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Prolactin is a potential physiological modulator of swine ovarian follicle function

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ABSTRACT

Apart from the well established role of prolactin (PRL) in the control of mammary development and lactation, this hormone appears to possess a variety of physiological functions and evidence exists about its expression in many extra-pituitary sites.

This experimental work was undertaken to gain knowledge about PRL and its receptor presence in the porcine antral follicle. In particular, we investigated the expression and local production of PRL in follicular fluid, theca and granulosa cells cultured in standard conditions and in hypoxia. Then, we also investigated its modulatory effect on several parameters mainly involved in granulosa cell function, namely redox status and steroidogenesis. In order to verify an involvement of PRL in the control of ovarian angiogenesis, a process strictly linked to follicle growth and development, we have verified possible PRL effects on granulosa cell production of Vascular Endothelial Growth Factor (VEGF) and nitric oxide as well as its modulatory role on the angiogenic activity of endothelial cells.

Our data demonstrate that in the swine PRL is expressed in both components of the antral follicle, theca and granulosa layers, and it is produced by granulosa cells. Moreover, the hormone represents a relevant modulatory factor on key processes underlying follicular growth and development, such as steroidogenesis and angiogenesis.

1. Introduction

The first and well established role of prolactin (PRL) is the control of mammary development and lactation in mammals. Apart from these effects, a wide variety of physiological functions has been recently suggested for PRL in different species [1]; moreover, evidence exists about its gene expression in many extra-pituitary sites [2] as well as its presence in many body fluids [3–5]. As regard to the ovary, PRL gene is expressed in the swine [6], bovine [7] and human species [8], but the biological relevance of this local production is still the subject of intense investigation.

The presence of both types of PRL receptors, the short and long isoforms, within the rat ovary [9] would suggest an involvement of this hormone in ovarian physiology. Normal corpus luteum function appears dependent on PRL at least in some species and in specific phases of the estrous cycle. PRL receptors also appear to mediate granulosa cell physiology and oocyte development; in particular, Porter et al. [10] have shown that PRL inhibits steroidogenesis in porcine granulosa cells and modulates FSH and LH receptor expression. However, the mechanisms of PRL action on ovarian function have not been clarified yet and need to be further investigated. On these bases, we have undertaken this experimental work to gain knowledge about PRL and its receptor in the swine antral follicle. In particular, the physiological presence, expression and local production of PRL were investigated in different components of ovarian follicle, such as follicular fluid, theca and granulosa cells cultured in standard conditions and in hypoxia. Moreover, we have also investigated about its modulatory role on several parameters mainly involved in granulosa cell function [11–13], in particular by studying possible PRL effects on the redox status as well as on the production of steroids, nitric oxide (NO) and Vascular Endothelial Growth Factor (VEGF). In addition, we also wanted to verify an involvement of PRL in the regulation of the angiogenic activity of endothelial cells, since different studies [14,15] suggest that this hormone plays a role in the control of angiogenesis, a process that in the ovary has been demonstrated to be strictly linked to follicle growth and development [16,17].

2. Materials and methods

All reagents used in this study were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.1. PRL in the swine antral follicle

2.1.1. Collection of ovaries

Swine ovaries were collected at a local slaughterhouse from 20 Large White cross-bred gilts, parity = 0. The stages of the estrous cycle were

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unknown. Ovaries were placed into cold PBS (4 °C) supplemented with penicillin (500 IU/ml), streptomycin (500 μg/ml) and amphotericin B (3.75 μg/ml), maintained in a freezer bag and transported to the laboratory within 1 h.

2.1.2. Follicles

Follicles were classified as healthy or atretic on the basis of morphological criteria and those with hemorrhagic, opaque or "milky" follicular fluid were excluded [18]. Healthy follicles were dissected from the ovaries and classified on the basis of their dimension into small (<3 mm), medium (3–5 mm) and large (>5 mm) according to Basini et al. [19]. Each follicle was cut open, oocytes were recovered and the remaining cumulus cells were removed by repeated pipetting with fine bore glass Pasteur pipettes with different diameters.

2.1.3. PRL quantification

2.1.3.1. Follicular fluid collection. Follicular fluids were collected by aspiration from the three follicle groups as described above. The fluids were pooled together during aspiration, obtaining on average 15–20 ml. Thereafter, pooled fluids were centrifuged at 300 ×g for 10 min to separate fluid component from the cellular ones. Supernatants were then frozen and stored at −20 °C until the assay.

2.1.3.2. Granulosa cell collection and culture. Granulosa cells were aseptically harvested by aspiration of large follicles (>5 mm) with a 26-gauge needle and released in medium containing heparin (50 IU/ml). During granulosa cell collection, a gentle scraping of the follicle wall was performed with the needle in order to collect also mural cells. Cells were then centrifuged for pelleting and treated with 0.9% prewarmed ammonium chloride at 37 °C for 1 min to remove red blood cells. Cell number and viability were estimated using a hemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%) of an aliquot of the cell suspension. Cells (10^6/ml) were seeded in 24-well plates in culture medium (CM) M199 supplemented with sodium bicarbonate (2.2 mg/ml), bovine serum albumin (BSA 0.1%), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), selenium (5 ng/ml) and transferrin (5 μg/ml). Cells were incubated at 37 °C under humidified atmosphere (5% CO_2, 19% O_2) for 24 h and then subjected for 18 h to normoxic (19% O_2), partial (5% O_2) or total hypoxic (1% O_2) conditions. Total and partial hypoxia were achieved employing Anaerocult® A mini and Anaerocult® C mini (Merck KgaA, Darmstadt, Germany), respectively. In both cases, the system consisted of plastic pouches and a paper gas generating sachet.

2.1.3.3. PRL assay. Swine PRL was measured in follicular fluids and granulosa cell culture media by a validated ELISA [20].

Briefly, a caprine secondary antibody (1:500) was absorbed to the wells and incubated with bovine serum albumin (BSA) in order to saturate all not specific binding sites. Thereafter, the sample and the primary anti-pPRL antibody (1:50,000; AFP5672099 provided by Dr. A.F. Parlow, National Hormone and Pituitary Program, Harbor-University of California-Los Angeles Medical Center, La Jolla, CA) are added. After incubation, the plate was decanted and previously biotinilated PRL was added to each well (20 ng/well), so that antigen binds to all free antibody binding sites. After addition of streptavidin conjugated to peroxidase and ABTS cromogen, absorbance was determined using the Victor reader (Multilabel Counter Victor, Perkin Elmer, Boston, USA) at a wavelength of 405 nm.

2.1.4. PRL and PRL receptor (PRL-R) expression

2.1.4.1. Isolation of theca and granulosa cells. Granulosa cells were separated from the follicles using a siliconized Pasteur pipette, centrifuged at 300 ×g for 10 min; at the end, total RNA was extracted. Theca tissue was peeled from each follicle using fine forceps, frozen in liquid nitrogen and powdered in a mortar.

2.1.4.2. RNA extraction and semiquantitative RT-PCR. Total RNA was extracted from oocytes, granulosa and theca preparation using Nucleospin ® RNA II (Macherey-Nagel Gmbh, Duren, Germany) according to the manufacturer's instructions. Total RNA was quantified by absorbance at 260 nm (Gen Quant Pro, Amersham Biosciences, Freiburg, Germany). Total RNA (2 μg) was reverse transcribed with High-Capacity cDNA Reverse Transcription kit (Applied Biosystem INC, Foster City, CA, USA).

Semiquantitative analysis of PRL expression was performed by nested PCR on 5 μl of cDNA. Two sequential runs of PCR were performed with two different sets of primers complementary to swine PRL cDNA. The primers used for the second run were designed to amplify a sequence target within the first run product. For the first amplification run, the following primer pair was used: forward 5′ ACAGGGTCGTCA CAGAAAGG 3′; reverse 5′ TCCTGCATACCCCTCACTTC (amplicon length: 401 bp). After a first 2 min denaturation step at 95 °C, the following PCR cycle was repeated for 40 times: 95 °C for 30 s, then 55 °C for 30 s and 72 °C for 30 s. A final elongation step was performed at 72 °C for 10 min. For the second PCR run, the following nested primers were used: forward 5′-CCTACTGCTGCTGGTGTCAA-3′, and reverse 5′-TTGG GCTTGCTCTTTGTCTT-3′ (amplicon length: 268 bp). The amplification was performed at the same conditions (cycle number, temperature and incubation) as described above.

In addition, a PCR was performed on cDNA in order to detect the presence of the transcript of the PRL receptor (PRL-R). The primers used were: forward 5′-ATCTGGTTGGCTCACACTCC-3′ and reverse 5′-CACGAAGATCCACATGGTTG-3′ with a resulting amplicon product of 244 bp. Reaction has been performed for 35 cycles. After a first 2 min incubation at 95 °C, we performed a denaturation step at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s. Reactions were terminated with a final elongation at 72 °C for 10 min. The PCR products were separated on 1.5% agarose gel stained with Gel Red dye (Biotium, Hayward, CA). Digital images were captured by Power Shot A610 camera (Canon, Tokyo, Japan).

Variability in mRNA amounts was assessed by amplifying swine actin using as an internal standard the primers pACT sense (5′-GAG ACC TTC AAC ACG CCG-3′) and pACT antisense (5′-GGA AGG TGG ACA GCG AGG-3′) (MWG Biotec, Ebersberg, Germany), with a resulting product of 685 bp.

2.1.5. Effects of PRL on swine granulosa cells

Granulosa cells were collected as previously described and seeded at different densities according to the different experimental protocols. After seeding, granulosa cells were treated with recombinant swine PRL (0.1, 1 and 10 nM) kindly provided by Prof. Farmer, Agriculture and Agri-Food Canada, Dairy and Swine Research and Development Centre, Quebec, Canada [21]; and incubated at 37 °C in humidified atmosphere (5% CO_2) for 48 h.

2.1.5.1. Granulosa cell steroid production. 10^4 cells/well were seeded in 96-well plates in 200 μl CM supplemented with 28 ng/ml androstenedione [22]. After a 48 h incubation, culture media were then collected, frozen and stored at −20 °C until progesterone (P4) and estradiol 17β (E2) determination by validated RadioImmunoAssays [23]. P4 assay sensitivity and ED_{50} were 0.24 and 1 nmol/l, respectively; E2 assay sensitivity and ED_{50} were 0.05 and 0.2 nmol/l. The intra- and inter-assay coefficients of variation were less than 12% for both assays.

2.1.5.2. Granulosa cell Vascular Endothelial Growth Factor (VEGF) production. 10^6 granulosa cells in 1 ml CM + 1% Fetal Calf Serum were seeded in 24-well plates and incubated for 48 h. VEGF in culture media was quantified by an ELISA (Quantikine, R&D System, Minneapolis, MI, USA). This assay, developed for human VEGF detection, has been