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Impaired insulin signaling pathways affect ovarian steroidogenesis in cows with COD

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ABSTRACT

Cystic ovarian disease (COD) represents an important cause of infertility in dairy cattle and is associated with multiple physiological disorders. Steroidogenesis, which is necessary to ensure normal ovarian functions, involves multiple enzymatic pathways coordinated by insulin and other proteins. We have previously shown that cows with COD have an altered insulin response. Therefore, in the present study, we evaluated further alterations in intermediates downstream of the PI3K pathway and pathways mediated by ERK as critical signals for the expression of steroidogenic enzymes in the ovaries of control cows and cows with spontaneous COD. To this end, we evaluated the gene and protein expression of pan-AKT, mTOR, ERK1/2, and steroidogenic enzymes by real-time PCR and immunohistochemistry. Steroid hormone concentrations were assessed at systemic and intrafollicular level. Results showed altered expression of intermediate molecules of the insulin signaling pathway, whose action might modify the synthetic pathway of steroidogenic hormones. Similarly, the expression of steroidogenic enzymes and the concentration of progesterone in serum and follicular fluid were altered. These alterations support the hypothesis that systemic factors contribute to the development and/or maintenance of COD, and that metabolic hormones within follicles such as insulin exert determinant effects on ovarian functionality in cows with COD.

1. Introduction

In dairy cattle, cystic ovarian disease (COD) is associated with multiple physiological disorders, including alterations in folliculogenesis, steroidogenesis, and ovulation, with many coexisting factors, such as stress, herd management, nutritional status, body condition and infectious disease (Silvia et al., 2002). Understanding the mechanisms underlying the development of COD is of great interest because this disease represents an important cause of infertility in dairy cattle and causes economic losses to the dairy industry due to the increase in the calving-to-conception and inter-calving intervals (Peter, 2004). However, key points of COD pathogenesis remain unknown.

Steroidogenesis, which is necessary to ensure the normal functions of the ovaries, involves multiple enzymatic pathways (Wood and Strauss, 2002; Silva et al., 2006). One of the limiting steps in the biosynthesis of steroid hormones involves the steroidogenic

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acute regulatory (StAR) protein, which facilitates the translocation of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. Another limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone in mitochondria, which is a step under hormonal regulation (Stocco et al., 2001). In the ovary, the conversion of cholesterol to pregnenolone is achieved by the intramitochondrial cholesterol side-chain cleavage enzyme commonly known as CYP11A1 (Strauss et al., 1999; Shih et al., 2011). Besides, a close correlation between the tissue-specific expression of the StAR gene and the tissue capacity to produce steroids suggests a specific role of StAR in steroidogenesis (Castillo et al., 2015). Both gene and protein expression of StAR is an intriguing issue, which includes different factors, hormones and kinases (Ivell et al., 2000; Chen et al., 2007; Castillo et al., 2015).

In ruminants, the delta 5 pathway is a predominant steroidogenic route, in which dehydroepiandrosterone (DHEA) and androstenedione are synthesized from pregnenolone by 17α-hydroxylase/C17-20 lyase cytochrome P450 (CYP17A1), which is primarily expressed in theca interna cells (Duggabatti et al., 2006; Lima et al., 2015), and 3-beta-hydroxysteroid dehydrogenase (3β-HSD), respectively (Conley and Bird, 1997; Sahmi et al., 2004). After both peroxidation and epoxidation reactions, androstenedione could be reduced by 17-beta-hydroxysteroid dehydrogenase (17β-HSD) to testosterone, and these two last steroids are finally converted in estrone and estradiol, respectively, through an aromatization catalyzed by cytochrome P450 aromatase A1 (CYP19A1), which is expressed mainly in granulosa cells (Wood and Strauss, 2002; Sahmi et al., 2004; Duggabatti et al., 2006; Padmanabhan et al., 2014; Lima et al., 2015).

The regulatory mechanisms involved in ovarian follicular development and steroidogenesis are still unclear. Fukuda et al. (2009) showed that luteinizing hormone (LH) stimulates CYP17 mRNA expression and androgen production in bovine theca cells via the activation of the phosphatidylinositol 3-kinase (PI3K/AKT) pathway. Follicle stimulating hormone (FSH) can also activate the PI3K pathway and a delicate interplay has been shown to exist between cAMP/protein kinase A (PKA) and PI3K signaling in the regulation of steroidogenesis by FSH and transforming growth factor (TGF) β1 in rat granulosa cells (Chen et al., 2007). Moreover, Silva et al. (2006) reported that, at physiological doses, FSH and insulin stimulate CYP19 mRNA and estradiol secretion predominantly through cAMP signaling. In bovine theca cells, it has been reported that an inhibition of ERK phosphorylation increases progesterone production and decreases androstenedione production concomitantly with a modulation of StAR and CYP17 expression (Tajima et al., 2005). In mouse cell lines, Duarte et al. (2014) demonstrated that mitochondrial StAR and mitochondrial active ERK and PKA are necessary for maximal steroid production.

We have previously found insights into the insulin response that might interact in ovarian physiology in sheep (Ortega et al., 2010) and cows with COD (Hein et al., 2015). In the latter study, we found that while the mRNA expression of insulin receptor (*IR*) and IR substrate-1 (*IRS1*) is higher in granulosa cells of control antral follicles than in follicular cysts, the *PI3K* mRNA levels are similar in all follicles evaluated. We also found that the gene expressions of *IR*, *IRS1* and *PI3K* are similar in theca cells and that the protein expression of IR is higher in control antral follicles than in the same structures in cows with COD and cysts. Our results also showed that IRS1 and PI3K protein expressions show the same pattern in granulosa cells of antral and cystic follicles. However, the subunit alpha p85 protein expression of PI3K is higher in theca cells from antral follicles than in cystic follicles (Hein et al., 2015). Accordingly, the lower gene and protein expressions of some insulin downstream effectors at an early stage of the signaling pathway could negatively influence the functionality of the ovaries and contribute to cyst development and maintenance. Therefore, in the present work, we aimed to study further alterations of downstream factors from both the PI3K pathway and pathways mediated by ERK as a critical signal in the expression of steroidogenic enzymes in the ovary of cows with COD.

2. Materials and methods

2.1. Ethical aspects

All procedures were performed with the approval of the Institutional Ethics and Security Committee of the Facultad de Ciencias Veterinarias – Universidad Nacional del Litoral, Argentina, protocol N° 134/12, and are consistent with the “Guide for the Care and Use of Agricultural Animals in Research and Teaching” (McGlone et al., 2010).

2.2. Animals

This study was performed in non-lactating Holstein cows from dairy herds of the milk-producing region of Santa Fe, Argentina, all of which had calved at least once. Cows were obtained at the end of lactation from local commercial farms and housed outside in an open lot, except during blood collection or ultrasound examinations (when they were moved to a stanchion barn). Cows were fed *ad libitum* with a diet based on alfalfa pasture, oat, or rye grass grazing supplemented with corn silage, alfalfa silage, corn grain, soybean expeller and hay, following the recommendations of the Nutrient Requirements of Dairy Cattle (Nutrient Requirements of Dairy Cattle, 2001). Besides, non-pregnant Holstein cows without macroscopic abnormalities in the reproductive system and with absence of corpora lutea (CL) in the two ovaries were collected from a local abattoir.

2.2.1. Groups evaluated

2.2.1.1. Control cows. Ovarian activity of cows with regular estrous cycles was synchronized with the G6G protocol (Bello et al., 2006), with some modifications (Díaz et al., 2015). The beginning of estrous was confirmed by rectal examination and ultrasonography, and designated as Day 0 of the cycle (Dobson and Smith, 2000; Ortega et al., 2008). Ovarian ultrasonographic examinations were performed using a real-time B-mode scanner equipped with a 5.0 MHz linear-array transrectal transducer (Honda HS-101V, Japan), as previously described (Rodríguez et al., 2013; Matiller et al., 2014). The growth and regression of

follicles > 5 mm and CL were daily monitored by ovarian ultrasonography (Amweg et al., 2013). Five of these synchronized control cows were ovariectomized (approximately on Day 18) when the dominant follicle reached a diameter greater than 10 mm, in the absence of an active CL following surgical protocols previously described (Marelli et al., 2014). Additionally, 10 synchronized control cows in proestrus were only aspirated to obtain follicular fluid (FF) rich in follicular cells. Follicles were aspirated using a digital ultrasound system (8300vet Chison, Chison Medical Imaging Co., China) equipped with a microconvex transducer of 5.0 MHz mounted on a transvaginal probe for follicular aspiration (Watanabe Applied Technology Ltd., Cravinhos, Sao Paulo, Brazil). FF samples were collected, cooled on ice and immediately transported to the laboratory and stored at −80 °C (Hein et al., 2015).

2.2.1.2. Spontaneous COD cows. Cows with spontaneous COD were diagnosed during the periodic reproductive control by rectal palpation and ultrasonography (HS-101V, Honda Co., Japan). A COD case was defined as a cow with a single cyst of 20 mm or more in diameter (i.e. more than the preovulatory follicle size), which persisted for at least 10 days in the absence of CL and lack of uterine tonicity (Silvia et al., 2002; Bartolome et al., 2005; Vanholder et al., 2006; Peter et al., 2009). Ten of these cows were ovariectomized and 15 were aspirated, following the protocols described above. Ovaries were removed as previously described (Marelli et al., 2014). Briefly, the procedure was done in sedated (150 mg of 10% ketamine hydrochloride, Alfasan, Santa Fe, Argentina) and epidurally anesthetized animals (2% lidocaine, Vetue, Santa Fe, Argentina). Before ovariectomy, follicles were aspirated to prevent the breakage of the cyst by using a digital ultrasound system 8300vet Chison equipped with a micro-convex transducer of 5.0 MHz mounted on a transvaginal probe for follicular aspiration (Watanabe Applied Technology Limited, Sao Paulo, Brazil). FF was refrigerated and immediately transported to the laboratory for processing. After cleaning the perineal area and disinfecting the vaginal canal with 5% chloroxylenol solution, the vaginal vault was separated from the rectum by using a vaginal tensor device to avoid their contact. A 5-cm longitudinal incision on the vaginal vault was performed with a hidden dismountable blade scalpel, followed by location and retraction of the uterus along with the ovaries into the vagina. Blood vessels were ligated by transfixion and finally the ovaries were removed. To prevent possible infections, animals were treated with antibiotics and anti-inflammatory drugs. After excision, the ovaries were refrigerated and immediately transported to the laboratory for processing as described above.

2.2.1.3. Cow samples from a local abattoir. As mentioned above, non-pregnant Holstein cows without macroscopic abnormalities in the reproductive system and with absence of active CL in the two ovaries were obtained from a local abattoir. The complete pairs of ovaries (n = 20) were collected within 20 min of slaughter and then refrigerated and immediately transported to the laboratory as previously described (Marelli et al., 2014). Follicular diameter was measured and FF from each follicle was stored separately until analysis. Antral follicles were classified into three categories according to their diameters: small (< 5 mm), medium (5–10 mm) or large (> 10 mm) (Parrott and Skinner, 1998). Follicular cysts (n = 11) were defined as follicles larger than 20 mm in diameter that occurred in the absence of a functional CL in either the right or left ovary following the description previously reported (Silvia et al., 2002; Ortega et al., 2008; Amweg et al., 2013).

2.3. Sample processing

The FF aspirated from control cows and cows with spontaneous COD was centrifuged for 10 min at 2000 × *g* to obtain pelleted granulosa cells (for other studies) and stored at −80 °C until hormonal determination to confirm the health status of follicles (Ortega et al., 2008; Amweg et al., 2013).

Blood samples were collected before follicular aspiration or ovariectomy of control cows and cows with spontaneous COD, and then processed, and stored at −80 °C until hormone analysis.

For RT-PCR and real-time PCR, samples of FF from reproductively healthy cows (control) and cows with COD obtained from the abattoir were processed. Granulosa cells were obtained by centrifugation of FF (aspirated with a syringe from the different control antral and cystic follicles) at 2000 × *g* for 10 min and immediately frozen at −80 °C until total RNA extraction. Theca cells from control and cystic follicles were washed with diethyl pyrocarbonate water (Genbiotech, Buenos Aires, Argentina) to remove granulosa cells and stored at −80 °C until total RNA extraction.

Samples used for immunohistochemistry (IHC) were previously obtained and histologically processed (Marelli et al., 2014; Amweg et al. 2013; Hein et al., 2015; Díaz et al., 2016). Briefly, ovaries obtained by ovariectomy were fixed in 4% buffered formaldehyde for 8 h at 25 °C after obtaining samples of follicular wall of control or cysts for molecular biology analysis. Then, the fixed tissues were dehydrated and embedded in paraffin wax. Sections (4 μm thick) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma–Aldrich, St. Louis, MO, USA) and assigned for staining with hematoxylin and eosin for preliminary observations of all ovarian structures (Amweg et al., 2013) or for use in IHC. Follicles were classified into primordial, primary, small preantral, large preantral, antral (Braw-Tal and Yossefi, 1997) and follicular cysts (Silvia et al., 2002). Only follicular cysts with a complete granulosa cell layer and without luteinization were analyzed.

2.4. Hormone assays

2.4.1. Estradiol, progesterone and testosterone determination

Estradiol, progesterone and testosterone levels in serum and FF were measured by electrochemiluminescence immunoassay (ECLIA) kits (Roche Diagnostics GmbH, Germany) in a COBAS e411 system (Roche Diagnostics), according to the manufacturer´s instructions, which were previously validated in bovine studies (Díaz et al., 2015). The assay sensitivity was 5.00 pg/mL for estradiol, 0.03 ng/mL for progesterone, and 0.02 ng/mL for testosterone.

Table 1
Primer pairs for real-time PCR.

Primer	Sequence (5 → 3)	Length, bp	Gene accession no.
StAR	F: GGAGGAGATGGCTGGAAGAAGGT R: TGCTGTAGCACTGGAATGGAAACA	174	NM_174189.2
3βHSD	F: ACCTGGGAGTGACAATGATGGGAA R: TCTGGTGGCGGAAGGCAGATAGTA	161	NM_174343.3
CYP17A1	F: CCAGTTGGCCAGGGAGGTG R: AACAGGGCAAAGGCATTCAGTG	168	NM_174304.2
CYP19A1	F: GGGCCTGTGCGGGAAAGTA R:TCGTCTGGATGCAAGGATAAGTCA	145	NM_174305.1
AKT	F: GCCGCCTGCCCTTCTACA R: TGATCTCCTTGGCGTCCTCAG	174	X61036.1
mTOR	F: ACAATAGCTCAGGCCGCATCGTCT R: GCTTCGGGGGCGTCAAATAACTTC	127	XM_002694043.5
GAPDH	F: CAC CCT CAA GAT TGT CAG CA R: GGT CAT AAG TCC CTC CAC GA	103	BC102589
β-actin	F: CGGAACCGCTCATTGCC R: ACCCACACTGTGCCCATCTA	290	BT030480

2.5. RNA extraction

Total RNA was isolated from granulosa and theca cells of different sized antral control follicles and cysts obtained from the abattoir, using TRIzol LS reagent (Invitrogen Life Technologies, CA, USA), according to the manufacturer's instructions with slight modifications (Rodríguez et al., 2013; Hein et al., 2015).

2.6. Reverse transcription

To avoid putative genomic DNA contamination, RNA samples were treated with DNAse (Invitrogen) according to the manufacturer's instructions and assessed for quantity with SPECTROstar Nano (BMG Labtech), aliquoted and stored at −80 °C. First-strand cDNA was synthesized using a master mix (Moloney Murine Leukemia Virus (MMLV) buffer, dithiothreitol, RNAout, MMLV reverse transcriptase, deoxyribonucleotide triphosphate and random primers (Invitrogen)). The reverse transcription conditions were as described previously (Rodríguez et al., 2013, 2011; Marelli et al., 2014). Briefly, they consisted of 10 min of annealing at 25 °C, 50 min of cDNA synthesis at 37 °C and 15 min of inactivation at 70 °C.

2.7. Real-time RT-PCR

An optimized real-time PCR protocol was used to analyze the mRNA expression of *3β-HSD*, *CYP17A1*, *CYP19A1*, *StAR*, *AKT* and *mTOR* in both granulosa and theca cells, using SYBR Green I (Invitrogen) technology in LightCycler (Pfaffl, 2001). To test cross-contamination of bovine granulosa and theca cells and confirm the mRNA purity, primer sequences for *CYP19A1* and *CYP17A1* were used (Table 1). Theca cells expressed *CYP17A1* and granulosa cells expressed only *CYP19A1*. Samples expressing both genes were discarded.

Transcript levels were measured by relative quantitative real-time PCR using a StepOne Real-Time PCR System (Applied Biosystems, Life Technologies, CA, USA) and results were expressed relative to the housekeeping gene selected. Previously, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and β actin were evaluated. The selection of the internal control gene was based on the Ct variance (as reflected by the standard deviation) among groups, choosing *GAPDH* as the housekeeping gene (Shibaya et al., 2007; Rey et al., 2010). An optimized protocol consisting of initial denaturation at 98 °C for 3 min, 40 cycles of denaturation at 98 °C for 5 s and annealing at 59 °C (*StAR*) for 20 s, 60 °C (*3β-HSD, CYP19A1*), for 10 and 15 s respectively, 60 °C (*mTOR, AKT*) for 20 s, 52 °C (*GAPDH*,) for 20 s, extension at 72 °C for 30 s (*StAR, 3βHSD*), 20 s (*CYP19A1*), 25 s (*mTOR, AKT*) and fluorescence reading at 80 °C (StAR), 82 °C(*3β-HSD*), 78 °C (*CYP19A1*), 83 °C (*mTOR*), 84 °C (*AKT*) and 74 °C (*GAPDH*) was used. All measurements for each sample were performed in duplicate and the coefficients of variation (CVs) were calculated as described by Bustin et al. (2009). All genes presented low overall variability (0.40–0.60% CV of Ct). The efficiency of the PCRs and relative quantities were determined from a standard curve as described previously (Hein et al., 2015). PCR efficiency and Ct were calculated manually using the StepOne software v2.3 (Applied Biosystems). Reactions with a coefficient of determination (R^2) higher than 0.98 and efficiency between 95 and 105% were considered optimized. The cDNA used for each real-time PCR reaction was previously quantified by the Qubit method (Invitrogen). About 10 ng cDNA was used for *StAR* in theca cells and 7.2 ng in granulosa cells, about 2.8 ng cDNA was used for *3βHSD* in both populations, 6 ng cDNA was used for *CYP19* in granulosa cells, around 6 ng cDNA was used for *mTOR* in theca cells and 12 ng in granulosa cells, and 4 ng cDNA was used for *AKT* in theca cells and 8 ng in granulosa cells. For amplification, 4 μL of cDNA was combined with a PCR reaction mix containing 5 X PCR buffer (Invitrogen) with 1:20,000 dilution of SYBR Green I, 200 mM dNTP,

Table 2
Antibodies, suppliers and dilutions used for immunohistochemistry (IHC) and western blot (WB).

Antibodies		Clone/ Source	Dilution	
			IHC	WB
Primary	Anti-StAR	Polyclonal (ab-3343, Abcam, Cambridge. UK)	1:75	1:500
	Anti-CYP17A1	Monoclonal clon 2C2 (given by Dr. C.R. Parker Jr., Univ. Of Alabama at Birmingham)	1:100	1:200
	Anti-3βHSD	Polyclonal (given by J.I. Mason PhD, FRC Path, Univ. Of Edinburgh)	1:750	1:3000
	Anti-CYP19A1	Polyclonal (SC-30086, Santa Cruz Biotechnology Inc., CA. USA)	1:300	1:1000
	Anti-p-mTOR	Polyclonal (ab-51044, Abcam, Cambridge. UK)	1:100	1:400
	Anti-pan-AKT	Polyclonal (ab-18785, Abcam, Cambridge. UK)	1:50	1:500
	Anti-p-ERK 1/2 (Thr 202)	Polyclonal (SC-101760, Santa Cruz Biotechnology Inc., CA. USA)	1:100	1:100
Secondary	HRP-anti rabbit- IgG	SC-2004 (Santa Cruz Biotechnology Inc., CA. USA)	–	1:7500
	HRP-anti mouse- IgG	SC-2005 (Santa Cruz Biotechnology Inc., CA. USA)	–	1:6000
	Anti-rabbit IgG	SC- 2040 (Santa Cruz Biotechnology Inc., CA. USA)	1:100	–
	Anti-mouse IgG	SC-2039 (Santa Cruz Biotechnology Inc., CA. USA)	1:100	–
	Biotinilated anti-rabbit/anti-mouse IgG2a	Polyclonal. CytoScan biotinilated link, CytoScan TM HRP detection system (Cell Marque, CA, USA)	Ready to use	–

400 nM forward primer, 400 nM reverse primer, and 1 U of Phire polymerase (Invitrogen) in a final volume of 20 µL. Negative DNA template controls were included in all the assays, and yielded no consistent amplification. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Fold change was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primer sequences used are described in Table 1.

2.8. 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 LASEGENE software (DNAStar, WI, USA).

2.9. Western blotting

The specificity of the primary antibodies against 3β-HSD, CYP17A1, CYP19A1, StAR, pan-AKT, phospho (p)-mTOR and pERK-1/2 (Thr 202) used in IHC (Table 2) was evaluated by western blot and a single band of the expected molecular mass was found in each case. Samples of follicular walls of antral follicles from the control group were homogenized in radioimmunoprecipitation assay lysis buffer with a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany). Proteins (40 µg/lane) were separated in 8–15 % SDS-PAGE gels, and transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) as previously described (Ortega et al., 2009). The membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST, Sigma–Aldrich Corp.) and then incubated overnight at 4 °C with specific primary antibodies. Bound antibodies were detected using anti-rabbit IgG or anti-mouse IgG peroxidase antibody after incubation for 90 min at room temperature. The immunopositive bands were detected by chemiluminescence using the ECL-plus system (GE Healthcare) on hyperfilm-ECL film (GE Healthcare).

2.10. Immunohistochemistry

Cytoplasmic protein expressions of pERK-1/2, pan-AKT, p-mTOR, StAR, CYP17A1, CYP19A1 and 3β-HSD were evaluated in follicles at different developmental stages in ovaries from control cows and cows with spontaneous COD. Also, nuclear pERK-1/2 protein expression was evaluated in the same structures, since its translocation to the nucleus is a critical step for the fulfilment of many cellular functions.

Protein expression was detected using polyclonal or monoclonal antibodies (specifications are shown in Table 2). A streptavidin-biotin immunoperoxidase method was performed as previously described (Rodríguez et al., 2015). Briefly, after deparaffinization, microwave pre-treatment (antigen retrieval) was performed by incubating the sections in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was inhibited with 3% (v/v) H_2O_2 in methanol, and nonspecific binding was blocked with 10% (v/v) normal goat serum. Slides were incubated with the corresponding antibodies (Table 2) for 18 h at 4 °C and then for 30 min at room temperature with biotinylated secondary antibody (Table 2). The antigens were visualized by the streptavidin-peroxidase method (Cell Marque, CA, USA), and 3,3-diaminobenzidine (Liquid DAB-Plus Substrate Kit, Invitrogen) was used as chromogen. Finally, the slides were washed in distilled water and counterstained with activated hematoxylin (Biopur, Argentina), dehydrated and mounted.

To verify the immunoreaction specificity, adjacent control sections were subjected to the same immunohistochemical method, replacing the primary antibody with rabbit non-immune sera. The specificity of the secondary antibodies was tested by incubation with anti-Ki-67 (polyclonal, rabbit anti-human Ki-67; Dako, Carpinteria, CA, USA), a primary antibody against human antigens with a

proven negative reaction to cattle tissues. To exclude the possibility of non-suppressed endogenous peroxidase activity, some sections were incubated with DAB reagent alone (Ortega et al., 2008). Positive controls were used as inter-assay controls to maximize the levels of accuracy and robustness of the method (Ranefall et al., 1998; Ortega et al., 2009, 2010).

2.11. Image analysis

Images were digitized using a color video camera (Motic 2000, Motic China Group, China) mounted on top of a conventional light microscope (Olympus BH-2, Olympus Co., Japan) and then analyzed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA) as described and validated previously (Ortega et al., 2009, 2010).

The methodological details of image analysis as a valid method for quantification have been described previously (Ranefall et al., 1998; Ortega et al., 2009). To obtain quantitative data for IHC staining in follicular walls, at least five sections were evaluated for each specimen and antibody. The average density (% of immunopositive area) of the antibodies was calculated from at least 50 images of each area (granulosa and theca cells) in each section as a percentage of the total area evaluated through color segmentation analysis, which extracts objects by locating all objects of a specific color (brown stain). These values were verified and normalized using the image software, with controls carried across various runs using the same region (verified by image comparison) for calibration. The percentage of immunopositive area was calculated separately for each follicular compartment (granulosa and theca cells). The main strength of the well-validated imaging approach used in this study is the visualization of *in situ* localization of proteins within cells of interest. The quantification of biological markers by using this approach has been successfully applied to quantify immunoreactivity in different tissues (Ranefall et al., 1998; Ortega et al., 2009).

2.12. Statistical analysis

The data were analyzed with a statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA). The distribution of data was tested for normality using the Kolmogorov-Smirnov test. Tests of homogeneity of variance between groups were conducted using Levene's test. The statistical significance of differences between groups of data from each follicular structure evaluated for protein and mRNA expression was assessed by one-way ANOVA, followed by Duncan's multiple range tests as multiple comparison tests. A value of $p < 0.05$ was considered significant. The t Student test was used to evaluate the levels of changes between two groups. Differences in protein expression were assessed separately in each follicular layer and the expression of markers of interest in the same follicular categories was compared between groups. Furthermore, analogous structures were compared with cysts, i.e. control antral follicles and spontaneous cysts. The results are expressed as mean ± SEM.

3. Results

3.1. Concentrations of estradiol, progesterone and testosterone in serum and follicular fluid

Steroid concentrations in serum and FF from control and COD cows are presented in Table 3. Mean estradiol and testosterone concentrations were similar both in the serum and FF of preovulatory follicles and spontaneous cysts from control and COD cows, respectively ($p > 0.05$). Mean progesterone concentrations were higher in the serum and FF of preovulatory follicles than in those of spontaneous cysts ($p < 0.05$).

3.2. Gene expression

All granulosa cell samples analyzed were positive for *CYP19A1* mRNA and negative for *CYP17A1* mRNA, whereas all theca cell samples analyzed were positive for *CYP17A1* mRNA and negative for *CYP19A1* mRNA.

The identity of the PCR products was confirmed by sequencing (100% homology with bovine sequences). All genes presented low overall variability (0.40–0.60% CV of Ct). GAPDH gene expression was similar between the different groups evaluated ($p > 0.05$) since no difference was obtained in the Ct average of GAPDH between groups.

Table 3
Steroid concentration in serum and follicular fluid of preovulatory follicles in control cow and spontaneous follicular cysts.

	Estradiol: *Serum* (pg/ml) FF (ng/ml)	Progesterone (ng/ml)	Testosterone (ng/ml)
	Serum		
Control (n = 15)	11.99 ± 1.44	0.94 ± 0.18[a]	0.03 ± 0.01
COD (n = 25)	18.05 ± 1.17	0.56 ± 0.08[b]	0.05 ± 0.01
	Follicular Fluid (FF)		
Control (n = 15)	360.53 ± 48.04	62.84 ± 5.84[a]	17.29 ± 2.04
COD (n = 25)	269.77 ± 24.49	38.54 ± 4.82[b]	22.76 ± 3.76

Values are expressed as mean ± SEM.
Within a column, means with distinct letters are significantly different ($p < 0.05$).

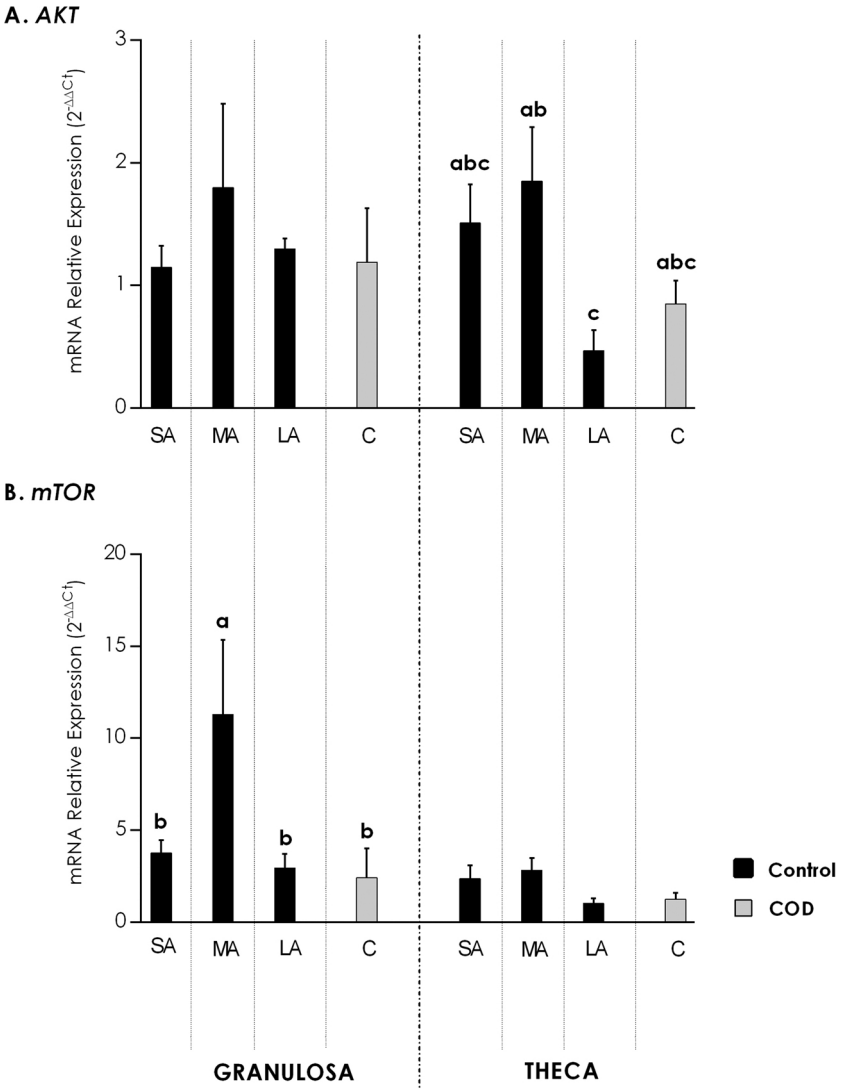

Fig. 1. Relative quantification of mRNA of *AKT* (A) and *mTOR* (B) by real-time PCR in granulosa and theca cells of small (SA), medium (MA), large (LA) antral and cystic (C) follicles from control cows (black bars) and cows with COD (gray bars). The $2^{-\Delta\Delta Ct}$ values are represented as means ± S.E.M. Bars with different letters are significantly different ($p < 0.05$).

3.2.1. AKT and mTOR gene expression

In granulosa cells, the levels of *AKT* mRNA were similar between all the control follicular structures and follicular cysts ($p > 0.05$, Fig. 1A). In theca cells, the levels of *AKT* mRNA were higher in medium than in large follicles of the control group ($p < 0.05$), and no differences were found between control follicles and cysts ($p > 0.05$).

In granulosa cells, the levels of *mTOR* mRNA were higher in medium antral follicles than in the other follicular structures in control and COD cows ($p < 0.05$), whereas in theca cells, *mTOR* mRNA levels were similar in the different follicles from both antral controls and cysts ($p > 0.05$, Fig. 1B).

3.2.2. StAR, CYP17A1, CYP19A1 and 3β-HSD gene expression

StAR and *CYP17A*1 mRNAs were detected only in theca cells, with similar levels in all the groups analyzed ($p > 0.05$, Fig. 2A–B). In granulosa cells, *StAR* was detected at low levels in a not significant number of antral follicles, and consequently, statistical analysis could not be performed. *CYP19A1* mRNA was detected only in granulosa cells, with lower levels in cysts and small antral follicles than in medium and large antral ones ($p < 0.05$, Fig. 2B). *3β-HSD* mRNA was detected both in granulosa and theca cells (Fig. 2C). In granulosa cells, *3β-HSD* mRNA levels were higher in medium antral follicles than in the other structures ($p < 0.05$), whereas in theca cells, they were higher in small antral follicles than in large antral follicles and cysts ($p < 0.05$).

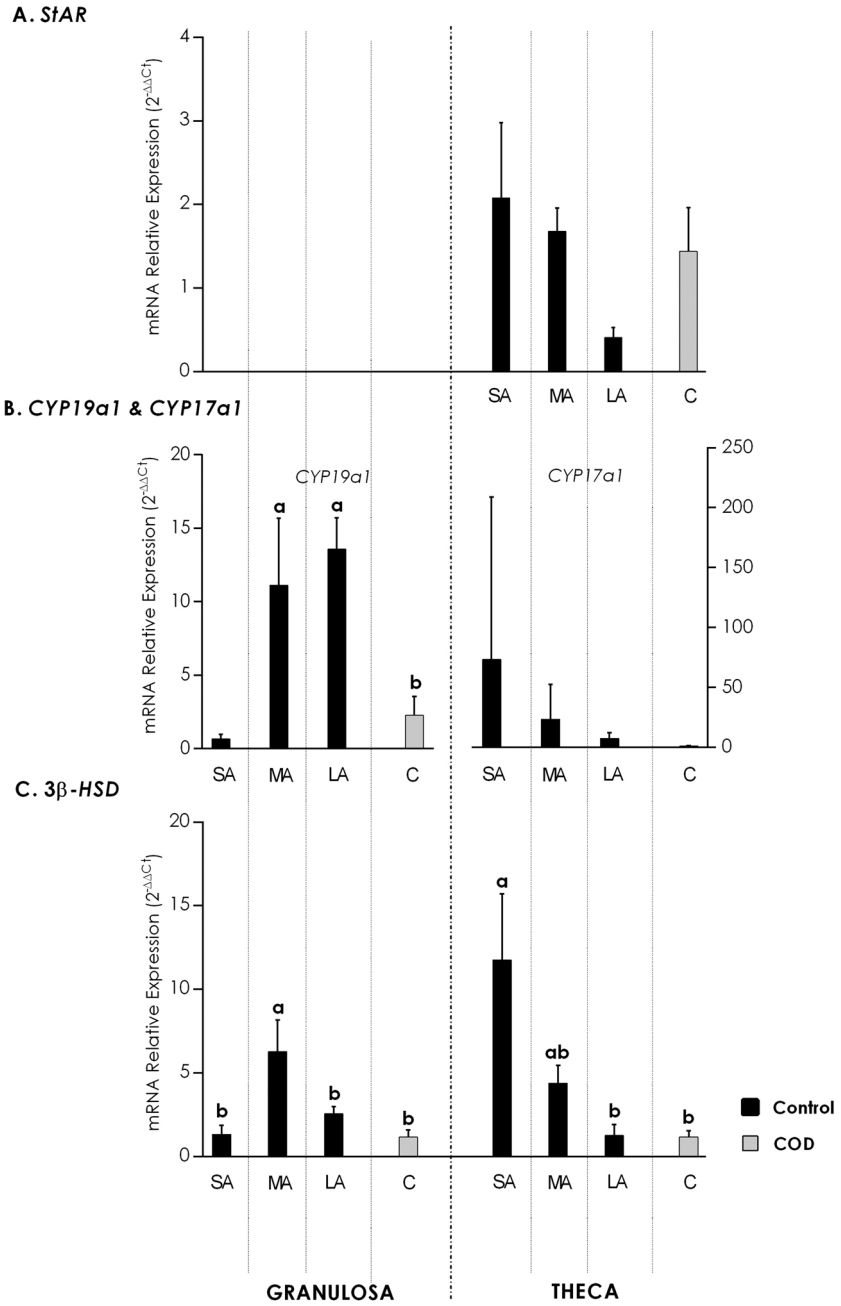

Fig. 2. Relative quantification of mRNA of *StAR* (A), *CY17A1/CYP19A1* (B) and *3β-HSD* (C) by real-time PCR in granulosa and theca cells of small (SA), medium (MA), large (LA) antral and cystic (C) follicles from control cows (black bars) and cows with COD (gray bars). The $2^{-\Delta\Delta Ct}$ values are represented as means ± S.E.M. Bars with different letters are significantly different ($p < 0.05$).

3.3. Protein expression

Target proteins were detected both in granulosa and theca cells of the different follicular structures evaluated with the exception of pERK-1/2, which was evidenced only in granulosa cells (Fig. 3).

3.3.1. pERK-1/2, pan-AKT and p-mTOR protein expression

pERK-1/2 expression was higher in granulosa cells of large preantral and antral follicles of the control group than in those from cows with COD ($p < 0.05$), and significantly higher in control antral follicles than in cystic follicles ($p < 0.05$) (Fig. 4A).

pan-AKT expression in granulosa cells was higher in large preantral follicles from cows with COD than in those from the control

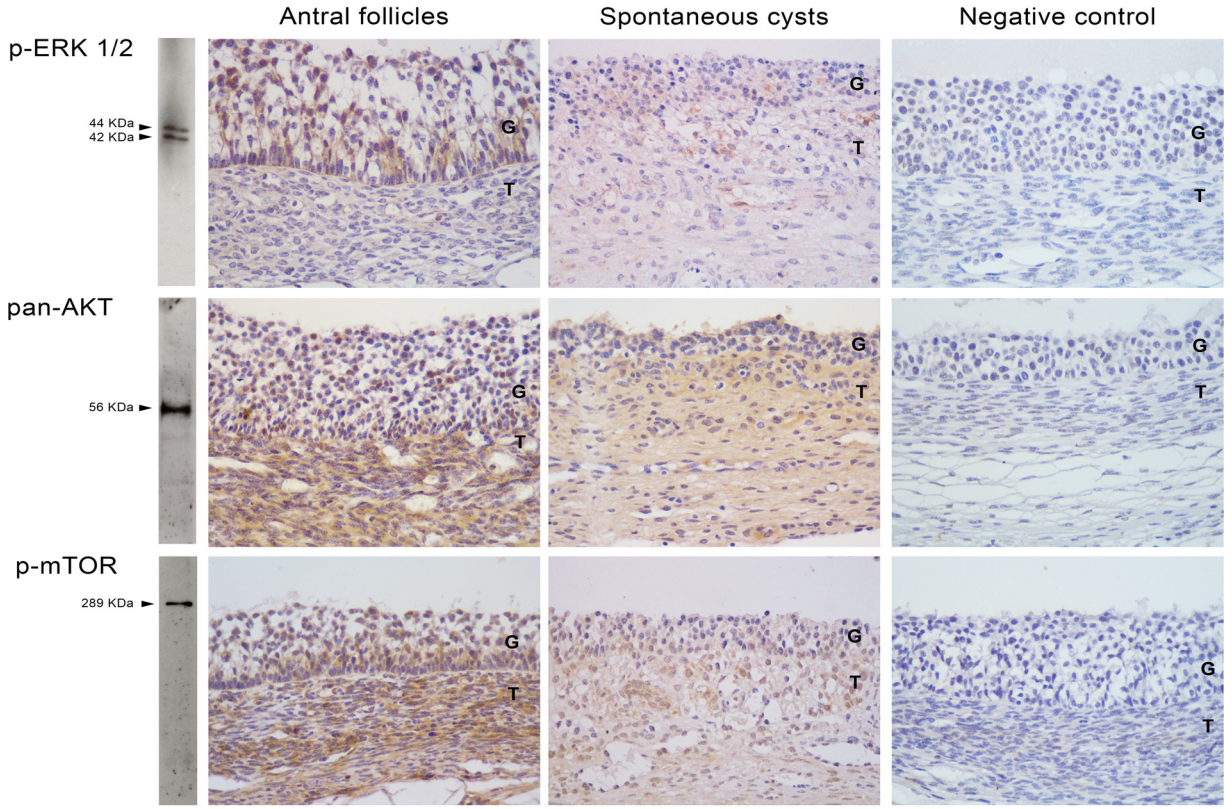

Fig. 3. Representative images of p-ERK 1/2, pan-AKT and p-mTOR immunostaining in antral follicles (control group) and cystic follicles from cows with spontaneous COD. Granulosa (G), theca interna (T). The specificity of the antibodies evaluated by western blot is shown in the left panel and a negative control of immunohistochemistry is shown in the right panel. Magnification: 400×.

group ($p < 0.05$), whereas that in theca cells was higher in control antral follicles than in follicular cysts ($p < 0.05$) (Fig. 4B).

p-mTOR expression in granulosa cells was higher in control small preantral than in those from cows with COD ($p < 0.05$) and higher in control antral follicles (as reference structure) than in follicular cysts ($p < 0.05$), whereas that in theca cells was similar in all the follicular structures evaluated ($p > 0.05$) (Fig. 4C).

3.3.2. StAR, CYP17A1, CYP19A1 and 3β-HSD protein expression

Immunohistochemical expression of StAR and 3β-HSD was detected in both granulosa and theca cells, while CYP17A1 expression was specifically detected in theca cells and CYP19A1 in granulosa cells (Fig. 5).

In most follicular categories, StAR protein expression was similar in both theca and granulosa cells from control cows and cows with COD ($p > 0.05$). In contrast, StAR protein expression was higher in granulosa cells of primary follicles from cows with COD than in those of control cows ($p < 0.05$, Fig. 6A).

CYP17A1 protein expression was higher in follicles from cows with COD than in those of the control group ($p < 0.05$), without differences in the other follicular categories ($p > 0.05$, Fig. 6B).

CYP19A1 protein expression in large preantral follicles was significantly higher in the control group than in the COD group ($p < 0.05$). CYP19A1 protein expression was also higher in cysts than in control antral follicles ($p < 0.05$, Fig. 6B).

3β-HSD protein expression in granulosa cells was greater in cysts than in control antral follicles ($p < 0.05$), whereas that in theca cells showed no significant differences ($p > 0.05$, Fig. 6C).

4. Discussion

Among the various signaling pathways involved in the insulin response in the ovary, the PI3K/AKT signaling pathway with its many branches appears to be the main non-gonadotropic regulator of the differentiation, growth and survival of follicles (Zheng et al., 2012; Dupont and Scaramuzzi, 2016). Downstream of PI3K activity, AKT phosphorylates several kinases or transcription factors involved in most of the main metabolic effects of insulin. Among these metabolic effects, the protein synthesis through mTOR, the stimulation of cell growth and differentiation, and the anti-apoptotic effects by phosphorylating Bad (an anti-apoptotic protein), exert determinant roles in ovarian functionality. Furthermore, through IRSs or Shc tyrosine phosphorylation, insulin activates the MAPK kinase pathway, which then phosphorylates and stimulates ERK-1/2 MAPK activity to regulate the expression genes involved in the

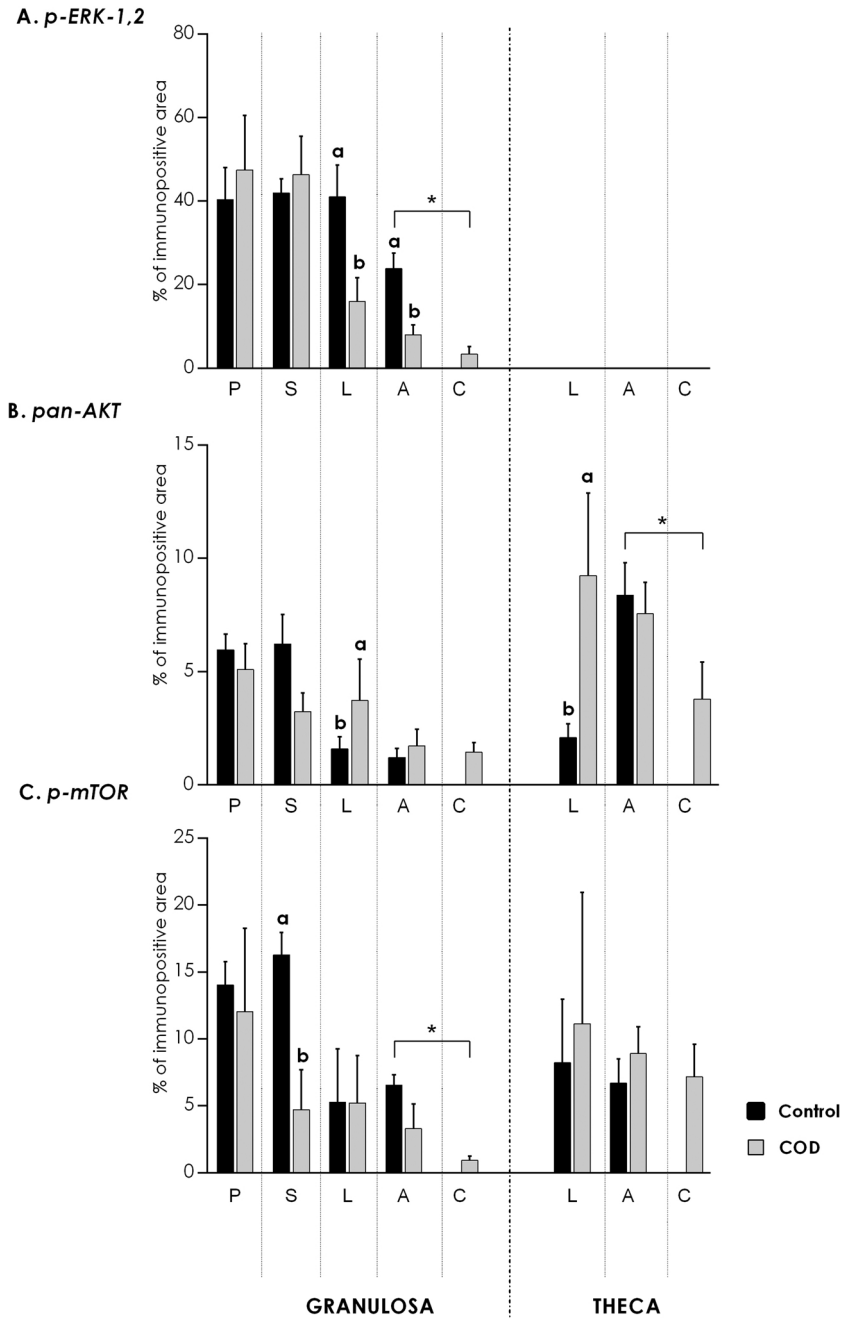

Fig. 4. Relative protein expression (measured as percentage of immunopositive area) of p-ERK 1/2, pan-AKT and p-mTOR in different follicular categories from control cows (black bars) and cows with COD (gray bars) in granulosa and theca interna cells of primary (P), small (S) and large (L) preantral, antral (A) and cystic (C, only in COD group) follicles. Values represent mean ± S.E.M. Bars with different letters within a category are significantly different ($p < 0.05$). Asterisks indicate differences between cystic follicles and antral follicles from the control group ($p < 0.05$).

PI3K pathway (Dupont and Scaramuzzi, 2016). So, and as we have previously obtained interesting results in these first steps related to ovarian functionality in cows with COD (Hein et al., 2015) as well as in a model of sheep treated to study ovarian dysfunctions (Ortega et al., 2010; Padmanabhan et al., 2014), in the present study, we decided to further evaluate the PI3K/AKT pathway.

We found highly relevant evidences relating the expression of molecular intermediates in the response to insulin with those of steroidogenic enzymes in cows with spontaneous COD. Although similar mRNA levels of *AKT* and *mTOR* were obtained, significant differences were detected in their protein expression. In theca cells, AKT protein expression was lower in cysts than in control antral follicles, in agreement with our previous results showing a similar pattern in its upstream intermediate PI3K (Hein et al., 2015), supporting a transitive behavior of this pathway in theca cells. PI3K promotes the generation of phosphatidylinositol-3,4,5-

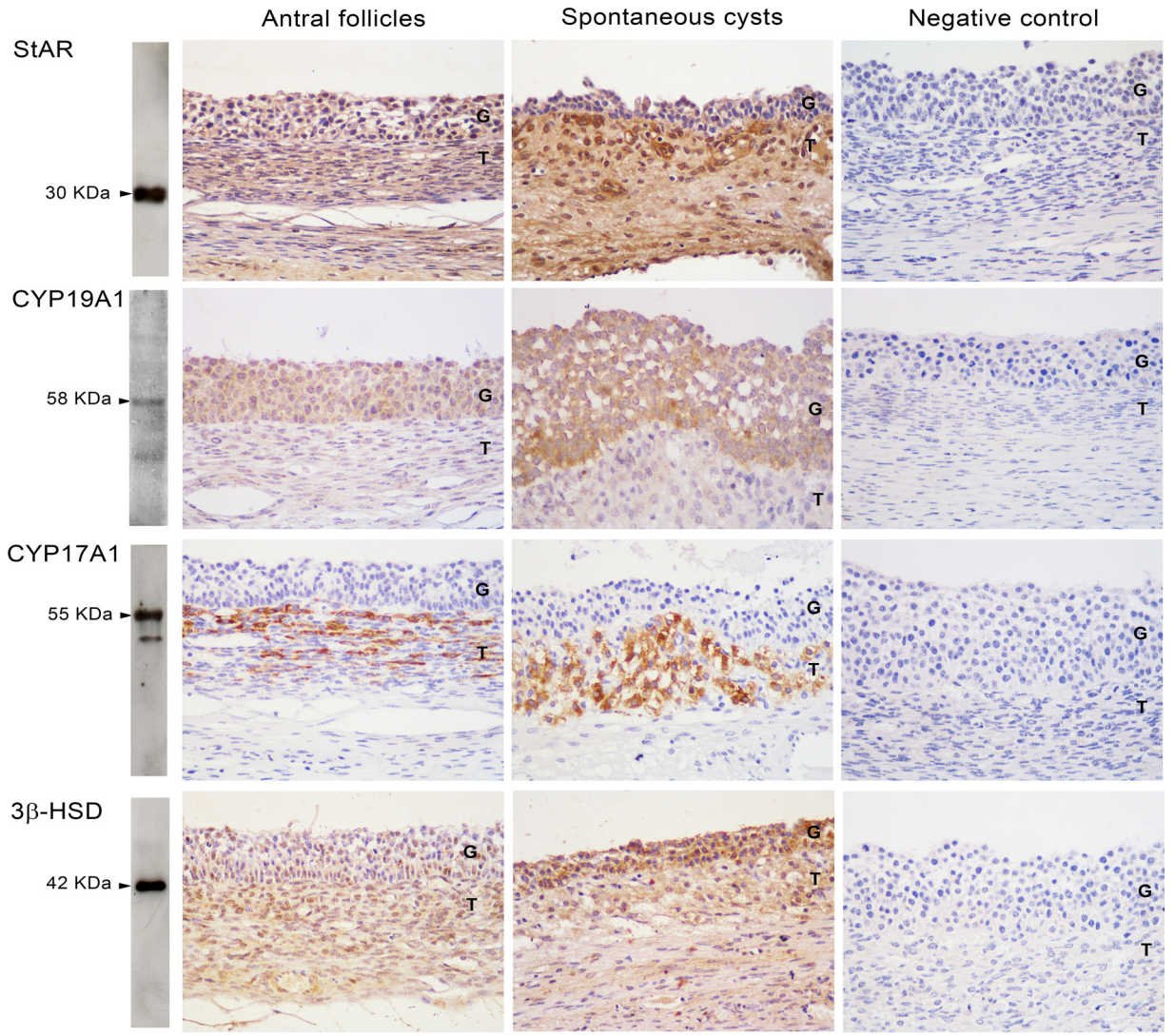

Fig. 5. Representative images of StAR, CYP17A1, CYP19A1 and 3β-HSD immunostaining in antral follicles (control group) and cystic follicles of cows with spontaneous COD. Granulosa (G), theca interna (T). The specificity of the antibodies evaluated by western blot is shown in the left panel and a negative control of immunohistochemistry is shown in the right panel. Magnification: 400×.

triphosphate, leading to activation of downstream kinases, including AKT, which in turn phosphorylate many other intermediates such as mTOR (Taniguchi et al., 2006). Indeed, it has been suggested that AKT is involved in mTOR activation induced by LH in bovine granulosa cells (da Rosa et al., 2016). Interestingly, in our study, while total AKT expression was not modified in granulosa cells, phosphorylated mTOR expression was lower in granulosa cells of cysts than in those of control antral follicles. Although mTOR activity has been suggested to be not essential for ovulation in cattle (da Rosa et al., 2016), it is involved in other processes associated with ovarian function (Palaniappan et al., 2013; Cheng et al., 2015; Dupont and Scaramuzzi, 2016) that could in turn be involved in cyst development or maintenance.

As previously described, PI3K/AKT activation leads cells through many biological functions (Marino et al., 2003; Fukuda et al., 2009). It has been demonstrated that, in the ovary, FSH and several growth factors activate the PI3K/AKT pathway in granulosa cells and cultured follicles (Hu et al., 2004; Carletti and Christenson, 2009). It has also been reported that, in cultured bovine theca cells, LH stimulates AKT phosphorylation and that activation of PI3K/AKT is involved in *CYP17A1* mRNA expression and androgen production (Fukuda et al., 2009).

Another important and well-documented insulin signaling pathway is the ERK pathway, where ultimately ERK-1 and ERK-2 are activated with the consequent protein synthesis stimulation and finally cell multiplication, differentiation (Mebratu and Tesfaigzi, 2009) and steroidogenesis modulation via the uptake of glucose (Scaramuzzi et al., 2015). Several authors have also studied intermediates of the insulin and IGF1 pathways using *in vivo* specific inhibitors of AKT and ERK and reported an inhibition of follicle growth and a reduction of FF estradiol concentrations in ewes (Ryan et al., 2008). Here, we report a significant reduction in pERK-1/2

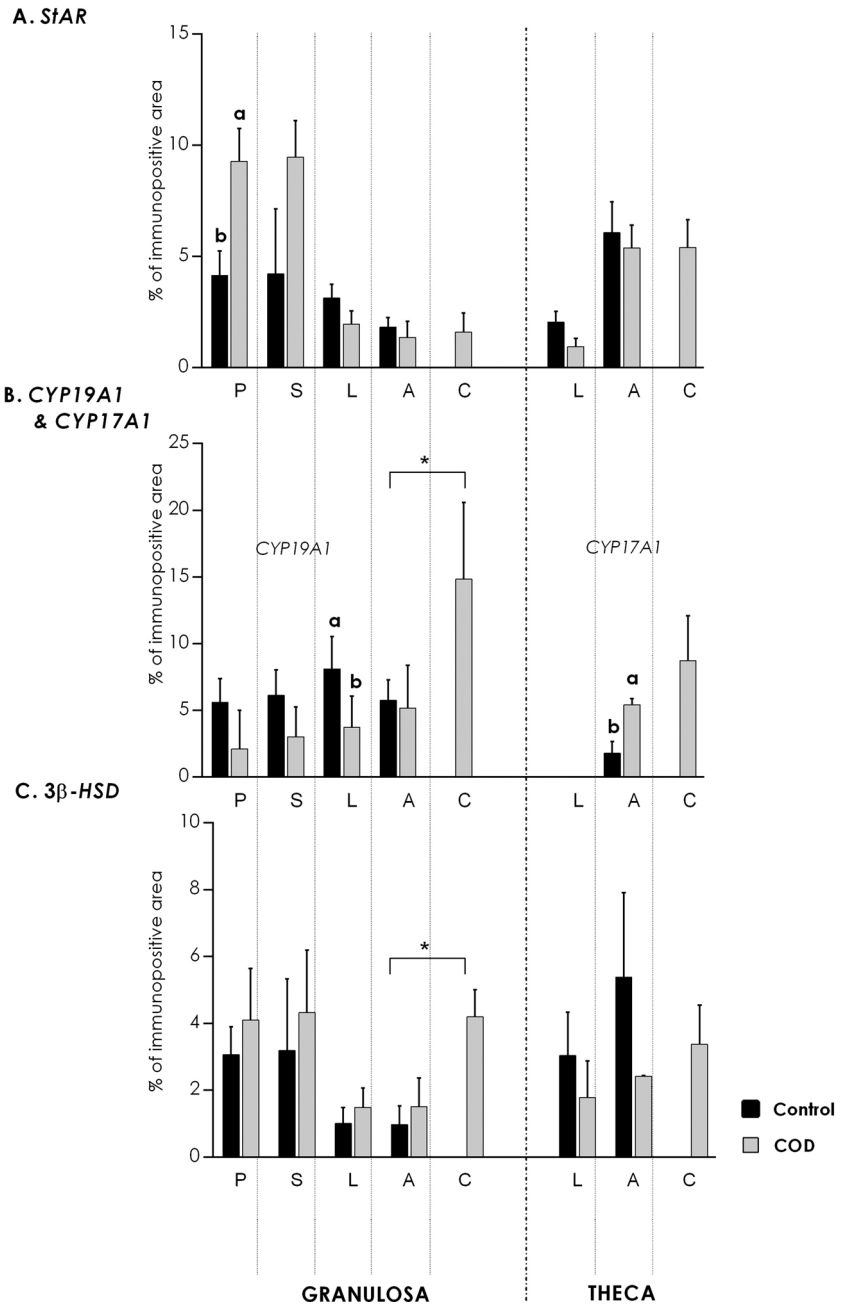

Fig. 6. Relative protein expression (measured as percentage of immunopositive area) of StAR, CYP17A1, CYP19A1 and 3β-HSD in different follicular categories from control cows (black bars) and cows with COD (gray bars) in granulosa and theca interna cells of primary (P), small (S) and large (L) preantral, antral (A) and cystic (C, only in the COD group) follicles. Values represent mean ± S.E.M. Bars with different letters within a category are significantly different ($p < 0.05$). Asterisks indicate differences between cystic follicles and antral follicles from the control group ($p < 0.05$).

expression in granulosa cells of cysts in comparison with that in control antral follicles. However, the expressions of proteins related to steroidogenesis evaluated were not modified (StAR and CYP17A1) or even increased (CYP19A1 and 3β-HSD). This could suggest that the intermediates of insulin signaling assayed might be exerting their modulation at the level of gene transcription or being targeted to other pathways. These key regulators like ERK-1/2 are induced by the LH surge (Fan et al., 2009; Sayasith and Sirois, 2015), which is known to induce dynamic changes in granulosa cell function by stimulating the expression of several genes (Carletti and Christenson, 2009). Indeed, both LH and FSH gonadotropins are regulators of later folliculogenesis and might be modulated by several external factors, such as insulin, and the opposite, i.e. the modulation of insulin action by gonadotropins, is also possible. In granulosa cells, insulin signaling through AKT1 and FOXO1 can enhance FSH-stimulated steroidogenesis (da Rosa et al., 2016). In the

present study, total pan-AKT was equally expressed in granulosa cells of antral and cystic follicles, but an increase in CYP19A1 was detected in cysts. This could be related to the high levels of estrogen previously detected in these structures in *in vitro* studies (Amweg et al., 2013) or during the first stages of persistence (Díaz et al., 2015). However, in the present study, cows with spontaneous cysts had levels of estrogen similar to those of control cows, in agreement with follicles with several days of persistence (Díaz et al., 2015). Differences in the expression of CYP19A1 and estrogen levels could be related to differences in CYP19A1 activity more than to its levels of expression. Monniaux et al. (2008) have detected similar levels of estrogen in cystic follicles of later stages to those of control cows. However, young cysts exhibited higher intrafollicular concentrations of estradiol, without any associated difference in mRNA expression of *CYP19A1* and *StAR* in granulosa cells (Monniaux et al., 2008). Other authors have proposed that ERK-1/2 could be involved as a negative regulator of CYP19A1 expression in mouse granulosa cells (Andric et al., 2010), a fact that could be supporting our results. It is important to note that CYP19A1 is one of the many ovarian genes that are down-regulated by the LH receptor in an ERK-1/2-dependent fashion (Fan et al., 2009; Andric et al., 2010). Previously, we found lower mRNA of *LH* receptor in cystic follicles (Marelli et al., 2014), a finding that could support the lower pERK-1/2 activation and the consequent higher expression of CYP19A1 herein detected.

Regarding 3β-HSD, an enzyme involved in progesterone synthesis and the first steps of cortisol, the granulosa cells of cysts expressed higher levels than antral follicles. In FF of cysts from cows with COD, we detected lower levels of progesterone; however, we have previously found an increase in cortisol concentration (Amweg et al., 2013). Richards and Pangas (2010) reported that MAPK ERK-1/2 phosphorylation is also necessary for LH-mediated progesterone secretion, and pointed out that, when ERK-1 and ERK-2 are disrupted in granulosa cells, global changes occur in ovary function and that, when ERK-1 and ERK-2 are activated, the FSH-regulated gene expression program is turned off, with effects on preovulatory follicle growth and differentiation.

Likewise, in granulosa cells, gonadotropins, particularly FSH, acting via mTOR and/or ERK, can enhance cell proliferation and protein synthesis, and FSH, acting via AKT1 and FOXO3a, can inhibit apoptosis (Dupont and Scaramuzzi, 2016). In this regard, the decreased levels of p-mTOR and pERK-1/2 detected could be related to the previously reported low proliferative and apoptotic rates in cystic follicles from cows with COD compared with controls (Salvetti et al., 2010).

In bovine theca cells, Fukuda et al. (2009) postulated that LH stimulates *CYP17A1* mRNA expression and androgen production via activation of the PI3K/AKT pathway. Our results showed that expression of total pan-AKT was lower in theca cells of cysts than in control antral follicles and that the levels of gene and protein expression of CYP17A1 were similar, therefore this expression could be compensated by the action of other signaling pathways.

The results herein obtained along with others previously reported (Hein et al., 2015) support the idea that maintaining optimal activities of PI3K and MAPK/ERK signaling within the ovary seems to be important for normal development and ovarian function, showing several variations in cystic follicles. Besides, in agreement with previous reports, the insulin pathway should be considered as a target for the development of preventive measures and treatments for ovarian diseases.

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Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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