC_{50} values were (in ng/well): 0.33, 0.60 and 0.72. Human PL and oPRL were only slightly or not at all active.

In order to evaluate the possible reason for the differences in their bioactivities, various hormones were assayed simultaneously by the Nb_2 lymphoma cell bioassay in which the bioactivity is transmitted through a lactogenic type of receptor [19]. Human GH, bPL, oPRL and the 20K variant of hGH were equally potent, while the activity of hPL was fourfold weaker. Bovine GH was not tested since it is inactive in this bioassay [19].

4. DISCUSSION

Our results clearly suggest the biological activity of bPL in 3T3-F442A preadipocytes to be mediated by its interaction with the growth hormone (somatogenic) receptor. This conclusion is based on the following experimental findings: (a) binding experiments revealed mutual competition, (b) only a single population of receptors was detected and the overall number of binding sites for hGH and bPL per cell was almost identical, (c) the M_r of the receptor or its partially degraded fragments was identical for both tracers and matched previously reported values [11,12]. Recent in vivo experiments in which recombinant bPL stimulated the growth of rats in a manner similar to bGH [15] further substantiate our conclusion.

Surprisingly however, the biological activity of bPL in 3T3-F442A preadipocytes was sixfold lower than that of hGH, despite bPL affinity is fivefold higher for the receptors. This lower biological activity could not be attributed to improper refolding of the recombinant bPL, since in the Nb_2 lymphoma cell bioassay, its activity was equal to hGH and its CD spectrum indicated ~60% of an α-helix structure, as expected from the properly folded hormone [21]. This paradoxical finding can be explained, however, by recent reports showing that hGH has two nonsymmetrical binding sites and acts as a cross-linker to form a complex with two molecules of the hGH receptor extracellular domain [22,23]. Structural homology between bPL and hGH hints at the possibility that bPL and other related hormones, such as PRLs, act in a similar manner. In recent experiments we have found that bPL can form a 1:2 complex with the cloned extracellular part of the bovine liver GH receptor, but its affinity for the binding of the second receptor molecule is lower than that of hGH (Vasdi, Sakal and Gertler, unpublished data). We have also suggested that dimerization of the lactogen receptor, which is structurally similar to the somatogenic receptor is the first event leading to the initiation of the mitogenic signal in Nb_2 lymphoma cells [24]. Thus binding, that is less effective in inducing dimerization may lead to diminished biological activity, despite higher affinity for the respective receptor. The discrepancy in the relative biological activities of bPL and hGH in the 3T3-F442A preadipocytes and Nb_2 cell bioassay may therefore result from minor structural differences in the binding domains of the hormones. The differences in the IC_{50} values found in the bioassay and the K_ds calculated

from the binding experiments also indicate that partial occupation of the receptors is sufficient to exhibit maximal biological effect.

In summary it is evident that bPL (and probably other PLs as well) are extremely versatile hormones, capable of transducing diverse biological signals through a minimum of two subfamilies (GH and PRL) of receptors. Additional biological activities, mostly unknown to date, may be transduced through an additional, unique receptor of bPL [10,25] that does not bind GHs and PRLs. Throughout pregnancy, bPL appears not only in the fetal but also in the maternal circulation, raising the as yet unanswered question of its in vivo mode of activity.

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