

Detection and activity of 11 beta hydroxylase (CYP11B1) in the bovine ovary

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Abstract

Glucocorticoids (GCs) such as cortisol and corticosterone are important steroid hormones with different functions in intermediate metabolism, development, cell differentiation, immune response and reproduction. In response to physiological and immunological stress, adrenocorticotrophic hormone (ACTH) acts on the adrenal gland by stimulating the synthesis and secretion of GCs. However, there is increasing evidence that GCs may also be synthesized by extra-adrenal tissues. Here, we examined the gene and protein expression of the enzyme 11 β -hydroxylase P450c11 (CYP11B1), involved in the conversion of 11-deoxycortisol to cortisol, in the different components of the bovine ovary and determined the functionality of CYP11B1 *in vitro*. CYP11B1 mRNA was expressed in granulosa and theca cells in small, medium and large antral ovarian follicles, and CYP11B1 protein was expressed in medium and large antral follicles. After stimulation by ACTH, we observed an increased secretion of cortisol by the wall of large antral follicles. We also observed a concentration-dependent decrease in the concentration of cortisol in response to metyrapone, an inhibitor of CYP11B1. This decrease was significant at 10⁻⁵ μ M metyrapone. In conclusion, this study demonstrated for the first time the presence of CYP11B1 in the bovine ovary. This confirms that there could be a local synthesis of GCs in the bovine ovary and therefore a potential endocrine responder to stress through these hormones.

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Introduction

Glucocorticoids (GCs) are steroid hormones synthesized in the adrenal cortex during emotional or physical stress. Their production is regulated by the hypothalamic–pituitary–adrenal (HPA) axis, more specifically, by the production of corticotropin-releasing hormone (CRH) in the hypothalamus and of adrenocorticotrophic hormone (ACTH) in the anterior pituitary (Dejager *et al.* 2014). ACTH acts on the adrenal gland to increase the production and release of corticosterone, the main physiological GC in rats and mice, and cortisol, the main GC in mammals like humans and cows including other kinds of rodents (for example, the guinea pig, cavies and capybara) (Nemeth *et al.* 2014, Magomedova & Cummins 2016). GCs regulate the basal activity of the HPA axis and modulate the stress response by acting mainly at the hypothalamus and pituitary gland, through a negative feedback on the secretion of CRH and ACTH respectively (Charmandari *et al.* 2005).

GCs act through the glucocorticoid receptor, a member of the nuclear steroid receptor superfamily (Nicolaidis *et al.* 2010), and are essential for normal physiological function due to their regulatory effects

on intermediate metabolism, development, cell differentiation, immune response and reproduction (Davies & MacKenzie 2003, Duong *et al.* 2012, Dejager *et al.* 2014, Magomedova & Cummins 2016). GCs are also anti-inflammatory agents that act to modulate the production of cytokines and prostaglandins in different reproductive processes: ovulation, luteolysis, embryo implantation, fetal growth and placental development (Hillier & Tetsuka 1998, Andersen 2002, Myers *et al.* 2007, Tetsuka *et al.* 2010, Duong *et al.* 2012, Majewska *et al.* 2012, Kuse *et al.* 2013).

In response to stress, ACTH binds to its specific melanocortin receptor 2 (MC2R), a G-protein-coupled receptor, and activates the adenylyl cyclase cascade, leading to cAMP production and cAMP-dependent protein kinase activation, triggering the internal signaling cascade that is essential for the expression of steroidogenic enzymes and production of cortisol (Xing *et al.* 2010, Novoselova *et al.* 2013, Asser *et al.* 2014). In this sense, the conversion of cholesterol to pregnenolone occurs via the action of the cholesterol side chain cleavage cytochrome P450 (CYP11A1). After the activation of several enzymes, the final step

in cortisol biosynthesis takes place in mitochondria and involves the conversion of 11-deoxycortisol to cortisol by the enzyme 11 β -hydroxylase P450c11 (CYP11B1) (Hu *et al.* 2010, Xing *et al.* 2010). This enzyme, which is a member of the cytochrome P450 (P450) superfamily, has been extensively studied in mammals (Schiffer *et al.* 2015). In cattle, studies have revealed that the bovine genome encodes five CYP11B1 genes. Three of these represent pseudogenes and the two functional genes encode two distinct CYP11B1 variants, but with only slight functional varieties (Schiffer *et al.* 2015).

Classically, it has been described that GCs are synthesized exclusively in the adrenal gland in response to stimulation by ACTH. However, several studies have shown that these hormones are also synthesized locally in other organs and tissues (Taves *et al.* 2011). In the gut, for example, CYP11B1 is expressed at low levels under basal conditions, and GCs are produced in reduced amounts, possibly fulfilling important functions in the regulation of immune homeostasis and epithelial layer integrity (Taves *et al.* 2011, Kostadinova *et al.* 2014). It has also been shown that epidermal keratinocytes express CYP11B1 and synthesize and secrete cortisol, probably to provide control of the initial proinflammatory response and prevent excessive inflammation upon tissue injury (Taves *et al.* 2011, Vukelic *et al.* 2011). CYP11B1 is also expressed in different cells of the brain and cardiovascular system (Davies & MacKenzie 2003, Schmidt *et al.* 2009, Taves *et al.* 2011).

The access of active cortisol to target tissues is regulated by the relative expression of two 11 β -hydroxysteroid dehydrogenase (11BHSD) enzymes: a bidirectional 11BHSD type 1 (11BHSD1), which mainly activates cortisone to cortisol, and 11BHSD type 2 (11BHSD2), which inactivates cortisol to cortisone (Krozowski *et al.* 1999, Tetsuka *et al.* 2003). In the ovary, both luteinized human granulosa cells (Michael *et al.* 1997) and cumulus cells (Simerman *et al.* 2015) generate cortisol from cortisone via 11BHSD1 action to regulate lipid metabolism in the maturing ovarian follicle, crucial for optimal oocyte maturation. Also, both 11BHSD1 and 11BHSD2 mRNAs have been identified in the bovine ovary (Tetsuka *et al.* 2003, Amweg *et al.* 2013), and their expression and activity have been associated with follicular development and maturation (Thurston *et al.* 2007, Tetsuka *et al.* 2010). This suggests that cortisol might be involved in regulatory mechanisms related to ovarian function associated with ovulation, steroidogenesis and luteal function (Tetsuka 2007, Amweg *et al.* 2011, 2013). In addition, although it has not been demonstrated that the ovary is able to produce GCs *de novo*, ACTH is able to stimulate the secretion of cortisol in bovine ovarian follicles *in vitro* (Amweg *et al.* 2011, 2013).

Considering this premise, the aim of the present study was to evaluate the expression of CYP11B1 in different components of the bovine ovary and to determine the *in vitro* activity of CYP11B1.

Materials and methods

Ethical aspects

All the procedures were carried out according to the 'Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching' (2010) and with the approval of the Institutional Ethics and Security Committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Argentina (Protocol N° 67/10).

Collection and preparation of tissues

Ovaries from 40 randomly cycling Argentinean Holstein cows, assessed visually as non-pregnant, with no apparent abnormality in the reproductive system, and with normal follicular activity (i.e. ovaries macroscopically showing different stages of follicles and without active corpora lutea) were collected at a local abattoir, within 20 min after death and quickly transported to the laboratory in conservative boxes with refrigerants over a period of time no longer than 60 min. Also, adrenal samples were collected from five cows and were used as positive controls.

In the laboratory, each pair of ovaries was washed, and then, the antral follicles were removed using scissors and scalpel dissection. Antral follicles from normal cycling cows were classified into three categories according to their diameters as described previously (Parrott & Skinner 1998): small (<5 mm, $n=15$), medium (5–10 mm, $n=15$) or large (>10 mm, $n=15$). Follicles with an obviously atretic appearance (avascular theca and debris in the antrum) were discarded. At the laboratory, for RT-PCR and real-time PCR, granulosa cells were obtained by centrifugation of follicular fluid, aspirated with a syringe from the different antral follicles and immediately frozen at -80°C until total RNA extraction. The follicular fluid was stored at -20°C to corroborate the initial hormone levels in large antral follicles as described later. Theca cells of antral follicles were washed with diethyl-pyrocabonate (DEPC) water (Genbiotech, Buenos Aires, Argentina) to remove granulosa cells and stored at -80°C until total RNA extraction.

For Western blot, small samples from antral follicles ($n=10$) and adrenal glands ($n=5$) were immediately frozen at -80°C .

For tissue culture, ovaries ($n=6$) were processed as detailed below.

Total RNA extraction

Total RNA was isolated from granulosa and theca samples after treatment with TRIzol LS Reagent (Invitrogen, Life Technologies Corporation), according to the manufacturer's instructions, with slight modifications. Also, adrenal samples were processed as positive controls. Briefly, 50–100 mg of tissue or pellet of granulosa cells was homogenized with 750 μL of TRIzol LS Reagent (Invitrogen) and incubated for 10 min at 25°C . Then, RNA was obtained by vigorously homogenizing with chloroform and incubating for 15 min at 4°C . After centrifugation at 12,000g, the aqueous phase was incubated overnight with an equal volume of isopropyl alcohol at -20°C and centrifuged at 12,000g to obtain the pellet of RNA, which was then washed with 75 vol/vol ethanol

for 10 min at 4°C. Alcohol was replaced by DEPC water (Genbiotech) and prewarmed at 60°C. The extracted RNA was DNase treated with deoxyribonuclease I (Invitrogen) to eliminate contaminating DNA and was stored at -80°C for further use.

Reverse transcription

First-strand cDNA was synthesized in a 20 µL reaction mixture using 1 µg of total RNA samples treated with DNase I (Invitrogen). The reaction tubes contained random hexamers (1 µg/L), dNTPs (25 mM), reaction buffer 5×, dithiothreitol (0.1 M), ribonuclease inhibitor (40 U/µL) and M-MLV reverse transcriptase (200 U/µL) (Invitrogen). The reverse transcription conditions were as described in a previous study (Amweg et al. 2011).

PCR primer design

Bovine sequences for *CYP11B1*, cytochrome P450 aromatase (*CYP19A1*) and cytochrome P450 17hydroxylase/17,20-lyase (*CYP17A1*) were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/Entrez/index.htm>). Specific primers were designed using the PrimerSelect program in the LASERGENE software (DNASar, Madison, WI, USA). The primers were purchased from Invitrogen, and the sequences are summarized in Table 1. Oligonucleotide primers and amplification products were tested using the Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/BLAST>) software to confirm gene specificity and determine exon locations, assuring that they were not designed from any homologous regions coding for other genes. The primer sequences for *CYP19A1* and *CYP17A1* were designed and validated to confirm the purity of mRNA in granulosa and theca cells respectively (no cross-contamination) (Lagaly et al. 2008). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene (Shibaya et al. 2007).

End point PCR

An optimized end point PCR protocol was used to analyze the mRNA expression of *CYP19A1* and *CYP17A1*. PCR was carried out in a final volume of 25 µL containing 10 ng cDNA (previously quantified by the Qubit method (Invitrogen)),

1.5 mM MgCl₂, 0.5 µM forward primer, 0.5 µM reverse primer, 0.2 mM dNTP, Taq buffer 10× and 2 U Taq polymerase (5 U/µL) (Invitrogen). The reaction conditions were as described in a previous study (Amweg et al. 2011). Only granulosa samples positive for *CYP19A1* mRNA and negative for *CYP17A1* mRNA and theca samples positive for *CYP17A1* mRNA and negative for *CYP19A1* mRNA expression were used to detect *CYP11B1* mRNA expression. In addition, the negative control performed using water instead of cDNA controls was negative to the reaction and the positive control using adrenal tissue was positive for *CYP11B1* mRNA.

Real-time PCR

An optimized real-time PCR protocol was used to analyze the mRNA expression of *CYP11B1*, using SYBR Green I (Invitrogen) technology in a LightCycler Detection System (Pfaffl 2001). Transcript levels were measured by relative quantitative real-time PCR using a StepOne Real-Time PCR System (Applied Biosystems, Life Technologies Corporation). For *CYP11B1*, the following standardized protocol was used: initial denaturation at 98°C for 3 min, followed by 40 cycles of denaturation at 98°C for 5 s, annealing at 58°C for 15 s, extension at 72°C for 20 s and fluorescence reading at 80°C for 10 s. A master mix of the reaction components was prepared using SYBR Green I (Invitrogen). Each PCR reaction was performed in a final volume of 20 µL containing 4 µL cDNA and 16 µL of a reaction mixture: forward primer (20 µM), reverse primer (20 µM), 0.2 mM of each dNTP, 1 µL SYBR Green I, 4 µL buffer 5× and 0.05 µL enzyme Phire (Hot Start II DNA Polymerase, Thermo Fisher Scientific Company). For *GAPDH*, the following standardized protocol was used: initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 52°C for 20 s, extension at 72°C for 30 s and fluorescence reading at 74°C for 10 s. Each PCR reaction was performed in a final volume of 20 µL containing 4 µL cDNA and 16 µL of a reaction mixture: forward primer (20 µM), reverse primer (20 µM), 0.2 mM of each dNTP, 1 µL SYBR Green I, 2 µL buffer TAQ 10×, 50 mM Cl₂Mg and 0.32 µL Taq DNA Polymerase (Invitrogen). All measurements for each sample were obtained in duplicate. The efficiency of the PCR and the relative quantities of cDNA were determined from a six-point standard curve. Standard curves were constructed from 50% serial dilutions of a cDNA mixture of granulosa

Table 1 Primer sequences, regions of the target genes and conditions used for real time RT-PCR.

Name	Sequence (5'–3')	Gene accession no.	Amplicon position	Amplicon size (bp)	Annealing temp. (°C)	No. cycles
<i>CYP11B1</i>						
Forward	CCCTGACAGCCTGAACCTTA	NM_174638	656–767	112	58	40
Reverse	CCTCCACATGTTGGTGCTCA					
<i>GAPDH</i>						
Forward	CACCCTCAAGATTGTCAGCA	BC102589	492–594	103	52	35
Reverse	GGTCATAAGTCCCTCCACGA					
<i>CYP17</i>						
Forward	GGAGGCGACCATCAGAGAAGTGC	NM_174304	1105–1423	319	60.8	35
Reverse	CAGCCGGGACATGAAGAGGAAGAG					
<i>CYP19</i>						
Forward	TAAACAAAGCGCCAATCTCTACG	BTCYP19	8–348	341	55.4	35
Reverse	GGAACCTGCAGTGGGAAATGA					

and theca cells, and PCR efficiency was calculated using the StepOne software v2.2 (Applied Biosystems). In standard curves, R₂: 0.99 for *CYP11B1* and R₂: 0.98 for GAPDH. About 20 ng cDNA, previously quantified by the Qubit method (Invitrogen), was used. The mRNA expression levels of genes were recorded as cycle threshold (C_t) values that corresponded to the number of cycles at which the fluorescence signal can be detected above a threshold value. The C_t was calculated manually using StepOne v2.2 (Applied Biosystems). Negative DNA template controls were included in all the assays and yielded no consistent amplification. The purity of the product was confirmed by melting curve analysis to obtain a specific temperature dissociation product of 86.76°C and 85°C for *CYP11B1* and GAPDH respectively. Random samples were subjected to agarose gel electrophoresis. Fold change was determined using the 2^{-ΔΔC_t} method (Livak & Schmittgen 2001).

Nucleotide sequencing

The specificities of the PCR products were checked by direct sequencing to ensure amplification of the correct sequences using the Macrogen Sequencing Service (Macrogen, Korea). The resulting sequences were verified using the MegAlign tool in the LASERGENE software (DNASar). Homology with bovine sequences was 98%.

Production of CYP11B1 antibody

To evaluate CYP11B1 protein expression, an affinity-purified rabbit polyclonal antibody was generated using previously described protocols (Monje et al. 2009). The antigen was expressed in *Escherichia coli* JM109 (Stratagene Corp.) as a glutathione-S-transferase fusion protein, using a pGEX4T-3 vector (Stratagene). The 255-bp region formed between bases 370 and 624 was amplified from the coding sequence of bovine *CYP11B1* (NM_174638.3). This region encodes the peptide GHKCGVLLNGPQWRDLRLNPDVLSLPALQKYTPLVDGVARDFSQTLKARVLQNARGSLTLGHRAQLFRYTIEASTLVLYGER belonging to the antigenic region of the molecule. The antibody was purified using antigen-linked affinity chromatography (Hi-Trap NHS activated HP column; GE Healthcare). The homology between the target peptide of the antibody and the corresponding bovine protein was tested with the BLAST software to determine the peptide location and to confirm antigen specificity.

Western blot

To determine CYP11B1 protein expression, bovine ovarian tissue and adrenal samples were homogenized in lysis buffer with a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets; Roche), as previously described (Salveti et al. 2012). The proteins were separated by SDS-PAGE (12% resolving gel) as described previously (Ortega et al. 2009, Salvetti et al. 2010). Proteins were transferred to nitrocellulose membranes (GE Healthcare), blocked for 5 h in 5% wt/vol nonfat milk in Tris-buffered saline that contained 0.05% vol/vol Tween 20 (Sigma-Aldrich) and then incubated overnight at 4°C with specific CYP11B1 polyclonal primary antibody at

a 1:1000 dilution. After washing, membranes were treated for 1 h with the corresponding goat anti-rabbit IgG peroxidase-conjugated antibody (Santa Cruz Biotechnology), at a 1:7500 dilution. The immunopositive bands were visualized with a chemiluminescent detection kit (ECL Prime Western Blotting Detection Reagent, GE Healthcare).

Functionality of CYP11B1 in vitro: secretion of steroids by the follicular wall

In a laminar flow hood, ovaries were rinsed, and large antral follicles were dissected using a scalpel. Follicular fluid from each selected follicle was aspirated and stored separately at -20°C for 17β-estradiol, progesterone and testosterone determination to confirm their health status by the hormone levels.

Each large antral follicle was sectioned into five fragments of 100 mg each (one fragment destined to culture basal and four fragments to be treated with ACTH). The fragments were cultured on 24-well (1 mL medium/well) culture plates (Nunc plate, Applied Scientific, South San Francisco, CA, USA) and incubated in RPMI-1640 buffer (Gibco, ThermoFisher Scientific) with ascorbic acid 1% (Gibco, ThermoFisher Scientific) and antibiotic/antimycotic 1% (Gibco, ThermoFisher Scientific). The culture medium of four fragments was supplemented with 100 nM ACTH (Laboratorios ELEA, Buenos Aires, Argentina). This concentration has been described for a high cortisol secretion response in antral follicles in *in vitro* follicular wall cultures (Amweg et al. 2011). Additionally, three fragments were treated alternatively with 0.1, 1 or 10 μM metyrapone (Sigma-Aldrich), an inhibitor of CYP11B1. As a control (basal secretion), tissues were incubated without the supplementation of ACTH or metyrapone. Each set of cultures (n=6) was performed under identical conditions. The fragments were incubated for 7 h at 37°C. Then, the culture medium was aspirated from each well and maintained at -20°C until assayed.

Hormone assays

The concentrations of estradiol, progesterone, testosterone and cortisol in culture medium and of estradiol, progesterone and testosterone in follicular fluid were measured by electrochemiluminescence immunoassay kits (Roche Diagnostics GmbH) in a cobas e411 system (Roche

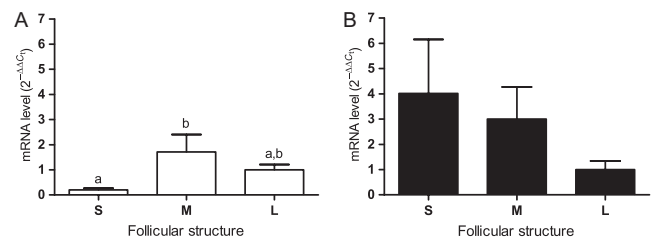


Figure 1 Relative quantification of CYP11B1 mRNA by real-time PCR in granulosal (A) and theca (B) cells of small (S), medium (M) and large (L) antral follicles. The 2^{-ΔΔC_t} values are represented as means ± S.E.M. Bars with different letters are significantly different (P < 0.05).

Diagnostics), according to the manufacturer's instructions and previous validation (Díaz *et al.* 2015). The assay sensitivity was 5 pg/mL for estradiol, 0.03 ng/mL for progesterone, 0.02 ng/mL for testosterone and 0.308 µg/dL for cortisol. According to the manufacturer's specifications, cortisol has 7% cross-reactivity with cortisone. The concentrations of 17-hydroxyprogesterone in culture medium were measured by a commercially available competitive RIA without extraction (Immunotech, Marseille, France) with a sensitivity of 0.05 ng/mL.

Steroid concentrations in follicular fluid showed that all the large antral follicles used in the culture assays were estrogen active (estrogen:progesterone ratio >1) and non-atretic (estrogen:testosterone ratio >1).

Statistical analyses

The statistical software package SPSS 11.0 for Windows (SPSS) was used to analyze the results of steroid concentrations in culture medium. The differences between groups of data were assessed by one-way ANOVA, followed by Duncan's multiple range tests. Regression analysis was used to determine the concentration–response effects. For all statistical analyses, $P < 0.05$ was considered significant. Results are expressed as means ± S.E.M.

Results

CYP11B1 gene expression

CYP11B1 mRNA expression was compared in real-time PCR. GAPDH mRNA expression was similar in the different groups evaluated ($P > 0.05$), which confirmed that the cDNA quantities used were equivalent.

CYP11B1 mRNA was expressed in granulosa and theca cells in small, medium and large antral follicles (Fig. 1). CYP11B1 mRNA expression in granulosa cells was lower in small than that in medium antral follicles ($P < 0.05$; Fig. 1A). In contrast, no significant differences in CYP11B1 mRNA expression were observed in the different follicular categories analyzed in theca cells ($P > 0.05$; Fig. 1B).

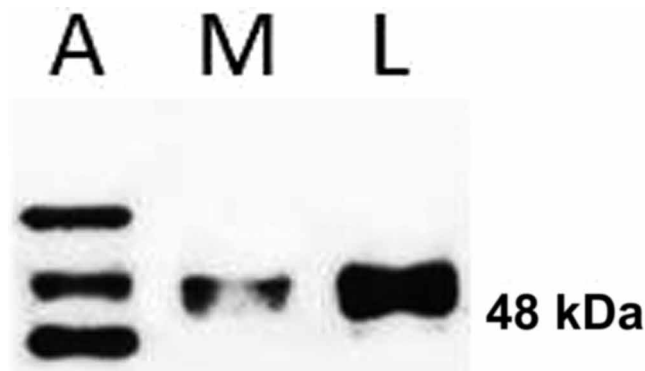


Figure 2 Representative image of a western blot showing expression of CYP11B1 of medium (M) and large (L) antral follicles and adrenal gland (A). The molecular weight of the mature form detected in follicular walls is indicated.

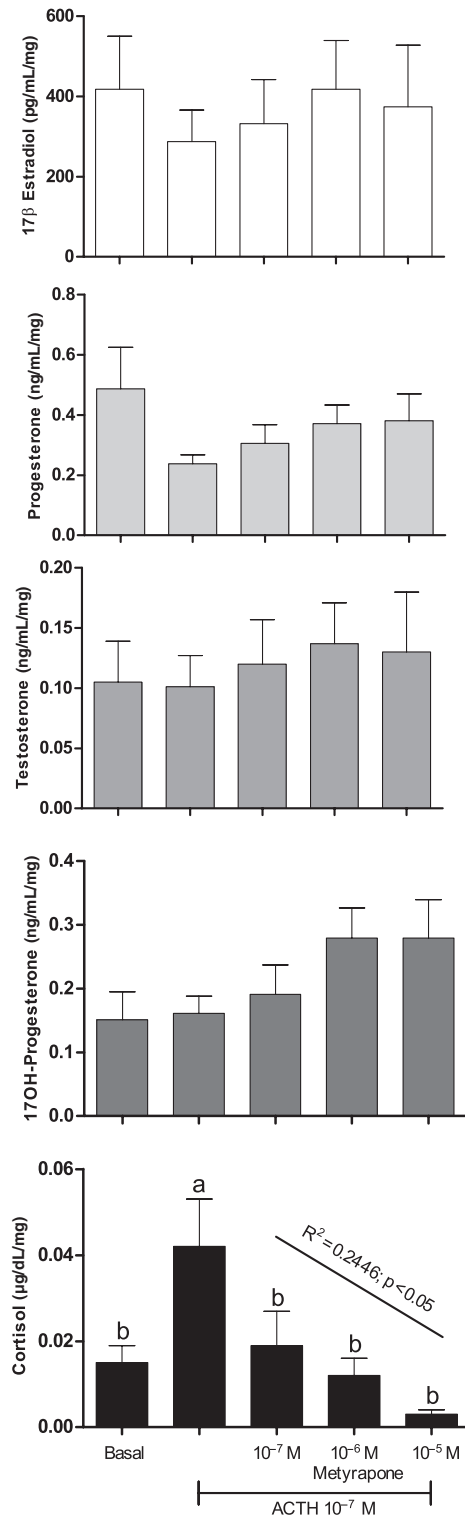


Figure 3 Effects of ACTH on the secretion of estradiol, progesterone, testosterone, 17-hydroxyprogesterone and cortisol by the follicular wall of large antral follicles. Bars represent the mean ± S.E.M. Bars with different letters are significantly different ($P < 0.05$). Concentration–response effects are also indicated with a regression line.

CYP11B1 protein expression

The expression of CYP11B1 by Western blot in bovine ovarian tissue and adrenal samples is showed in Fig. 2. The CYP11B1 antibody recognized a band of 48kDa, corresponding to the mature form of CYP11B1 enzyme, confirming the translation of mRNA detected by real-time PCR. On the other hand, in samples of bovine adrenal, a pattern of bands was obtained (Fig. 2) in agreement to that described by Morohashi (Morohashi *et al.* 1990).

Functionality of CYP11B1 in vitro

Steroid concentrations in the culture medium from the follicular wall of antral follicles are presented in Fig. 3. After 7 h of incubation, basal concentrations of secreted steroids were detected in all samples, with a significantly higher ($P < 0.05$) concentration of cortisol in the culture medium of ACTH-stimulated follicular wall portions relative to the control and those treated with different concentrations of metyrapone ($P < 0.05$). In response to metyrapone, the concentration of cortisol decreased in a concentration-dependent manner. This decrease was significant ($P < 0.05$) at 10^{-5} μM metyrapone (Fig. 3).

Discussion and conclusions

In this study, we found clear evidence that mRNA and protein for CYP11B1 are expressed in bovine ovaries. This suggests that the ovary is an extra-adrenal site of GC synthesis. In this sense, Murata and coworkers (Murata *et al.* 2011) and Raghuvver and coworkers (Raghuvver *et al.* 2011) reported that CYP11B1 is expressed in gonads during sexual differentiation in fish. Also, Yazawa and coworkers (Yazawa *et al.* 2008) reported the expression of CYP11B1 mRNA and protein after human chorionic gonadotropin (hCG) treatment in murine testes and ovaries. On the other hand, Fru and coworkers (Fru *et al.* 2006) found no expression of CYP11B1 in the ovaries of rhesus macaques. These results differ from those found in our work in cattle. This could be because synthesis and metabolism of cortisol in the ovary of rhesus macaques may differ from those of cattle. 11-Deoxycortisol was not detectable in follicular fluid before hCG administration or at 3 h after hCG administration, and CYP11B1 mRNA was not detected before or after hCG administration. However, we showed that CYP11B1 mRNA was expressed in granulosa and theca cells in small, medium and large bovine antral follicles and that protein was expressed in medium and large antral follicles. Furthermore, a significant decrease in cortisol concentration was observed after inhibition by metyrapone. This is the first description of the expression of this key enzyme in the cortisol metabolism in the bovine ovary.

Ovulation involves active remodeling of the extracellular matrix (EMC) to facilitate follicle rupture

prior to luteinization, to increase blood flow and vascular permeability, and to release the oocyte (Richards *et al.* 2008, Zackrisson *et al.* 2011). This process is considered similar to an inflammatory response, in which expression of many proinflammatory genes, including cytokines such as interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor- α (TNF- α), is increased (Field *et al.* 2014). In addition, specific components of the ECM and basal membrane can be altered through cleavage by matrix metalloproteinases (MMPs), such as collagenase (i.e. MMP-1, -8 and -13) or gelatinase (MMP-2 and -9), whose activities are inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Smith *et al.* 2002). Ovulation is a form of natural injury in which gonadotropin induces an inflammatory cascade within the ovary that terminates in proteolytic breakdown of the follicle wall and follicular rupture (Rae *et al.* 2009). The generation of anti-inflammatory GCs during ovulation would help to limit the inflammatory process in the ovary (Hillier & Tetsuka 1998, Andersen 2002, Michael *et al.* 2003), and their actions could be due to a paracrine regulation of these hormones that may play an important physiological role in ovarian homeostasis. However, extreme situations could affect the normal ovulatory mechanism, altering the estrous cycle and blocking ovulation. In this sense, it has been demonstrated that ACTH increases endogenous cortisol secretion, thus inducing follicular persistence and cyst formation in cattle (Dobson *et al.* 2000, Salvetti *et al.* 2010, Amweg *et al.* 2013).

ACTH is produced by cleavage from its precursor pro-opiomelanocortin, and released into the circulation to act on peripheral sites, mainly the adrenal glands, to stimulate CYP11B1 transcription and GCs production. A significantly higher concentration of cortisol was observed in the culture medium of follicular wall stimulated with ACTH, considering that a percentage could be due to the inert metabolite cortisone due to 7% of cross-reactivity of immunoassay. These results are consistent with those found previously *in vitro* by our group, where we showed that ACTH is able to stimulate the secretion of cortisol by the wall of ovarian follicles (Amweg *et al.* 2011, 2013). Moreover, we found the presence of MC2R in the bovine ovary, suggesting that ACTH may have an important role as a modulator of gonadal function via MC2R (Amweg *et al.* 2013). However, these results did not allow determining whether the increase in cortisol concentration was due to *de novo* synthesis or to conversion from cortisone by 11BHSD. Nevertheless, the results of the present manuscript suggest that the bovine ovary could have *de novo* synthesis of GCs.

GCs have potent anti-inflammatory and immunosuppressive activity. Their action could not only be a product of the modulation between active and inactive forms of corticosteroids as had been described previously (Thurston *et al.* 2007, Tetsuka *et al.* 2010),

also there is evidence that they are locally produced in extra-adrenal organs (Davies & MacKenzie 2003, Taves *et al.* 2011, Vukelic *et al.* 2011, Kostadinova *et al.* 2014). Studies in the intestinal mucosa suggest that the intestinal expression of steroidogenic enzymes and the synthesis of GCs could be triggered in response to strong immune cell activation. This suggests a direct relationship between the activation of immune cells, the release of inflammatory mediators and the induction of steroidogenesis in the intestinal epithelium (Kostadinova *et al.* 2014). Thus, a contribution to the maintenance of local immune homeostasis and the regulation of inflammatory processes might be effective (Kostadinova *et al.* 2014). On the other hand, epidermal keratinocytes synthesize and secrete cortisol *in vitro* and *in vivo* and express CYP11B1, suggesting that epidermal synthesis of cortisol *in situ* constitutes a negative feedback mechanism to prevent the inflammatory response caused by epidermal injury (Vukelic *et al.* 2011).

CYP11B1 is required for the final step of GCs synthesis. Thus, we evaluated *in vitro* the functionality of this enzyme through metyrapone, a specific inhibitor of 11-hydroxylase activity, used to diagnose/treat hypercortisolism as well as to test the functional integrity of the HPA axis (Parthasarathy & Balasubramanian 2008). We found significantly higher concentration of cortisol in the culture medium of ACTH-stimulated follicular wall relative to the control, and a concentration-dependent decrease in the concentration of cortisol, due to the inhibition by metyrapone. The results obtained in the *in vitro* study showed that the bovine ovary is able to respond to the ACTH released as a consequence to stress and also that the ovaries have steroidogenic enzymes responsible for the synthesis and secretion of GCs. Systems activated by stress can influence reproduction at the hypothalamus, pituitary gland or gonads (Tilbrook *et al.* 2000). Particularly, cortisol can affect reproduction through its actions at the hypothalamic level, affecting the secretion of gonadotropin-releasing hormone in the pituitary gland by inhibiting the secretion of gonadotropins, especially LH, and in the ovaries, by affecting the ovarian functions (Tilbrook *et al.* 2000, Tetsuka *et al.* 2010, Ralph *et al.* 2016). Based on the results here obtained, we propose that CYP11B1 could synthesize cortisol which is then able to exert local anti-inflammatory actions in the bovine ovary. Also, these actions could be additionally regulated in the ovary by the availability of active cortisol via 11BHS1 (Tetsuka *et al.* 2010). In this sense, we have previously demonstrated that all follicular stages in the bovine ovary express 11BHS1 and 11BHS2 (Amweg *et al.* 2013) with increased expression of 11BHS1 in granulosa as follicles mature (Amweg *et al.* 2013).

In the bovine ovary, the high expression of TNF- α found in the granulosa cells of primary and preantral bovine follicles and low expression in the granulosa

cells of antral and atretic follicles (Baravalle *et al.* 2015) could be related to the presence of CYP11B1 able to synthesize cortisol, as we demonstrated in ACTH-stimulated bovine ovary. In addition, previous studies have demonstrated a high expression of glucocorticoid receptor in the granulosa cells from atretic follicles in relation to that in primary, secondary and tertiary follicles, as well as to that in the theca interna cells of all follicular categories (Amweg *et al.* 2016). In this sense, after the interaction with glucocorticoid receptor, GCs are able to inhibit the synthesis of multiple inflammatory proteins through suppression of the genes that encode them (Cosío *et al.* 2005, Barnes 2006).

In conclusion, this study demonstrated that CYP11B1 is present in the bovine ovary. This confirms that there could be a local synthesis of GCs in the bovine ovary and therefore a potential endocrine response to stress through these hormones. An increase in the local concentration of GCs in the ovary could play a protective mechanism to control the deleterious effects of excessive production of inflammatory mediators. Moreover, through their anti-inflammatory effects, local GCs may be involved in the regulatory mechanisms related to ovulation, ovarian steroidogenesis and luteal function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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