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Expression of progesterone receptor and progesterone-induced blocking factor in the spleen during early pregnancy in ewes

Ling Yang*, Runqing Guo, Xiaolei Yao, Jinkun Yan, Ying Bai, Leying Zhang

Department of Animal Science, College of Life Science and Food Engineering, Hebei University of Engineering, Handan 056021, China

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ABSTRACT

The spleen plays a key role in immune regulation. Progesterone (P4) exhibits different immunological effects through binding to different types of receptor. In the present study, splenic samples were obtained at day 16 of non-pregnancy and days 13, 16, and 25 of pregnancy to study the expression of P4 receptor (PGR) and P4-induced blocking factor (PIBF) in ewes through quantitative real-time PCR, Western blot and immunohistochemistry analysis. Our results showed that the relative expression levels of PGR and PIBF mRNA increased from day 13–16 in pregnant ewes, but decreased from day 16–25, and the 26 kDa isoform of PGR and the 22 kDa variant of PIBF were down-regulated in the spleens at day 25 of pregnancy compared with expression at day 16 of the oestrous cycle and days 13 and 16 of pregnancy. The immunohistochemistry test results confirmed that PGR and PIBF proteins were localized in the cytoplasm of cells in the capsule, trabeculae and splenic cords. This paper reports for the first time that the 26 kDa isoform of PGR and the 22 kDa variant of PIBF were down-regulated during early pregnancy, which may be essential for normal pregnancy in ewes.

1. Introduction

The primary signals for maternal recognition of pregnancy act on the endometrium, prevent the release of prostaglandin F2α (PGF2α) in the endometrium (Bazer et al., 1991; Roberts et al., 1992), corpus luteum (CL) (Sakumoto et al., 2014) and peripheral blood mononuclear cells (PBMCs) (Yang et al., 2016b) through lymph circulation and blood circulation in ruminants (Bott et al., 2010; Antoniazzi et al., 2013; Yang et al., 2014), to prolong the life span of the CL (Bazer et al., 2009; Brooks et al., 2014). Progesterone (P4) is a steroid hormone, primarily produced by the CL in females, which is critical to pregnancy, embryogenesis, and immunomodulation in mammals (Hughes, 2012; Micks et al., 2015).

P4 traditionally acts through the P4 receptors (PGR), which results in gene transcription and translation into proteins. Additionally, P4 also causes rapid stimulatory effects through intracellular signalling pathways independent of gene transcription (Boonyaratanakornkit and Edwards, 2004). Expression of PGR mRNA and protein is down-regulated between day 10 and 12 in the endometrial luminal epithelium and day 12 and 16 in the glandular epithelium in cyclic ewes; however, the expression of PGR mRNA is up-regulated from day 11–17 and then is down-regulated in the endometria from day 17–25 in pregnant ewes (Spencer and Bazer, 1995). The expression of PGR mRNA changes significantly in the region of the oviduct, and the expression level is high in the isthmus, but the pregnancy status or stage of oestrous cycle does not influence the expression of PGR mRNA in the bovine oviduct (Saint-Dizier et al., 2012). Our previous study shows that the expression of PGR mRNA is varied in PBMC from non-pregnancy to pregnancy in cattle (Yang et al., 2016b). Many PGR isoforms occur. PGR-A is a truncated form of PGR-B and has different transcription activation properties and responsiveness compared to PGR-B (Lonard and O'Malley, 2012). The truncated PGR-C variant inhibits PGR-B transactivation in human myometrial cells (Condon et al., 2006), and the functions of different PGR isoforms are distinct (Condon et al., 2006; Zakar and Mesiano, 2011).

P4-induced blocking factor (PIBF) is a molecule with inhibitory effects on cell-mediated immune reactions, is synthesized by P4 receptor-positive T lymphocytes in response to P4 during pregnancy, and acts on lymphocytes to induce up-regulating production of type 2 cytokines with a shift of type 1 to type 2 cytokine in pregnant women (Raghupathy et al., 2009). PIBF exerts an immunomodulatory function, and contributes to the maintenance of pregnancy in mice, and there is a characteristic feature of PIBF production for normal pregnancy (Polgár et al., 2004). PIBF controls myometrial contractility through inhibiting prostaglandin synthesis, and reduces the deleterious effect of high natural killer (NK) activity through suppressing peripheral NK activity during pregnancy (Szekeres-Bartho and Polgar, 2010). Many PIBF isoforms are expressed in fetuses and in placental, decidual, and uterine

* Corresponding author.
E-mail address: yangling@hebeu.edu.cn (L. Yang).

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samples in pregnant mice, and different PIBF isoforms have different functional attributes (Bogdan et al., 2014).

As the largest lymphoid organ in the mammalian body, the spleen has an abundant and diverse population of immune cells, which play an important role in immune regulation (de Porto et al., 2010). A combination of the innate and adaptive immune system occurs in the spleen in a uniquely organized way, which involves many immune cells (Mebius and Kraal, 2005). The effects of P4 are exerted, including immunomodulation, on the maternal uterus through an endocrine mode and play a pivotal role in the maintenance of pregnancy. Pregnancy exerts extreme effects on the female immune system, and the first signs of pregnancy recognition are observed via the immune system even before implantation (Chelmonska-Soyta et al., 2014). T-cell lymphopoiesis is blocked through a PGR-dependent paracrine mechanism, and thymic stromal PGR is required for normal fertility in mice (Tibbetts et al., 1999). The expression of cofilin-1, F-actin capping protein subunit alpha and malate dehydrogenase proteins is deeply down-regulated in splenic $CD4^+$ lymphocytes at the preimplantation period of pregnancy in mice (Chelmonska-Soyta et al., 2014). We recently reports that PGR and PIBF are expressed in ovine lymph nodes, limited to the subcapsular sinus and trabeculae in the cortex and medullary sinuses (Yang et al., 2017). Uterine PGR gene expression is regulated in a tissue- and cell type-specific manner during early pregnancy in sheep (Spencer and Bazer, 1995). Nevertheless, it is still unclear whether P4 exerts any effect on the spleen during early pregnancy in sheep. In the present study, the spleens from non-pregnant and early pregnant ewes were used to compare the expression of PGR and PIBF in splenic immunomodulation.

2. Materials and methods

2.1. Animals and experimental design

Small-tail Han ewes approximately 18 months of age with normal oestrous cycles were observed daily for oestrus using a vasectomized ram at the farm of Handan Boyuan Animal Husbandry Co., Ltd. in China. The ewes mated twice with intact rams at 12-h intervals after the detection of sexual receptivity. The experimental protocol was approved by the Hebei University of Engineering Animal Ethical Committee, and humane animal care and handling procedures were followed throughout the experiment. The day of coitus was counted as day 0 of pregnancy or non-pregnancy. The ewes were randomly divided into four groups (n = 6 for each group), and ewes assigned to the non-pregnant group were not mated with an intact ram. The spleens were obtained from ewes on days 13, 16 and 25 of pregnancy and on day 16 of non-pregnancy at the time of slaughter. Pregnancy was confirmed by observation of conceptus in the uterus. Splenic transverse pieces (0.5 cm^3) were fixed in fresh 4% (w/v) paraformaldehyde in PBS buffer (pH 7.4), and the remaining portions of spleens were frozen in liquid nitrogen for subsequent quantitative real-time PCR (qRT-PCR) and protein analysis.

2.2. RNA extraction and qRT-PCR assay

The total RNA from the splenic samples was extracted using the TRIzol method. The cDNA was synthesized with a FastQuant RT Kit (Tiangen Biotech Co., Ltd., Beijing), and a SuperReal PreMix Plus Kit (Tiangen Biotech) was employed for the qRT-PCR. The primer sequences of PGR, PIBF and GAPDH were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (Table 1). The relative levels of mRNA expression were calculated using an internal control gene (GAPDH). The relative expression value was set as 1 for the group of day 16 of non-pregnancy.

Table 1
Primers used for qRT-PCR.

Gene	Primer	Sequence	Size (bp)	Accession number
PGR	Forward	CAACAGCAAACCTGATACCT	183	XM_
	Reverse	CCATCCTAGTCCAAATACCATT		012169084
PIBF	Forward	CCAGGCAGCTAATTGAACGG	189	XM_
	Reverse	GGGCTAGTACCTGCTTCTGG		004012184
GAPDH	Forward	GGGTCATCATCTCTGCACCT	176	NM_
	Reverse	GGTCATAAGTCCCTCCACGA		001190390.1

2.3. Western blot

The total proteins of the splenic samples were extracted with RIPA Lysis Buffer (Biosharp, BL504A). The protein concentration was measured using a BCA Protein Assay Kit (Tiangen Biotech, PA115) with bovine serum albumin as the standard. An equal amount of total proteins (10 μg/lane) was separated using 12% SDS-PAGE, and the proteins were transferred to 0.22 μm polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). PGR and PIBF were detected with Western blot using rabbit anti-PGR polyclonal antibody (Santa Cruz Biotechnology, Inc., sc-538, 1:1000) and rabbit anti-PIBF polyclonal antibody (Santa Cruz Biotechnology, sc-99129, 1:1000), respectively. Secondary goat anti-rabbit IgG-HRP was diluted to 1:2000. Pro-light HRP chemiluminescence detection reagent (Tiangen Biotech) was used to detect the immunoreactive bands. Sample loading was monitored with the GAPDH antibody (Santa Cruz Biotechnology, sc-20357) at a dilution of 1:1000, and secondary rabbit anti-goat IgG-HRP was diluted to 1:2000. The intensity of blots was quantified using Quantity One V452 (Bio-Rad Laboratories), and the relative levels were calculated using GAPDH.

2.4. Immunohistochemistry analysis

The fixed splenic samples were embedded in paraffin, and the paraffin-embedded sections were deparaffinized in xylene and rehydrated via ethanol. The rehydrated sections were quenched endogenous peroxidase activity with 3% H_2O_2, and non-specific binding was reduced with 5% normal goat serum in PBS. Immunocytochemical localizations of PGR and PIBF in the spleen were performed using rabbit anti-PGR polyclonal antibody (sc-538, 1:100) and rabbit anti-PIBF polyclonal antibody (sc-99129, 1:100), respectively. For a negative control, non-immune goat serum was used in place of the primary antibody. A DAB kit (Tiangen Biotech) was used to visualize the antibody binding sites in the tissue sections, and the nucleus was stained with hematoxylin. Finally, the images were captured on a light microscope (Nikon Eclipse E800, Japan) with digital camera DP12, and the intensity of stained cells was analyzed through the images. The immunostaining intensity of the different splenic samples was scored by two different investigators in a blinded fashion, according to the following scale: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining (Yu et al., 2013).

2.5. Statistical analyses

The relative expression values for the qRT-PCR assay were calculated using the $2^{-\Delta\Delta Ct}$ analysis method (Wong and Medrano, 2005). The data for the relative expression levels of PGR and PIBF mRNA, PGR and PIBF isoforms were analyzed as a completely randomized design with at least three animals per group using the Proc Mixed models of SAS (Version 9.1; SAS Institute, Cary, NC). For spleens from different stage of gestation or pregnancy status, the model contained the random effect of ewe and fixed effects of stage of gestation, pregnancy status and the interaction of stage of gestation and pregnancy status. The comparisons among the relative expression levels of different groups were performed