Theriogenology 93 (2017) 71–77

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Analysis of LH receptor in canine ovarian follicles throughout the estrous cycle

Monica De los Reyes [a, *], Jaime Palomino [a], Victor H. Parraguez [b], Fernando Ramirez [a]

[a] Laboratory of Animal Reproduction, Faculty of Veterinary Sciences, University of Chile, Casilla 2 Correo 15, Santiago, Chile
[b] Laboratory of Animal Physiology, Faculty of Veterinary Sciences, University of Chile, Casilla 2 Correo 15, Santiago, Chile

ARTICLE INFO

Article history:
Received 31 July 2016
Received in revised form
15 January 2017
Accepted 15 January 2017
Available online 18 January 2017

Keywords:
Luteinizing hormone
Receptor
Dogs
Follicular growth

ABSTRACT

The aim of this study was to determine the mRNA LHR and LHR protein expression pattern in the canine ovarian follicles at different stage of development throughout the estrous cycle. Dog ovaries were obtained from 1-6y bitches at proestrus/estrus, anestrus and diestrus stages following ovariohysterectomy. Follicular cells were mechanically recovered from follicles distributed into four types (preantral, small antral, medium antral and large antral). Total RNA extraction was performed and the evaluation of gene expression levels was achieved by relative quantification q-PCR analysis. Intrafollicular amounts of LHR were assessed by western blot method. All results were evaluated by ANOVA. The expression levels of mRNA LHR in follicular cells were observed in every stage of development, however this gene expression varied over the estrous cycle. LHR transcripts increased ($P < 0.05$) from preantral to antral stage. There were not differences in LHR gene expression among follicles at preantral stages; however, at antral stages the lowest ($P < 0.05$) LHR mRNA expression was found at anestrus and the highest ($P < 0.05$) at proestrus/estrus. The LHR protein was also detected in dog follicles in all reproductive phases with patterns varying with stage of follicular development over the reproductive cycle. The antibody against human LHR revealed two bands at ~90 and ~67 kDa, probably representing the matured protein and its precursor respectively. Both bands LHR appeared already at preantral follicles increasing ($P < 0.05$) with growth. A high proportion of LHR was presented as immature forms in all follicles stages during different phases of the estrous cycle. In conclusion, the gene and protein of LHR are differentially expressed in dog follicles over the estrous cycle, increasing with growth and the precursor protein is the most predominant LHR form present in canine follicles.

© 2017 Elsevier Inc. All rights reserved.

1. Introduction

Luteinizing hormone (LH) plays a major role in reproductive processes. This pituitary gonadotropin regulates follicle/oocyte maturation, ovulation, as well as optimal fertilization and corpus luteum (CL) development [1]. In the ovary, granulosa, theca, and luteal cells are capable of secreting steroid hormones, such as estrogens, testosterone and progesterone in response to LH [2,3]. The type and amount of hormone released vary according to the reproductive-physiological status of the follicle and CL [4,5].

This gonadotropin exerts its effects through binding to its receptor (LHR), which is one of the seven transmembrane domain G-protein-coupled receptor (GPCR) superfamily [6]. It has been demonstrated in many species that at the ovarian tissue LHR is mainly expressed in theca cells, but it is also present in granulosa cells of preovulatory follicles [7,8]. The interaction LH-LHR activates adenylate cyclase, phospholipase C and ion channels, which in turn control cellular cyclic AMP, inositol phosphates, Ca^{2+} and other secondary messengers [9,10]. Through this pathway, the final differentiation of the granulosa cells and the enzymes responsible for androgen production in the theca cells are enhanced [11,12]. However, alternative second messenger pathways, which may not include stimulation of cAMP levels also exists [13,14]; but, none seems to be as effective as cAMP at mediating the induction of steroids synthesis [15].

In several species the LHR expression levels increase with follicle growth in response to follicle stimulating hormone (FSH), estradiol and paracrine factors, reaching maximum levels prior to ovulation [16]. At this time, granulosa cells also express sufficient receptors for LH. Therefore, the activity of LH increases, supporting the

* Corresponding author.
E-mail address: mdlreyes@uchile.cl (M. De los Reyes).

http://dx.doi.org/10.1016/j.theriogenology.2017.01.029
0093-691X/© 2017 Elsevier Inc. All rights reserved.

growth and maturation of larger ovarian follicles and the oocyte as a result of specific LH granulosa cell receptors [17,18]. In dogs, the estrous cycle is longer than that of other mammals, with a non-seasonal anestrus of about 3–12 months [3]. The LH pulses appear to occur in all stages of the estrous cycle, including anestrus [4]. In fact, termination of the anestrus involves selection of LH-sensitive follicles, and this final follicles selection is caused by an increased frequency of high-amplitude LH pulses at the end of anestrus [19], since the responsiveness to gonadotropins increase circulating basal LH concentrations [20]. This period of increased LH pulsatility has been reported as an important determinant in the start of a new follicular phase [3,21].

It has even been suggested that changes in LH secretion may be more important than changes in FSH secretion in the initiation of a follicular phase leading to ovulation [21]. However, the endocrine mechanisms by which a cohort of follicles is recruited in anestrus for further development in proestrus is unclear, as it is not known when this sensitivity to LH begins in those follicles. Expression of both LHR gene and LHR protein may be involved in the process of recruitment and selection of anestrous follicles, but unlike the evidences in rodents and other mammals, the canine LHR expression pattern is poorly understood.

Luteinization process begins in preovulatory follicles prior to ovulation in the bitch [22]; therefore, canine follicular cells may increase receptors for LH before the end of proestrus. An early acquisition of LH receptors on granulosa cells has been reported in dogs around the period of LH surge, by measuring the binding of radioactive labeled porcine gonadotrophins by autoradiography [23]. However, there are not available data regarding the precise follicular stage at which LHR gene and LHR protein start to become expressed. Therefore, the objective of this study was to analyze the messenger LHR gene expression and LHR protein on canine follicles cells from preantral to preovulatory follicles throughout the estrous cycle.

2. Material and methods

All animals used in the present study were treated according the protocols approved by the Chilean Bioethics Committee of the National Foundation for Scientific and Technological Research (FONDECYT).

2.1. Ovaries and follicles collection

Canine ovaries were collected from clinically healthy bitches of different breeds aged from 1 to 6 years at anestrus (n = 64), proestrus/estrus (n = 42) and diestrus (n = 43) stages after neutering. Immediately after surgery, the stage of estrous cycle was assessed according the ovarian structures (type of growing follicles and CL) [24] and also by measurements of progesterone obtained from blood samples on the day of surgery, according previous reports [25]. Blood samples (2 mL) were collected without anticoagulant, centrifuged at 3000 rpm for 10 min (Eppendorf Centrifuge 5415 D, Hamburg, Germany) and the plasma was stored at −20 °C. Plasma progesterone concentration was assessed by enzyme-linked immunosorbent assay (ELISA) [26], (PHomo Microplate Reader®, Autobio Labtec Instruments, Zhenghaidong, China) using a progesterone canine kit (Prog ELISA Kit, MyBioSource®, San Diego, CA, USA) [25]. Sensitivity of the assay was 0.33 ng/mL. The mean intra- and inter assay precision was 7.2% and 8%, respectively. Dilution curves of samples were proved parallel to the standard curve.

Only the ovaries with no visual abnormalities were used for experiments. The ovaries were washed in phosphate buffer solution (PBS) (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.47 mM; KH_2PO_4; pH 7.4). The growing follicles were observed by stereomicroscope (Leider MZ-730-J6 American Scientific, Portland, OR, USA), the preantral and antral follicles were classified as: a) preantral corresponding to follicles which contain oocytes surrounded by granulosa cells and an intact basal membrane without an antral cavity within the granulosa, and, antral follicles at different sizes of: b) Small antral ~0.2–0.39 mm; c) medium antral ~0.4–5.9 mm and d) large antral or preovulatory ~6–10 mm [25]. Selected follicles were trimmed off the surrounding connective tissue and follicular cells from each follicle were retrieved manually using 1 mL-gauge needle (Nipro Corporation, Miami, FL USA) after puncture for release of intra-follicular contents. The method we used for the isolation of the granulosa and theca cells was used in previous studies [24,25].

The follicular cells were pooled in each essay, classified according the estrous cycle phase (anestrus, proestrus/estrus and diestrus) and follicle type (preantral, small antral, medium antral or large antral). The diameter of each individual follicle was measured using a graticule in the eyepiece of the stereomicroscope before releasing the follicular cells, excluding the oocyte. Follicles with signs of degeneration, such as debris in their isolated cells or non healthy-looking oocyte, were excluded for the experiments.

After isolation, follicles for further q-PCR analysis were transferred to RNAlater® (Ambion™Invitrogen™ Eugene, OR, USA) and subsequently stored at −20 °C until total RNA isolation. For Western Blot analysis, samples were put in PBS medium and then stored at −80 °C. For each bitch, the number and classification of estrous phase was recorded prior to pooling.

2.2. RNA extraction, reverse transcription, and quantitative real-time PCR

Evaluation of gene expression levels was achieved by relative quantification RT-PCR analysis. Follicular samples were thawed at room temperature (22 °C) for about 5 min and then homogenized in each vial. Total RNA was extracted from follicles cells by affinity columns using the Gene JET™ RNA Purification Kit (Thermo Scientific, Eugene, OR, USA), according to the manufacturer's instructions. Due to the fragility of the RNA, the extraction was performed in a laminar flow chamber under ribonuclease-free conditions using refrigerated racks (0–5 °C). After extraction, RNA was dissolved in RNase-free media and the concentration of the total RNA was determined by fluorometric measurements in a Qubit® 2.0 Fluorometer (Invitrogen™ Eugene, OR, USA), using the quantification kit Qubit® RNA Assay (Molecular Probes® Invitrogen™).

RNA samples were stored at −80 °C until use. Reverse transcription (RT) was performed using the enzyme conjugate SuperScript II™ First-Strand Synthesis System (Invitrogen™, Eugene, OR, USA) after a DNAse treatment. The complementary DNA (cDNA) concentration was also determined by fluorometry with the Kit ssDNA Qubit® Assay (Molecular Probes®, Invitrogen™, Eugene, OR, USA). cDNA samples were stored at −20 °C.

PCR reactions were performed using the Maxima SYBR Green/ROX qPCR Master mix Kit (Thermo Fisher Scientific ™, Waltham, MA, USA), according to the manufacturer's instructions. Amplification was performed using the Two Steps real-time Eco ™ PCR system (Ilumina®, San Diego, CA, USA). All samples were run in duplicates using 10 ng of complementary DNA (cDNA) in an 18-mL total reaction volume.

Control samples without reverse transcriptase and without the template, were included in each plate. The following thermal profile was used: 1 cycle at 95 °C for 10 min for initial denaturation and 40 cycles at 95 °C for 15 s (denaturation), and 60 °C for 60 s (annealing) and 72 °C for 30 s (extension).

All samples were run in duplicates; the β-actin RNA and Histone

Download English Version:

https://daneshyari.com/en/article/5523507

Download Persian Version:

https://daneshyari.com/article/5523507

Daneshyari.com