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Original Research

Immunohistochemical Localization of Prolactin Receptors Within the Equine Ovary

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ABSTRACT

Prolactin (PRL) is hypothesized to stimulate follicular growth through a physiological action at the ovary. Immunohistochemistry was used to identify PRL receptors (PRLrs) within the equine ovary. Prolactin receptors were detected in anestrous (n = 3), winter cycling (n = 2), summer follicular (n = 3), and luteal phase (n = 3) ovaries. Prolactin receptors were detected in follicles of all stages, in corpus luteum and on oocytes. Staining intensity did not differ ($P > .05$) between primordial and preantral follicles but was greater ($P < .001$) in antral follicles than in primordial or preantral follicles. Greater PRLr staining ($P < .001$) occurred in winter cycling primordial follicles (1.58 ± 0.09) than anestrous (0.67 ± 0.10) and summer luteal phase primordial follicles (1.16 ± 0.12; $P < .01$) but not in primordial follicles during the summer follicular phase ($P > .05$). Prolactin receptor staining in preantral follicles during anestrus was lower ($P < .05$) than for all other reproductive states. Winter cycling and summer luteal phase preantral follicles stained most intensely, and both had greater PRLr staining than preantral follicles from anestrous ovaries ($P < .001$) or summer follicular phase ovaries ($P < .001$). Prolactin receptor staining of antral follicles was the most intense of all follicular sizes and did not vary ($P > .10$) between reproductive states. Prolactin receptor staining was also detected in luteal tissue. In conclusion, PRLrs were detected in all stages of follicular growth with staining intensity highest in large antral follicles, indicating a possible mechanistic role for PRL during late stage follicular growth and perhaps ovulation.

1. Introduction

One of the first reports of prolactin (PRL) action in ovaries demonstrated binding of PRL to granulosa and theca cells of porcine ovaries [1]. Moreover, PRL receptors (PRLrs) have been observed in ovarian follicular cells of mice [2], pigs [3], sheep [4], deer [5], and humans [6], as well as in luteal tissue in cow [7] and horse [8] ovaries. Furthermore, PRLr knockout mice exhibited failure of embryonic implantation, reduced number of mature oocytes, and low fertilization rates [9]. Knockout females also displayed a reduced number of primary follicles [9].

Prolactin exerts its action on target tissues by binding to transmembrane receptors. Three different isoforms of the PRLrs, long, intermediate, and short, have been identified and differ based on the length of the portion extending into the cytoplasm [10]. Clark et al [11] detected both short and long PRLr messenger RNA (mRNA) in both ovarian follicular cells and luteal cells of rat ovaries. A lesser quantity of short PRLr mRNA was present throughout the estrous cycle compared with long PRLr mRNA. Short PRLr mRNA was more abundant in cumulus cells surrounding the oocyte, whereas long PRLr mRNA was more abundant in granulosa cells of preantral follicles [11].

Exogenous PRL administered to seasonally anovulatory mares was reported to stimulate early follicular growth and hasten the date to first ovulation [12,13]. Additionally, an increase in circulating PRL, achieved through the use of

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dopamine antagonists, has also been shown to enhance follicular activity during transition with varying results during anestrus [14–16]. The presumptive mechanism of PRL action is direct stimulation of follicular tissue, most likely through its membrane-bound receptor.

The aim of this study was to determine if PRLrs were located on equine ovarian follicle cells and if PRLr content differed between different stages of follicular growth as well as between different reproductive states of the ovary.

2. Materials and Methods

2.1. Animals

A single anestrous season ovary or winter cycling ovary from each of five light horse mares of unknown age or reproductive history was collected at an abattoir in February. Anestrus was defined as the presence of small ovaries lacking luteal structures and with follicles less than 20 mm in diameter. Winter cycling was defined as ovaries obtained during February possessing luteal structures. In June and July, ovaries from six estrous cycling mares were collected. Ovaries were bisected longitudinally at the midline through the ovulation fossa. One half of each ovary was immersed in approximately five volumes of 10% buffered formalin solution for transport and storage. In preparation for immunohistochemistry (IHC), ovarian halves were further bisected longitudinally two or three times, creating three or four sections, respectively. Ovarian sections were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffinized blocks were cut at 5 μm on a rotary microtome and mounted on positively charged amino silane slides where they were allowed to dry overnight at 23°C. Three anestrous, three summer follicular phase, three summer luteal phase, and two winter cycling ovaries were used for IHC ($n = 11$). A follicular phase ovary was defined as an ovary bearing follicles greater than 25 mm in the absence of active luteal tissue. A luteal phase ovary was defined as an ovary bearing a corpus luteum (CL).

Mammary tissue from a lactating mare was used as positive control (courtesy of Janet F. Roser, PhD, University of California, Davis).

2.2. Immunohistochemistry

To carry out IHC, mounted sections were deparaffinized (5 minutes, 60°C–80°C) and transferred to two changes of clearing solvent (CitraClear; StatLab, Lewisville, TX) for 2 minutes each to remove any remaining paraffin. Tissue was subsequently transferred to three changes of 100% ethanol (EtOH) for 2 minutes each, then two changes of 95% EtOH for 2 minutes each, and then distilled water for 2 minutes. To retrieve antigens, tissue was steamed in Trizma buffer (pH 10.0) using a rice cooker at 95°C for 20 minutes and allowed to cool for 20 minutes (Trizma base, #T1503 and Trizma HCl, #T3253; Sigma Chemical, St. Louis, MO). Slides were then rinsed in distilled water for 5 minutes. To block for endogenous peroxidase, slides were immersed in 0.3% H_2O_2 and methanol for 30 minutes at 23°C and then rinsed in distilled water for 5 minutes. Tissue was additionally rinsed in phosphate-buffered saline (PBS) for 5 minutes. To decrease nonspecific staining, tissue was incubated in normal goat serum for 30 minutes at 23°C and then lightly shaken to remove the serum. Tissue was incubated with an anti-PRLr monoclonal mouse antibody at 1:500 (U5, #MA1-610; Thermo Fisher Scientific, Rockford, IL) for 1 hour at 23°C and then for overnight at 4°C. Primary antibody was omitted from negative control tissue. This tissue was instead incubated in normal goat serum. All the tissue was rinsed three times in PBS for 5 minutes each and then incubated in biotinylated goat anti-mouse IgG (BA-9200; Vector Laboratories, Burlingame, CA) at 23°C for 1 hour. Tissue was rinsed again three times in PBS for 5 minutes each and then incubated in avidin–biotin complex (Vectastain ABC Kit; Vector Laboratories) at 23°C for 1 hour. Tissue was rinsed one time in PBS for 5 minutes and developed using 3,3-diaminobenzadine (Kit A; Vector Laboratories) for 15 minutes and then rinsed once in distilled water. Prepared tissue was counterstained with hematoxylin for 2 minutes and rinsed again in distilled water. Slides were quickly dipped three times in acid alcohol and immediately rinsed in distilled water. Slides were dipped in lithium carbonate for 1 minute and rinsed in distilled water for 1 minute. Tissue was transferred to three changes of 100% isopropyl alcohol for 2 minutes each and coverslip mounted using a mounting medium (Clearium; Leica Microsystems, Richmond, IL).

2.3. Evaluation and Statistical Analysis

A minimum of 10 follicles from each follicle stage, or every structure in that follicle stage found on the ovary in cases where fewer than 10 were present, were assessed from each ovary. Although the location of the follicles within the ovary was noted, structures were not selected based on location. Table 1 summarizes the number of structures analyzed in follicle stage by ovary classification.

Slides were visualized by a single observer using an Olympus light microscope at ×200. Primordial, preantral, and antral follicles were counted and graded based on PRLr

Table 1
Mean staining ± standard error of the mean for prolactin receptors in follicles of different stages and within each reproductive state of the ovary.

Stage of Follicle Growth	Reproductive State of Ovary			
	Anestrus	Winter Cycling	Follicular Phase	Luteal Phase
Primordial	0.67 ± 0.10^{a} (43)	1.58 ± 0.09^{c} (53)	$0.69 \pm 0.16^{a,b}$ (16)	1.16 ± 0.12^{b} (31)
Preantral	0.45 ± 0.10^{a} (47)	1.59 ± 0.14^{c} (22)	1.00 ± 0.10^{b} (43)	1.51 ± 0.09^{c} (49)
Antral	1.00 ± 0.46^{a} (2)	1.80 ± 0.29^{a} (5)	2.10 ± 0.21^{a} (10)	2.00 ± 0.16^{a} (18)

The values within parentheses are the number of follicles in each developmental category analyzed for prolactin receptor staining. Different superscript letters next to the mean indicate differences within the stage of follicle growth between different reproductive states of the ovary.