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Relationship between concentrations of progesterone, oxytocin, noradrenaline, gene expression and protein level for their receptors in corpus luteum during estrous cycle in the cow

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ABSTRACT

The aim of these studies was to evaluate of the relationship between luteal concentrations of oxytocin (OT), noradrenaline (NA), progesterone (P4), oxytocin receptors (OT-R) and β_2-adrenoreceptors (β_2-R) gene expression and their protein level throughout the estrous cycle in cattle. Corpora lutea (CL) collected during days 1–5, 6–10, 11–16 and 17–21 of the estrous cycle were used in these studies. Concentrations of P4, OT and NA were determined in tissue extracts. Gene expression and protein level for OT-R and β_2-R were investigated by RT-PCR and Western blot, respectively. Luteal concentration of P4 was higher ($P<0.01$) on days 6–10 and 11–16 than during days 1–5 and 17–20 of the estrous cycle. Concentration of OT was the highest on days 1–5 and 6–10 of the estrous cycle. This concentration decreased ($P<0.01$) during days 11–16 reaching the lowest level ($P<0.001$) on days 17–20 as opposed to days 1–10 of the estrous cycle. Expression of OT-R mRNA was lower on days 6–16 ($P<0.05$) followed by its increase on days 17–20 as opposed to the expression observed on days 1–5. Expression of OT-R mRNA was negatively correlated ($P<0.05$) with the profile of OT-R protein level. This latter parameter was the lowest on days 17–20 and 1–5, and the highest on days 6–10 and 11–16. Oxytocin concentration was negatively correlated ($P<0.05$) with expression of OT-R mRNA and positively correlated ($P<0.001$) with OT-R protein level. This protein level was only correlated with P4 ($r=0.59$; $P<0.05$). Concentration of OT was positively correlated with level of P4 ($P<0.001$). Concentration of NA was the highest on days 1–5 of the estrous cycle, whereas it was similar or lower ($P<0.05$) on days 6–21. Expression of mRNA for β_2-R was the lowest on days 1–5 and was highly increased ($P<0.05$) on days 6–16. The expression was the highest ($P<0.001$) on days 17–21 compared to all others group of the estrous cycle. Protein level for β_2-R was the highest on days 1–5 and decreased ($P<0.05$) on days 6–10 and 11–16. The expression was the lowest on days 17–20 compared to the β_2-R protein level in CL from all others stages of the estrous cycle. Protein level for β_2-R was positively correlated ($P<0.05$) with the OT concentration. Expression of mRNA for β_2-R was negatively correlated ($P<0.001$) with level of β_2-R protein. No correlation was found between β_2-R mRNA expression and NA concentration or between NA and P4 concentrations. Results presented in this study suggest an evident relationship between OT and NA and may play an important role in the regulation of luteal steroidogenesis during all stages of the estrous cycle.

1. Introduction

The main function of the corpus luteum (CL) is the production of progesterone (P4). This hormone is crucial for the duration of the estrous cycle and for a successful pregnancy. The mechanism regulating secretory function of the bovine CL involves many factors that are produced both within and outside the CL. It was also found that treatment of bovine luteal cells with P4 increased 3β-hydroxysteroid dehydrogenase (3β-HSD) activity [1]. In addition, this treatment also stimulated mRNA expression for StAR protein (steroidogenic acute regulatory protein), cytochrome P450scc, and 3β-HSD on days 6–16 of the estrous cycle [2]. Thus, P4 can itself regulate its own synthesis.

Oxytocin (OT) and noradrenaline (NA) are also produced within the bovine CL and they affect CL function. Oxytocin gene is actively transcribed in the bovine CL [3] in large luteal cells [4]. Its direct action on the luteal cells is mediated by the membrane OT receptor (OT-R) [5]. Although, OT can stimulate the release of uterine prostaglandin F2α (PGF2α) and in turn this prostaglandin affects the secretion of OT [6]. It does not seem to be necessary to initiate luteolysis in cattle [7,8]. However, this OT stimulation may support luteolysis as a regulator of the amplitude of pulsatile PGF2α

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secretion [9]. Moreover, OT can acutely stimulate the release of P4 from microdialyzed bovine CL in *in vitro* conditions. This effect was evident mostly during the early luteal phase (days 5–7) followed by its decrease on days 8–12 and 15–18 [10]. Thus, OT can be a luteotropic factor in a newly formed CL.

In CL, noradrenaline is synthesized from dopamine (DA) and plays a significant role in regulation of CL [11,12]. Within a few minutes, after NA administration, increased secretion of OT and P4 is observed [8]. This observation was confirmed by increase in activity of cytochrome P450scc, 3β-HSD [13] and peptidyl-glycine α-amidating mono-oxygenase (PGA) enzyme. PGA participates in post-translational processing of OT synthesis in *in vitro* studies [14]. Noradenaline affects the luteal cells via β_2-subtype receptors, which predominate in mediating the stimulatory effect of NA on steroidogenesis [15].

Considering the importance of OT and NA for CL steroidogenesis, receptors for these hormones are equally important as an integral part of the luteal cells responsiveness upon these hormones stimulation. Therefore, the aim of these studies was to evaluate of the relationships between luteal concentrations of OT, NA and P4, and OT-R and β_2-R mRNA expression. In addition, we were trying to determine their protein level throughout the estrous cycle in cattle.

2. Materials and methods

2.1. Corpora lutea collection

Corpora lutea ($n = 4$ for each stage of the cycle) from healthy non-gravid cows and heifers were harvested at a commercial slaughterhouse. Immediately after collection, CL were frozen in liquid nitrogen, transported to the laboratory and stored at −80 °C until further use. Four stages of CL growth (days 1–5, 6–10, 11–16, and 17–20) were determined according to Ireland et al. [16]. The deeply frozen tissues were homogenized with a vibratory mill (Retsch MM-2). The tissue powder was divided into individual portions for determinations of OT, P4, NA and isolation of RNA and protein.

2.2. Hormone determination

In extracts from CL tissue, progesterone and OT concentrations were determined by EIA, according to Prakash et al. [17]. A reader plate (Multiscan EX, Labsystem, Finland) was used for the measurement of absorbance at 450 nm. Data were corrected for procedural losses. Progesterone was extracted from the CL tissue with diethyl ether [18], then was subjected for enzyme immunoassay (EIA) determination. Recovery of P4 averaged 90%. The final dilution of progesterone labelled with horseradish peroxidase and P4 antiserum (IFP4) was 1:60 000. The range of the standard curve was 0.1–25 ng/ml and the sensitivity of the procedure was 0.15 ng/ml. Intra-assay coefficient of variation was 7.7%.

Oxytocin concentration was determined by EIA using biotin-labelled OT and streptavidin-peroxidase. This hormone was extracted from the tissue with 1% of acetic acid and recovery of extraction averaged 85%. Oxytocin antiserum (R-1) was used at the final dilution of 1:25 000, and biotinylated OT at 1:50 000. The standard curve ranged from 3.9 to 1000 pg/ml. Intra-assay variation for OT was 7.9% and the sensitivity of the procedure was 20 pg/ml.

Tissue concentrations of NA were determined by high performance liquid chromatography (HPLC) with electrochemical detection (HP 1049A; Hewlett-Packard), as described previously by Kotwica et al. [11]. Briefly, NA was extracted from the powdered luteal tissue with a 10-fold excess amount of 0.1 M trichloroacetic acid (TCA) containing 0.01% $Na_2S_2O_5$ and 0.0003% ascorbic acid. Then, the suspension was centrifuged for 20 min at 14 000 × *g* and the supernatant was collected and diluted with Tris buffer, pH 8.6. The 3, 4-dihydroxybenzylamine (DHBA) was used as an internal standard and was added to plasma samples. Catecholamine standards were prepared in 0.5 ml of 0.1 M TCA containing 0.01% $Na_2S_2O_5$ and 0.0003% ascorbic acid. The mobile phase consisted of 0.05 M citrate–sodium phosphate buffer (pH 3.5) containing 0.3 mM octane sulfonate, 0.1 mM EDTA, 15% methanol and 1% acetonitrile. Recovery of the DBHA as internal standard was 71%. Final data were corrected for procedural losses.

2.3. RNA isolation and reverse transcription

Total RNA was extracted from homogenized tissue in accordance with the procedure described by Chomczynski and Sacchi [19] using the Total RNA Kit (A&A Biotechnology, Poland) as given in the manufacturers' instruction. The isolated RNA was stored at −80 °C for further analysis. The purity and concentration of the RNA were determined by measuring the absorbance at 260 nm and 280 nm wavelength. One microgram of RNA was treated with DNase and subjected to reverse transcription for 60 min at 42 °C in 20 μl of reaction mixture. The reaction mixture contained the following components: RT-buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 5 mM dithiothreitol; Fermentas, Vilnius, Lithuania); 10 mM of each dNTP; 500 ng of anchored oligo $(dT)_{23}$ primers; and 200 U of reverse transcriptase (Fermentas, Vilnius, Lithuania). The reaction was terminated by heating for 10 min at 70 °C.

2.4. Real-time PCR procedure

Real-time PCR was performed by means of the Applied Biosystems 7300 Real-time PCR System (Applied Biosystems, USA) performed using Power SYBR Green PCR Master Mix (Applied Biosystems, USA). The oligonucleotide primers and expected product sizes used for PCR amplification of OT-R, β_2-R and GAPDH as a housekeeping gene are depicted in Table 1. Real-time PCR (25 μl) included cDNA (800 ng for OT-R and β_2-R and 100 ng GAPDH), 12.5 μl of QuantiTect SYBR Green PCR Master Mix, 0.2 mM of both PCR primers for each studied gen. The standard curves used in quantification were obtained from serial dilutions (5×10^{-2}–5×10^{-9} ng) of product from previous real-time PCR reaction. The PCR for each pair of primers was carried out as follows: initial denaturation (10 min at 95 °C), followed by 40 cycles of denaturation (15 s at 95 °C), annealing and extension (1 min at 60 °C). After the PCR, melting curves were acquired by stepwise increased in the temperature from 60 °C to 95 °C to ensure that a single product was amplified in the reaction. The data obtained from the reaction were normalised to GAPDH products to obtain arbitrary units of relative amount of the PCR product.

2.5. Western blot analysis

Radioimmunoprecipitation assay buffer (RIPA) with protease inhibitors was used for protein sample preparation (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). Proteins obtained from the CL tissue (50 μg) were electrophoresed in 10% SDS-PAGE and transferred to Immobilon PVDF membrane (Millipore, Billerica, USA). Thereafter, the membrane was blocked with 5% non-fat dry milk in TBST buffer (100 mM Tris–HCl; 0.9% NaCl and 0.05% Tween 20). The membranes were incubated overnight in 4 °C with anti-rabbit OT-R antibodies ($n = 4$) at concentration 6 μg/ml (Alpha Diagnostic International, San Antonio, USA) and anti-goat β_2-R antibodies ($n = 4$) at concentration 0.3 μg/ml (Everest Biotech, Oxfordshire, UK). Membranes were washed three times (10 min each) with TBST buffer and subsequently treated with anti-rabbit IgG (dilution 1:35 000)