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Expression patterns of Toll-like receptors in the ovine corpus luteum during the early pregnancy and prostaglandin F2α-induced luteolysis

Mehmet O. Atli [a, *], Mehmet Kose [a], Mustafa Hitit [b], M. Salih Kaya [c], Faruk Bozkaya [d]

[a] Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Dicle University, Diyarbakır, Turkey
[b] Department of Genetics, Faculty of Veterinary Medicine, Kastamonu University, Kastamonu, Turkey
[c] Department of Physiology, Faculty of Medicine, Yildirim Beyazit University, Ankara, Turkey
[d] Department of Genetics, Faculty of Veterinary Medicine, Harran University, SanliUrfa, Turkey

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ABSTRACT

The aim of this study was to elucidate the expression profiles of Toll-like receptors (TLRs) in the ovine corpus luteum (CL) during early pregnancy and prostaglandin F2α (PGF2α)-induced luteolysis. For this purpose, multiparous Anatolian Merino ewes were selected and randomly allotted into cyclic (including those in the induced luteolysis group, n = 20) and pregnant (n = 12) groups. All of the ewes were scheduled to be slaughtered for predetermined days/hour during the estrous cycle, early pregnancy, and PGF2α induced luteolysis. The CLs were collected from both cyclic and pregnant ewes on days 12 (C12 and P12; n = 8) and 16 (C16 and P16; n = 8) and pregnant ewes on day 22 (P22; n = 4). For the induced luteolysis model, ewes were injected with PGF2α on day 12 of the estrous cycle and CLs were collected at 1 h (PG1h; n = 4), 4 h (PG4h; n = 4), and 16 h (PG16h, n = 4) after injection. Quantitative polymerase chain reaction (qPCR) was used to evaluate the expression profiles of *TLR2*, *TLR4*, *TLR6*, *TLR8*, and *TLR10*, while free-floating in situ hybridization and immunohistochemistry were used to define the spatial localization of TLR2, TLR4, and TLR7 in the CL. Data were then analyzed by one-way ANOVA and were considered statistically significant when P values were lower than 0.05. Expression of *TLR2* was upregulated in both early and late stages of luteolysis ($P < .05$). An upregulation of *TLR4* was detected at PG16h, while *TLR6* was decreased at PG4h ($P < .05$). Expression of *TLR7* and *TLR8* was significantly increased during early pregnancy, at both PG16h and regressed groups (C16, $P < .05$). In contrast, *TLR10* was downregulated during PGF2α-induced luteolysis and on P16 ($P < .05$). TLR4 and TLR7 proteins were particularly localized in endothelial cells on C12/PG0h, but prominent signals corresponding to TLR4 and TLR7 were detected in luteal cells at PG16h. The results suggest an involvement of TLRs in the luteolytic mechanism in ovine CL, as indicated by differential expression levels of TLRs during PGF2α-induced luteolysis. Moreover, the present study indicates that early pregnancy-mediated changes in TLR expression in the CL may contribute to the establishment and maintenance of ovine pregnancy.

1. Introduction

In many species, including ruminants, progesterone released from the corpus luteum (CL) during early pregnancy regulates vital functions in the uterus, such as endometrial nutrient secretion and immune modulation of the endometrium for the developing embryo [1,2]. If pregnancy occurs, the CL is protected from luteolytic prostaglandin F2α (PGF2α) pulses by embryonic interferon-τ (IFN-τ) [3,4]. If an animal is mated, but pregnancy does not occur, the CL needs to regress to allow for a new fertile estrous cycle. The regression of the CL in ruminants is initiated by the rhythmic release of PGF2α in high concentrations from the non-pregnant uterus [5,6]. The CL is composed of multiple cell types, including steroidogenic cells (large and small luteal cells) and non-steroidogenic cells (endothelial cells, pericytes, fibrocytes, and immune cells) [7,8]. Due to the heterogeneous structure of the CL, understanding the communication among the various cells during luteolysis is still an ongoing field of research. For example, immune cells in the CL were previously thought to play a phagocytic role following luteolysis [9], the immune cell population and

* Corresponding author. Department of OBGYN, Faculty of Veterinary Medicine, Dicle University, Diyarbakir, Turkey.
E-mail addresses: moatli@hotmail.com, moatli@dicle.edu.tr (M.O. Atli).

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chemokines in the CL are affected during functional luteolysis in the early response to PGF2α [10–12]. Moreover, different regulations of immune components were also demonstrated in the CL during early pregnancy [13].

Toll-like receptors (TLRs), as both transmembrane and intracellular proteins, are an important part of the innate immune system and also exert roles in activation of the acquired immune system [14]. Secreted or released endogenous molecules, such as reactive oxygen species (ROS), heat shock proteins, and products from both intracellular and extracellular degradation, as well as molecules produced by an embryo, can activate the TLR system [15,16]. Therefore, activation of TLRs can be observed in both physiological and pathological processes such as pro/anti-inflammatory responses [17]. Different TLR expression patterns regulated by pregnancy have been indicated in ruminants [18]. Moreover, during early pregnancy, expression profiles of TLRs dramatically changed in both peripheral blood leukocytes (PBLs) and trophoblast cells of ewes [15]. It is also known that the expression of many pro/anti-inflammatory molecules is affected during the maintenance and regression of the CL [19]. In previous studies, it has been emphasized that expression of TLRs is regulated in parallel with the changing physiology of the CL [20,21]. Earlier studies investigating TLRs in the CL used slaughterhouse material, which could not provide information about the exact time profiles for TLR regulation in the CL at different physiological stages. Therefore, the present study investigates TLR expression patterns in specific pregnancy/cycle days and during experimental luteolysis in the CL. Specifically, the expression profiles of TLRs in the ovine CL were elucidated by comparing the mRNA levels of *TLR2*, *TLR4*, *TLR6*, *TLR7*, *TLR8*, and *TLR10* during early pregnancy and PGF2α-induced luteolysis. Moreover, temporal and spatial cell-specific localizations of TLR2, TLR4, and TLR7 in the CL were also evaluated at certain times of the cycle and during pregnancy. Efficiency of PGF injection was tested by localization of *StAR* mRNA in both C12/PG0h and PG16h.

2. Material and methods

2.1. Materials

TRIzol® Reagent was purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). cDNA Synthesis Kit (RevertAid™ First Strand cDNA Synthesis Kit), RNAse-free DNAse I, qPCR Master Mix (2X) for Real-time PCR (Maxima™ SYBR), dNTP set and Taq DNA polymerase were purchased Thermo Fisher Scientific (Vilnius Lithuania). Gel extraction kit and RNEASY Mini kit were obtained from QIAGEN (Frederick, MD, USA). Digoxigenin 11-UTP, RNase inhibitor, T7 RNA polymerase, anti-DIG antibody, blocking reagent, and BM Purple AP substrate were purchased from Roche Applied Science (Mannheim, Germany). See-plaque-low melting agarose was purchased from LONZA (Rockland, ME, USA). Specific oligonucleotide primers were synthesized by Metabion International AG (Martinsried, Germany). DNAse and RNAse free sterile 8'strip 0.1 ml-tubes (Applied, Waltham, MA, USA) and 1.5 ml-tubes (TreffLab, Degersheim, Switzerland) were obtained. Antibodies for TLR4 and TLR7 have been achieved from antibodies-online.com (AA 780-830, USA), Santa Cruz Biotechnology (sc-30004, USA), respectively. Unless otherwise specified, all other chemicals and reagents were obtained from Merck (Darmstadt, Germany).

2.2. Animal materials and experimental design

All procedures were approved by the Bahri-Dağdaş Research Center Ethical Committee (Number: 29/01/2016-49-7). The study was performed during the breeding season of year 2014. Multiparous Anatolian Merino ewes were selected and randomly allotted into cyclic (including those in the induced luteolysis group, n = 20) and pregnant (n = 12) groups. Diets were formulated to meet the NRC (2007) nutritional requirements for 3- to 5- years-old, non-lactating sheep. Feed, water, and vitamin-mineral blocks were available ad libitum throughout the study. The estrous cycles were synchronized with progesterone impregnated intravaginal sponges (20 mg flugestone acetate, Chronogest CR, Intervet, Turkey), inserted for 10 days. Immediately after removing the sponges, estrus was observed three times daily in the presence of two teaser rams. The ewes then were allowed to complete their whole cycle without any application and observed for the new estrus by teaser rams. The ewes in the pregnancy group were allowed to mate (day 0) two times, 12 h apart, with by rams of proven fertility. The ewes in the cyclic group were observed at estrus (day 0). All of the ewes were scheduled to be slaughtered for predetermined days/hour during the estrous cycle, early pregnancy, and PGF2a induced luteolysis. The CL samples were collected from the cyclic ewes on days 12 (C12, n = 4) and 16 (C16, n = 4) and pregnant ewes on days 12 (P12, n = 4), 16 (P16, n = 4), and 22 (P22, n = 4).

For the induced luteolysis model, ewes were injected PGF2α (IM, 125 mcg/mL d-cloprostenol, Minoprost, Provet, TURKEY) on the 12th day of the estrous cycle, and the CL samples were collected at 1 h (PG1, n = 4), 4 h (PG4, n = 4), and 16 h (PG16, n = 4) after the injection. The CL samples collected at days 12 and 16 of the estrous cycle were also accepted as zero h (PG0h, no PGF2α injection, n = 4) and regressed group (C16, n = 4) for PGF2α-induced luteolysis model, respectively.

2.3. Tissue collection

The genital tract, including the ovaries, uterus, and cervix, was removed within 10 min of slaughter, put on ice and immediately transferred to the laboratory for examination and sampling. After washing the genital tract with Phosphate Buffered Saline (PBS), the uterine horns ipsilateral to the CL from the mated ewes were flushed with PBS, and the pregnancy was confirmed by observation of an embryo in the flushing fluid. Then, the CL was removed from the ovary, and the tissue was dissected. The CLs were snap frozen in liquid nitrogen, and stored at -80 °C for later isolation of RNA. A part of CL tissue was fixed in a 4% paraformaldehyde solution overnight at 4 °C and then dehydrated using serial dilutions of methanol (25%, 50%, 75%, and 100%). Dehydrated samples were stored in 100% methanol at -20 °C until evaluated by in situ hybridization or immunohistochemistry.

2.4. RNA isolation, RT reaction, and quantitative PCR

About 20 mg of CL tissue was homogenized in Trizol® by using a homogenizer (Slientcruzer M, Heidolph, Germany). Total RNA was then extracted using the manufacturer's protocol. The purity of RNA was evaluated by agarose gel (1%) electrophoresis and optical density at 260/280 nm of 2 ± 0.1 with NanoDrop ND-2000 (Thermo Scientific, Wilmington, DE, USA). Two μg of total RNA was treated with DNAse I to eliminate possible genomic DNA contamination and RNA was then reverse transcribed (RT) in the presence of oligodT primers using the Revert Aid First Strand cDNA Synthesis Kit according to the manufacturer's protocol. RT negative (all components of the RT kit without RNA) were also added into the analyses.

Oligonucleotide primers for *TLR2, TLR4, TLR6, TLR7, TLR8, TLR10*, and housekeeping genes (GAPDH, B-actin) were designed by using Primer3 from the sequences in NCBI gene database or from published primer sequence. The primer pair sequences and product sizes are shown on supplement file (Table 1). All PCR reactions