Research in Veterinary Science 92 (2012) 478–485

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Steroid receptor mRNA expression in the ovarian follicles of cows with cystic ovarian disease

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ARTICLE INFO

Article history:
Received 18 June 2010
Accepted 11 April 2011

Keywords:
Ovarian cyst
Cow
Steroid receptors

ABSTRACT

Steroid receptors have been demonstrated to be important intra-ovarian regulators of follicular development and ovulatory processes. The aim of the present study was to determine the expression of steroid receptor mRNA in ovarian follicular structures from cows with cystic ovarian disease (COD) compared with ovarian structures from regularly cycling cows using reverse transcription polymerase chain reaction (RT-PCR). The cystic follicles showed a higher *estrogen receptor α (ESR1)* mRNA expression in the theca and granulosa and a lower *estrogen receptor β (ESR2)* expression. The cystic follicles also showed a strong expression of *androgen receptor* mRNA in the granulosa. No changes were observed in total *progesterone receptor* mRNA, but a very significant increase in the B isoform was found in the granulosa of the cystic follicles. The findings of the current study provide evidence that an altered steroid signaling system may be present in bovine follicular cysts, and we suggest that in conditions characterized by altered ovulation, such as COD, changes in the expression of ovarian steroid receptors could play a fundamental role in the pathogeny of this disease.

1. Introduction

The etiopathogenia of cystic ovarian disease (COD) in dairy cattle is a complex process that involves dysfunctions in folliculogenesis and ovulation, and many factors such as stress, nutritional management and infectious disease can co-exist (Silvia et al., 2002).

Steroids play a key role in the growth, differentiation and function of female reproductive tissues, including the ovarian follicles (Drummond et al., 2002; Brosens et al., 2004). In this sense, locally produced androgens, estrogens and progesterone are involved in the regulation of several different follicular functions (Rosenfeld et al., 2001; Bramley et al., 2002; Drummond et al., 2002; Schams and Berisha, 2002; Brosens et al., 2004; Drummond, 2006; Kimura et al., 2007; Ortega et al., 2009).

Considering that the genomic effects of steroids are mediated via intracellular receptors (Beato and Klug, 2000; Brosens et al., 2004), and that steroids have the potential to upregulate or downregulate their own receptors (Drummond et al., 1999; Beato and Klug, 2000), the altered follicular dynamics and cellular differentiation observed in COD (Ortega et al., 2007, 2008; Rey et al., 2009; Velazquez et al., 2010) may be mediated via the altered expression of ovarian steroid receptors. Changes in receptor expression would result in altered steroid signaling culminating in alterations to cellular proliferation, apoptosis and differentiation (Rosenfeld et al., 2001; Drummond et al., 2002; Walters et al., 2008).

A critical role for steroids in regulating follicular growth has also been shown by the development of abnormal ovarian phenotypes associated with reduced fertility in mice lacking steroid receptors (Lydon et al., 1996; Drummond et al., 2002; Yeh et al., 2002). Some of these effects are related to changes in other hormones regulated by steroids, such as gonadotrophins, or follicular processes occurring late during follicular development. However, there is also growing evidence to support a direct intra-ovarian role for steroids, particularly estrogens and androgens, in regulating early follicular growth (Koering et al., 1994; Drummond et al., 2002; Britt et al., 2004; Jonard and Dewailly, 2004).

Steroids hormones act through specific receptors which are members of the nuclear receptor superfamily of transcription factors (Brosens et al., 2004). Estrogen receptors (ESR) are expressed as two structurally related subtypes in mammals, estrogen receptor α (ESR1) and estrogen receptor β (ESR2), which are encoded by two distinct genes (Kuiper et al., 1996). The existence of these two subtypes may partly explain the selective action of estrogen in different target tissues and in the same tissue at different phys-

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doi:10.1016/j.rvsc.2011.04.009

iological states (Mowa and Iwanaga, 2000; Wang et al., 2000). The progesterone receptor (PGR) has at least three isoforms (PGR-A, -B and -C), all originating from the same gene (Wei et al., 1990). The actions of androgens are mediated by androgen receptors (AR) (Brinkmann, 2001) encoded by a single copy gene in the X chromosome (Yong et al., 2000), with at least two isoforms originating from the same gene: AR-A and -B (Takeo and Yamashita, 1999).

The locations of steroid hormones receptors within ovarian follicles were confirmed by *in situ* hybridization (D'haeseleer et al., 2005). In bovine ovarian follicles, *ESR2* mRNA expression in granulosa cells decreases with an increase in follicular size (Manikkam et al., 2001). In contrast, mRNA expression of *ESR1* in the theca interna continuously increases during the final growth stages of the follicles, whereas no such increase occurs in granulosa cells (Schams and Berisha, 2002). A relatively high expression of ESR1 is found in thecal and stromal cells compared with granulosa cells (Van den Broeck et al., 2002). The expression of *PGR* mRNA in the follicles continuously increases to a maximum level in preovulatory follicles (Schams and Berisha, 2002), and a surge of gonadotrophin induces a rapid and transient increase in their expression in both theca and granulosa cells (Cassar et al., 2002; Jo et al., 2002). Also, *AR* mRNA expression in bovine follicles increases during early follicle development (Hampton et al., 2004).

It has been demonstrated that either estrogen or estrogen receptor imbalances/disturbances may result in the development of ovarian follicular cysts in cattle (Garverick, 1997; Salvetti et al., 2007), sheep (Ortega et al., 2009), humans (Shushan et al., 1996; Jakimiuk et al., 2002) and rodents (Salvetti et al., 2009). The expression of ESR1, ESR2 and PGR proteins has been analyzed in cows with COD by immunohistochemistry (Salvetti et al., 2007). However, there are no data available on the expression of steroid receptor mRNA in bovine cystic follicles. Since steroid receptors have been demonstrated to be important intra-ovarian regulators of follicular development and ovulatory processes, the aim of the present study was to determine the expression of *ESR1*, *ESR2*, *PGR* and *AR* mRNA in ovarian follicular structures from cows with COD compared with ovarian structures from regularly cycling cows using reverse transcription polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Collection and preparation of tissues

Ovaries from 65 random cows were collected at a local abattoir, within 20 min of death, from mixed breeds of *Bos taurus* cows assessed visually as being non-pregnant and without macroscopic abnormalities in the reproductive system. The complete ovaries were washed, refrigerated and immediately transported to the laboratory.

Each batch of ovaries was placed on ice and the antral follicles were removed using scissors and scalpel dissection. Before the ovaries were dissected, the follicular diameter was measured using callipers and the follicular fluid from each follicle was aspirated and stored separately at −20 °C for estradiol and progesterone determination. Follicles with an obviously atretic appearance (avascular theca and debris in the antrum) were discarded. Large antral follicles were obtained only the ovary couples without visible active corpora lutea.

Healthy follicles from normal cycling cows were classified into three categories according to their calculated follicle diameters, as described previously (Parrott and Skinner, 1998): small (<5 mm, $n = 15$), medium (5–10 mm, $n = 15$) or large (>10 mm, $n = 15$). The cystic follicles (>20 mm, $n = 20$) from COD animals were also used (Silvia et al., 2002). Follicles were hemisected in PBS and the granulosa cells (GC) were gently scraped into separate tubes containing approximately 20 ml of sterile PBS. The cell suspension was centrifuged at 400*g* for 10 min, the supernatant discarded and the GC pellets resuspended in Trizol LS reagent (Invitrogen, CA, USA). The follicular walls were further washed several times with PBS to remove the remaining GC. The surrounding stroma was also removed from the follicular walls, which was used as the thecal tissue samples (Sudo et al., 2007). All samples were snap-frozen in liquid nitrogen and stored at −80 °C until total RNA extraction.

2.2. Total RNA extraction

Total RNA was isolated from the samples after treatment with Trizol LS reagent (Invitrogen) according to the manufacturer's instructions with slight modifications. Briefly, 50–100 mg of tissue was homogenized with 750 µl of Trizol reagent (Invitrogen) and incubated for 5 min at 4 °C. The RNA was purified by vigorously homogenizing with chloroform and incubating for 15 min at 4 °C. After centrifugation at 12,000*g*, the aqueous phase was incubated with an equal volume of isopropanol for 30 min at −20 °C and centrifuged at 12,000*g* to obtain the mRNA pellet which was then washed with 75% ethanol for 10 min at 4 °C. The alcohol was replaced by DEPC-water pre-warmed to 55–60 °C. The extracted RNA was DNase treated with deoxyribonuclease I (amplification grade) (Invitrogen) to eliminate contaminating DNA, assessed for quality and quantity using a fluoroscopic method (Qubit, Invitrogen), aliquoted and stored at −80 °C until further use.

2.3. PCR primer design

2.3.1. Steroid receptor specific primers

The primers for ESR1, ESR2 and AR have been previously described (Pfaffl et al., 2003). For the PGR primer design, bovine sequences of PGR were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Entrez/index.htm) and two specific primers were designed spanning at least two mRNA-splicing sites using the PrimerSelect program in the LASERGENE software (DNAStar, WI, USA). The *PGRb* primer set flanks part of the NH2-terminal region of the B isoform (62 bp).The *PGRab* primer set flanks part of the common region of the A and B isoforms (131 bp) and the *PGRtotal* primer set flanks the hormone-binding domain for all A, B and C isoforms (339 bp) (Fig. 1).

All primers were purchased from Invitrogen and the sequences are summarized in Table 1. Oligonucleotide primers and amplification products were tested using BLAST (http://www.ncbi.nlm.nih.-

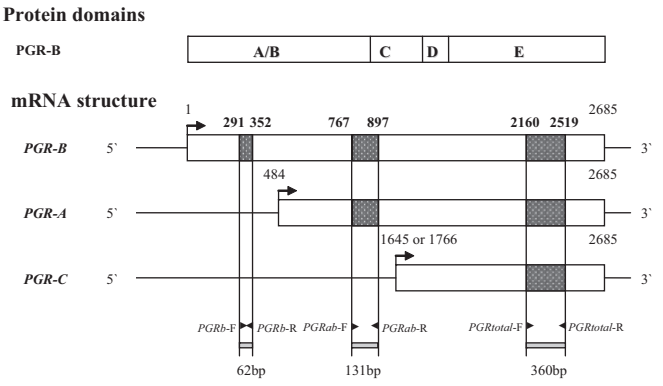

Fig. 1. Diagrammatic scheme and functional domains of the bovine progesterone receptor (PGR) and its hypothetical variants. The progesterone receptor A isoform (PGR-A) is an NH2-terminally truncated naturally occurring variant of the B isoform. The progesterone receptor C isoform (PGR-C) is also an NH2-terminally truncated transcriptional product, but it is much smaller than PGR-A. Locations of PCR primers used to generate fragments of the different isoforms are indicated (Adapted from (Fang et al., 2002)).