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Prolactin acts on the hypothalamic–pituitary axis to modulate follicle-stimulating hormone gene expression in the female brushtail possum (*Trichosurus vulpecula*)

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ABSTRACT

Brushtail possums exhibit a distinct preovulatory pattern of prolactin (Prl) secretion suggesting that Prl is involved in normal reproductive function. In some mammals, Prl is essential for corpus luteum (CL) function and/or modulation of steroidal effects on hypothalamic–pituitary activity. The aim of this study was to test the effects of biologically active recombinant possum Prl (recPosPrl) on both pituitary gland and CL function in possums. To confirm biological activity, administration of recPosPrl-N2C1 (10 μg) resulted in an 18-fold stimulation ($P < 0.05$) of progesterone (P_4) production by possum granulosa cells *in vitro*. Based on these findings, minipumps containing either recPosPrl-N2C1 ($n = 10$) or saline ($n = 8$) were inserted into lactating female possums. The expression levels of pituitary-derived *PRL*, *LHB*, *FSHB* and *GNRHR* and CL-derived *LHR* mRNA were quantified. Following a resumption of reproductive activity, no differences in ovulation incidence or plasma Prl concentrations were observed. Plasma Prl levels were less variable ($P < 0.001$) in Prl-treated possums, confirming a self-regulatory role for Prl in this species. There was a marked down-regulation ($P < 0.001$) of *FSHB* mRNA at the mid-luteal stage in Prl-treated possums, whereas mean *PRL*, *LHB*, *GNRHR* and *LHR* mRNA expression levels were not different between experimental groups. Plasma P_4 concentrations were not different ($P = 0.05$) in Prl-treated possums, although tended to be higher in the peri-ovulatory and early-luteal phase. We conclude in the brushtail possum that Prl is self-regulated via a short-feedback loop common to all mammals studied and is able to modulate *FSHB* expression probably at the level of the hypothalamus and/or pituitary gland.

1. Introduction

The regulation of prolactin (Prl) secretion is unique amongst the anterior pituitary gland hormones. Unlike many endocrine hormones, the main regulator (dopamine) of Prl secretion acts to suppress rather than to stimulate its release [5,30,33]. The major feedback loop is Prl itself acting back at the level of hypothalamus to regulate the synthesis [43], turnover [26] and secretion [2] of dopamine into the pituitary portal blood system. The best known target tissue for Prl is the mammary gland however Prl has also been reported to play a role in multiple other biological processes such as reproduction, osmoregulation, growth and development, metabolism, brain function and behaviour, immunoregulation and protection [7,30,32,68,72].

Excessive levels of Prl in many mammalian species including humans [44,49,65] or ablation of Prl receptors in rodents [36] have been implicated in several reproductive disorders. It is well established that Prl exerts direct inhibitory effects on both the hypothalamic–pituitary axis [12,37,58,69] and on gonadotrophic action at the level of the ovary [46,52,53,55]. Whilst the effects of the direct actions of Prl on follicular development are less clear, it is well established that Prl is directly involved in the normal corpus luteum (CL) function in many mammalian species [6,29,31,36].

In newly-lactating tammar wallabies, suckling-induced increases in circulating Prl concentrations directly suppress the growth of, and steroid production by, the subsequent CL [40] halting the development of the embryo at the blastocyst stage. Reactivation of the CL of pregnancy requires a decline in circulating Prl as occurs at weaning [62]. Interestingly, the quiescent wallaby CL contains an abundance of Prl receptors which significantly decline after reactivation, whilst numbers of LH receptors remain low [66,71]. The CL of the brushtail possum however is thought to be largely autonomous, relying on little, if any, support from the pituitary gland. Nonetheless, the possum CL also exhibits high expression levels of *PRLR*, but this is exceeded by an extremely high

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affinity for LH [25,45,71]. Expression of Prl receptor in the possum is also extensive in the anterior pituitary gland (J.L. Crawford, unpublished observation) and on granulosa and thecal cells of antral follicles [45]. Therefore, it is possible that Prl may have a direct role on pituitary and/or ovarian function in the brushtail possum.

The affects of elevated levels of Prl on the incidence of ovulation, and on the function of the pituitary gland and CL in the brushtail possum is not known. Therefore the aims of this study were to (1) produce a biologically active form of recombinant possum Prl (recPosPrl) as assessed by P_4 production by possum granulosa cells *in vitro* and; (2) to determine the effects of sustained recPosPrl administration throughout an oestrous cycle on the incidence of ovulation *in vivo*, on mRNA expression levels of GnRH receptor (*GNRHR*), LHβ-subunit (*LHB*), FSHβ-subunit (*FSHB*) and Prl (*PRL*) in the pituitary gland, and of *LHR* in the CL, and on plasma P_4 concentrations in the female brushtail possum.

2. Materials and methods

All experimental procedures were approved by the AgResearch Wallaceville Animal Ethics Committee in accordance with the Animal Welfare Act of NZ, 1999.

2.1. Animals and management

All possums were live-captured in the Wellington region (latitude 41°S) of New Zealand. At the time of capture, each animal was screened for health status and only healthy animals were included in this study. Animals were housed in the AgResearch Wallaceville possum facility under a group housing system [51]. A mixed diet of fresh fruit and vegetables, bread and cereal-based pellets was provided along with selective browsing of *Pinus radiata* branches. Fresh water was always available. At the end of each experiment, possums were euthanased by asphyxiation using CO_2.

2.2. Production of recombinant possum Prl

The methods for amplification of possum Prl by PCR and the sub-cloning of confirmed possum Prl DNA fragments into either pET23a or pET23b vectors (Novagen, Madison, WI, USA) have been described previously [17]. The latter vector adds a 6-Histidine tag (*his*-tag) to the C-terminus of the expressed protein to facilitate purification.

Using a modified version of a previously described method [11], single colonies of BL21 (DE3) pLysS bacteria transformed with either pET23a/posPrl(N2C1) or pET23b/posPrl(H1B1) were cultured in 25 mL Luria broth containing ampicillin (200 μg/mL) overnight at 37 °C. Then, an aliquot of culture (20 mL) was diluted 25 times in the same medium and incubated at 37 °C with agitation until the OD_{600nm} reached 0.5. Recombinant protein expression was induced by incubating for 3 h with isopropyl-β-D-thiogalactoside (final concentration 0.4 mM) and cells were pelleted by centrifugation at 3000*g* for 10 min. The pellet was then re-suspended in 15 mL of ice-cold wash buffer (50 mM Tris–HCl, pH 7.5) and stored overnight at −20 °C prior to protein purification.

The cells were thawed, and after addition of enzyme inhibitors (Complete™ Protease Inhibitor Cocktail tablet; Roche, Roche Products NZ Ltd., Auckland, NZ), were lysed by sonication (four 30 s cycles). Inclusion bodies were collected by centrifugation at 10,000*g* for 10 min, washed (wash buffer and centrifugation at 10,000*g* for 10 min), solubilised in buffer (8 M urea, 300 mM NaCl, 0.1 M $NaPO_4$, 0.01 M Tris, 0.1% Tween 20, 1% BME; pH 8.0) by passage through an 18 g needle and incubated for 1 h at room temperature on a shaker. Any remaining precipitated protein was separated by centrifugation at 10,000*g* for 10 min.

The supernatant containing recPosPrl without a *his*-tag (N2C1) was dialysed at 4 °C in 12,000–14,000 MWCO dialysis tubing (SERVA Electrophoresis, NSW, Australia) against 6 M urea, 50 mM Tris–HCl (pH 8.5) for 24 h with two changes of buffer. The protein was then concentrated to a suitable column-loading volume using a stirred-cell concentrator (Amicon, Inc., MA, USA) and MWCO 10,000 Da membrane (Millipore; Millipore Australia Pty., Ltd., NSW, Australia) under nitrogen gas. Protein was measured using a BCA Protein Assay Reagent Kit (Pierce Chemical Company, Rockford, IL, USA). All buffers and samples were filtered through a 0.45 μm filter (Millipore) prior to applying to a Sephadex G100 column previously equilibrated with 6 M urea, 50 mM Tris–HCl, pH 8.5. The column was run at 0.15 mL/min and 1.4 mL fractions were collected. The supernatant containing the recombinant Prl with a *his*-tag (H1B1) was purified on a Nickel Agarose column (QIAGEN, Auckland, NZ) following the manufacturer's recommendations.

For each batch (N2C1 and H1B1), fractions containing purified recPosPrl were pooled and dialysed in 12,000–14,000 MWCO dialysis tubing (SERVA Electrophoresis) against 50 mM NH_4HCO_3 at 4 °C for 3 days with six changes of buffer. The re-folded protein solution was re-salted 0.15 M with NaCl prior to removal of *Escherichia coli* endotoxins on a Detoxi-Gel (Pierce Chemical Company, Rockford, IL, USA) in accordance with the manufacturer's instructions. Fractions containing recPosPrl were pooled and loaded on a SDS–PAGE gel under non-reducing conditions to confirm refolding (decreased molecular weight compared to the original material in 6 M urea solution). The bioactivity of each of the batches of recPosPrl (N2C1 and H1B1) was subsequently tested in primary cell cultures of possum granulosa cells.

2.3. Experimental design

2.3.1. Assessment of bioactivity

To test the bioactivity of recPosPrl-N2C1 and -H1B1, their effects were compared to that of ovine Prl (oPrl) and oLH (positive control), on progesterone (P_4) production by possum granulosa cells *in vitro* as previously described [17]. In brief, granulosa cells were isolated from ≥0.3 mm diameter follicles immediately following euthanasia and recovery of the ovaries. Cells were then washed, checked for viability using a trypan blue exclusion test (ranged from 60% to 80%), seeded in 24-well plates at a density of 1.5×10^5 cells/well, and thereafter cultured at 37 °C in an atmosphere of 5% CO_2 in humidified air. After 48 h, 0.5 mL of culture media was removed from each well so that results could be normalised for pre-treatment P_4 production. Fresh culture media (0.5 mL) was added alone (negative control) or supplemented with 10 μg of either batch of recPosPrl (H1B1 and N2C1), 10 μg of oPrl or 1 ng of oLH. After 24–48 h, media were collected from all wells for measurement of post-treatment P_4 production by RIA.

2.3.2. In vivo experiments

Eighteen lactating adult female possums with pouch young were divided into two groups. All animals underwent surgery whereby indwelling catheters were placed in a jugular vein [21]. Osmotic minipumps (model 2ML4, 2.5 μL/h for 28 days; Alzet, Alza Corporation, Palo Alto, CA, USA) were loaded with either saline (control; $n = 8$) or 2.94 mg of recPosPrl-N2C1 ($n = 10$) and inserted ip under general anaesthesia (*Zoletil 100*; Virbac, Techvet Laboratories, Auckland, NZ) using aseptic techniques. On the day of surgery, pouch young were removed (RPY) to induce cyclical oestrous activity in the adult females. Oestrous cyclicity was monitored in each possum by analysing the cytology of urine samples collected on a daily basis. The day of influx of epithelial cells was estimated to be the day of oestrus [21,24]. Blood samples were collected via the jugular catheter every 1–2 days from Day 0 after RPY, until 10 days after oestrus or mating (influx of epithelial cells and/or