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Factors regulating the bovine, caprine, rat and human ovarian aromatase promoters in a bovine granulosa cell model

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ABSTRACT

The ovarian promoter of the primate and rodent genes encoding cytochrome P450 aromatase (*CYP19A1*) are robustly responsive to forskolin in luteinized cell models, whereas the ruminant ovarian promoter is minimally active. We explored this discrepancy by investigating the activity of the bovine ovarian promoter in two bovine granulosa cell models, luteinizing and non-luteinizing cells in vitro. In non-luteinizing cells, both FSH and IGF1 increased abundance of transcripts derived from the ovarian promoter. Comparison of the activity of promoters of several species in response to transcription factors (forskolin, NR5A2, FOXL2) in luteinizing cells demonstrated that a rat ovarian promoter-luciferase reporter was regulated mainly by forskolin (18-fold increase over basal expression) and addition of NR5A2 or FOXL2 had no further effect. Activity of a human promoter was significantly increased by NR5A2 plus forskolin (153-fold) compared with forskolin alone (71-fold over basal); addition of FOXL2 did not significantly increase promoter activity. Forskolin alone provoked minor activation of caprine and bovine promoter reporters (3-fold over basal), and addition of NR5A2 increased activity (7- to 11-fold). When forskolin, NR5A2 and FOXL2 treatments were combined, the activity of the caprine and bovine promoters increased to 20- and 34-fold, respectively. These data suggest that a major reason why *CYP19A1* is not expressed in luteinized cells (and the corpus luteum) of ruminants may be the stimulatory effect of FOXL2, which does not appear to be the case in the human and rat.

1. Introduction

Oestrogens are crucial for fertility in females, as they are necessary for granulosa cell proliferation, growth of the oocyte and acquisition of luteinizing hormone (LH) receptors (Knecht et al., 1985; Britt et al., 2004; Quirk et al., 2006). Loss of the enzyme responsible for the production of oestrogens, cytochrome P450 aromatase (CYP19A1), renders mice infertile (Fisher et al., 1998). Expression of ovarian *CYP19A1* is stimulated by follicle-stimulating hormone (FSH) in human, rat and bovine granulosa cells (Steinkampf et al., 1987; Fitzpatrick and Richards, 1991; Silva and Price, 2000), and by insulin-like growth factor-1 (IGF1) in humans and cattle (Steinkampf et al., 1988; Spicer and Aad, 2007) although in rodents IGF1 may augment FSH-stimulated *CYP19A1* expression rather than act alone (Zhou et al., 1997).

FSH and IGF1 act through multiple pathways. FSH increases intracellular cAMP levels and activates protein kinase A (PKA), which in turn activates cAMP response element binding protein, a major transcription factor for FSH target genes (reviewed in Hunzicker-Dunn and Maizels, 2006). In rodent granulosa cells, PKA has also been shown to activate ERK1/2 and phosphoinositide 3-kinase (PI3K)/Akt pathways (Zeleznik et al., 2003; Hunzicker-Dunn et al., 2012), although these may not be as important in bovine granulosa cells (Silva et al., 2006). IGF1 acts through the PI3K/Akt and ERK1/2 pathways independently of PKA in rodent and bovine granulosa cells (Gonzalez-Robayna et al., 2000; Mani et al., 2010).

Transcription of *CYP19A1* is driven by tissue-specific promoters, and there are numerous species differences in the number and structure of the *CYP19A1* promoters. In rats, two promoters are described, in humans there are nine promoters, and in sheep and cattle there are five and seven promoters, respectively. In all species, the proximal promoter, also named PII in humans and P2 in

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ruminants, is the ovarian promoter (Vanselow et al., 2001; Bulun et al., 2003; Pannetier et al., 2006); in cattle, P2 derived transcripts normally represent over 80% of all *CYP19A1* transcripts detected in granulosa cells and a distal promoter, P1.1, is a minor contributer to granulosa cell *CYP19A1* transcripts (Lenz et al., 2004; Hamel et al., 2005). In the corpus luteum, the major promoter used is PII in humans and rodents (Means et al., 1991; Stocco, 2004) whereas in ruminants P1.1 is the main luteal promoter (Lenz et al., 2004).

The proximal end of the ovarian promoters contains a number of fairly well conserved regulatory elements. These include two completely conserved binding sites for the orphan nuclear transcription factors NR5A1/NR5A2 (Stocco, 2008), and the forkhead box family member FOXL2. In humans and rodents, the proximal promoter contains a cAMP response element (CRE)-like sequence (CLS) that confers responsiveness to cAMP pathways (Fitzpatrick and Richards, 1993; Michael et al., 1997). In the cow, however, there is a single base pair deletion in the CLS of P2 that renders this promoter unresponsive to cAMP in reporter assays with luteinized bovine granulosa cells (Hinshelwood et al., 1997). The goat and buffalo P2 sequences also have a CLS with this deletion, but in reporter assays both promoters appear active (Pannetier et al., 2006; Monga et al., 2012). Another intriguing species difference involves the activity of FOXL2, which is a repressor of PII in humans and mice (Kuo et al., 2011, 2012), but which stimulated activity of the goat P2 (Pannetier et al., 2006).

Considering the apparent difference in promoter activities between bovine, caprine and human promoters, the objectives of the present study were to determine if FSH/cAMP regulates both P1.1 and P2 in non-luteinized bovine granulosa cells, and to compare the regulation of human, rat, goat and cow PII/P2 activities in luteinized bovine granulosa cells with the specific objective of determining the effects of NR5A2 and FOXL2, alone and in combination.

2. Materials and methods

2.1. Oestrogenic bovine granulosa cell culture

All materials were obtained from Invitrogen Life Technologies (Burlington, ON, Canada) unless otherwise stated. Bovine ovaries were obtained from adult cows, irrespective of stage of the oestrous cycle, at an abattoir and transported to the laboratory at 30 °C in phosphate-buffered saline (PBS). Follicles between 2 and 5 mm diameter were isolated from the ovaries and granulosa cells were collected by rinsing the follicle wall with MEM containing penicillin (100 IU/ml) and streptomycin (100 μg/ml). The cells were washed twice by centrifugation at 980*g* for 20 min each and suspended in MEM containing Hepes (15 mM), sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), BSA (0.1%; Sigma–Aldrich, Oakville, ON, Canada), penicillin (100 IU/ml), streptomycin (100 μg/ml), transferrin (2.5 μg/ml), non-essential amino acid mix (1.1 mM), insulin (10 ng/ml) and androstenedione (A4; 10^{-6} M). The number of cells was counted with a hemocytometer and the viable cells were assessed by the dye exclusion method using 0.1% Trypan Blue. Cells were placed in 24-well tissue culture plates (Sarstedt Inc.; Montréal, QC, Canada) at a density of 1×10^6 viable cells per well in 1 ml medium. Cultures were maintained at 37 °C in 5% CO_2 in air for 6 days with medium changes every 2 days.

The effect of gonadotrophins was tested by culturing cells with graded doses of FSH (AFP-5346D, National Hormone & Peptide Program, Torrance CA, USA) or IGF1 (R3 analog; Sigma–Aldrich) for 6 days of culture. To investigate intracellular pathways used to control *CYP19A1* promoter use, on day 4 of culture the cells were pretreated for 1 h with pathway inhibitors in medium devoid of FSH or IGF1, and then FSH (1 ng/ml) or IGF1 (10 ng/ml) was added for 2 days before recovery of RNA for PCR. The inhibitors were GF-109203X (3 μM), a protein kinase C (PKC) pathway inhibitor; LY-294002 (20 μM), a phosphoinositide 3-kinase (PI3K) inhibitor; PD-98059 (50 μM), a mitogen-activated protein kinase (MAPK) inhibitor or H89 (10 μM), a protein kinase A (PKA) pathway inhibitor.

The direct effect of FSH and IGF1 on MAPK and Akt activation was measured by Western blotting. Cells were cultured as above with FSH (1 ng/ml) or IGF1 (10 ng/ml), and on day 5 of culture cells were challenged with addition of FSH or IGF1, in 5 μl medium devoid of supplements, for 15 min. Controls received medium alone. Proteins were extracted in RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% NP-40 1 mM sodium orthovanadate and protease inhibitor cocktail).

2.2. Western blot

Samples (15 μg) were separated on 10% SDS–polyacrylamide gels and electrophoretically transfered to nitrocellulse membranes. Westerns for total ERK1/2, phospho-ERK1/2, total Akt and phospho-Akt (Cell Signaling Technology, Danvers MA, USA) were performed as described (Jiang et al., 2011). Bands were revealed by chemiluminescence and autoradiography. Semiquantitative analysis was performed with ImageJ software (NIH).

2.3. Total RNA extraction and real-time RT-PCR

After treatments, the culture medium was removed and total RNA was extracted using

Trizol according to the manufacturer's instructions. Total RNA was quantified by absorbance at 260 nm. Reverse transcription was performed on 1 μg DNase-treated total RNA in the presence of 1 mmol/l oligo(dT) primer and a primer specific for exon 3 of the *CYP19A1* gene (primer 3A in (Fürbass et al., 1997), 4 U Omniscript RTase (Qiagen, Mississauga, ON, Canada), 0.25 mmol/l dideoxynucleotide triphosphate (dNTP) mix, 19.33 U RNase Inhibitor (GE Healthcare, Baie D'Urfé, QC, Canada) in a volume of 20 μl at 37 °C for 1 h. The reaction was terminated by incubation at 93 °C for 5 min.

Real-time PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems, Streetsville, ON, Canada) with Power SYBR Green PCR Master Mix. Forward primers were located within the 5′UTR derived from *CYP19A1* promoters 1.1 and 2 and the common reverse primer was located in exon 3 as previously described (Fürbass et al., 1997). Thermal cycling conditions were 3 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C. Samples were run in duplicate and were expressed relative to the histone *H2AFZ* as housekeeping gene (Jiang et al., 2011). Data were normalized to a calibrator sample using the ΔΔCt method with correction for amplification efficiency (Pfaffl, 2001). Amplification efficiencies were 96% and 95% for P2 and P1.1, respectively.

2.4. Transient transfection of granulosa cells

Primary bovine granulosa cells were collected as described above and were seeded into 24-well tissue culture plates (Sarstedt Inc., Newton, NC, USA) at a density of 0.5×10^6 viable cells in 500 μl medium (as above but with 2% FBS and without insulin). Cultures were maintained at 37 °C in 5% CO_2, 95% air with a medium change on day 2. On day 4 of culture, at approximately 70% confluency, cells were transfected with 0.8 μg vector and 2 μl of Lipofectamine for 4 h at 37 °C. The *CYP19A1* promoter vectors used were the rat 245 bp rat proximal promoter (Cai et al., 2007), 1000 bp human promoter PII (kindly provided by Dr. Stocco, University of Illinois at Chicago), and 507 bp of the goat P2