Animal Reproduction Science 156 (2015) 64–74

Contents lists available at ScienceDirect

Animal Reproduction Science

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

Impaired insulin signaling pathway in ovarian follicles of cows with cystic ovarian disease

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ARTICLE INFO

Article history:
Received 4 January 2015
Received in revised form 26 February 2015
Accepted 27 February 2015
Available online 9 March 2015

Keywords:
Insulin signaling pathway
Cystic ovarian disease
Ovaries
Dairy cows

ABSTRACT

Cystic ovarian disease (COD) is an important cause of infertility in dairy cattle. Follicular cell steroidogenesis and proliferation in ovulatory follicles is stimulated by hormones such as insulin and its necessary post-receptor response. The aim of this study was to determine the expression of insulin receptor (IR), IR substrate-1 (IRS1) and phosphatidylinositol 3-kinase (PI3K), key intermediates in the insulin pathway, in control cows and cows with spontaneous COD and ACTH-induced COD. *IR* and *IRS1* mRNA levels were greater in granulosa cells and lower in follicular cysts than in control tertiary follicles. *PI3K* mRNA levels were similar in all follicles evaluated, whereas the expression of *IR*, *IRS1* and *PI3K* was similar in theca cells. Protein expression of IR was higher in control tertiary follicles than in the same structures in animals with COD and with cysts. IRS1 and PI3K protein expression showed the same pattern in tertiary and cystic follicles. However, the protein expression of subunit alpha p85 of PI3K was greater in theca cells from tertiary follicles than in cystic follicles. These results provide new insights into the insulin response in cows with COD. 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 ovaries and contribute to follicle persistence.

1. Introduction

Milk production in dairy cows has increased over the past decades. Unfortunately, infertility associated with metabolic and reproductive disorders during the early postpartum period in high-yielding dairy cows has increased, causing considerable economic loss (Opsomer et al., 1999; Royal et al., 2000; Lucy, 2007). An important cause of infertility in dairy cattle is cystic ovarian disease (COD), which has been defined as the presence of one or more follicular structures of at least 20 mm in diameter in the ovaries, which persist in the absence of luteal tissue, interrupting the normal reproductive cycle (Silvia et al., 2002; Peter, 2004; Vanholder et al., 2006). Cystic ovarian disease pathogenesis is not fully understood, mainly because different factors contribute to cyst formation in bovines (Vanholder et al., 2006). However, a hypothalamic unresponsiveness to estradiol appears to be one of the underlying causes of follicular cyst development (Gümen et al., 2002).

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http://dx.doi.org/10.1016/j.anireprosci.2015.02.010
0378-4320/

Follicular cell steroidogenesis and proliferation in ovulatory follicles are stimulated by metabolic hormones such as insulin and insulin-like growth factor-1 (IGF1). IGF1 is essential for the growth of the dominant follicle, stimulating estradiol-17B secretion, whereas insulin is needed to promote follicular maturation (Bossis et al., 1999; Spicer et al., 2002; Silva et al., 2006; Kawashima et al., 2007). Therefore, estradiol-17B enhanced by IGF1 and modulated by insulin levels induces an LH surge with the consequent ovulation of the dominant follicle (Butler et al., 2004; Kawashima et al., 2012; Walsh et al., 2012). Insulin and IGF1 concentrations are reduced by the dietary restriction and negative energy balance of the peripartum period, (Spicer et al., 1990; Lucy et al., 1992; Beam and Butler, 1998; Diskin et al., 2003; Francisco et al., 2003). Lower insulin concentrations and a lack of LH response have been reported in postpartum anestrous cows (Sinclair et al., 2002; Lucy, 2007). Probably, an absence of LH receptors in granulosa cells, which are dependent on the combined actions of FSH and estradiol-17B, could contribute to this lack of responsiveness (Bao and Garverick, 1998; Webb et al., 1999; Marelli et al., 2014). Follicular estradiol-17B is dependent on LH-stimulated production of androgens from theca cells, which in turn appears to be enhanced by insulin and IGF1 (Diskin et al., 2003). Therefore, low plasma insulin concentrations could not only reduce androgen and estradiol production, but also compromise the ability of follicles to acquire LH receptors, all of which finally alters the ovarian function.

Insulin exerts its biological activities by binding to the extracellular portion of its receptor complex (IR). In women, IRs are widely distributed throughout all ovarian compartments (el-Roeiy et al., 1994; Willis and Franks, 1995; Shimizu et al., 2008), whereas in cattle, they are mainly expressed in granulosa cells (Shimizu et al., 2008; Bossaert et al., 2010).

Insulin signaling is mediated by a complex, highly integrated network that regulates distinct biological effects. In the presence of insulin, the substrate tyrosine kinase activity of the IR initiates a cascade of cellular phosphorylation reactions that regulate protein interactions and enzyme activities. Substrates of the IR include the insulin receptor substrate proteins-1 and 2 (IRS1, IRS2), which serve as docking molecules binding to and activating cellular kinases involved in signaling pathways. The metabolic effects of insulin are regulated by activation of the phosphatidylinositol 3-kinase (PI3K) pathway, in which the regulatory PI3K subunit binds to phosphotyrosine residues on IRS1 (Taniguchi et al., 2006; Youngren, 2007). Insulin-mediated activation of PI3K increases production of 3-phosphorylated phosphoinositide lipids (PIP3), which serve as second messengers to recruit Akt to the plasma membrane (Datta et al., 1999). Once properly localized in the membrane, Akt becomes activated by phosphorylation and in turn phosphorylates a number of downstream targets which finally regulate transcription factors and cell growth (Puig et al., 2003). The adequate activation of insulin signaling pathway is determinant both in steroidogenesis and in multiple metabolic pathways. Insulin can enhance FSH-stimulated estradiol production in bovine granulosa cells (Spicer et al., 1993; Gutiérrez et al., 1997) and modulate the expression of the enzyme cytochrome P450 aromatase and the secretion of estradiol in the absence of FSH (Gutiérrez et al., 1997; Silva et al., 2000). FSH stimulates PI3K and other downstream kinases with potential modulation of aromatase activity in bovine cells (Silva et al., 2006). Therefore, considering the relevance of several targets in the insulin action for an adequate ovarian function, in the present study, we aimed to evaluate the expression of downstream effectors in the insulin pathway in cows with COD.

2. Materials and methods

2.1. Induced cystic follicles and controls

All procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, 2010), and the protocol was approved by the ethics and safety committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Santa Fe, Argentina). The model of ACTH-induced ovarian follicular cysts used in the present study has been previously described (Dobson and Smith, 2000; Ortega et al., 2008; Salvetti et al., 2010; Salvetti et al., 2012; Amweg et al., 2013) and has been optimized to evaluate the target proposed with a time controlled cyst formation. Briefly, ten nulliparous Argentine Holstein heifers (18–24 mo old; maintained under standard husbandry conditions) with regular estrous cycles according to prior detection of estrus, rectal palpation and ultrasonography were used. Their estrous cycles were synchronized using the Ovsynch protocol, as described previously (Ortega et al., 2008; Rodríguez et al., 2011). The time of ovulation was monitored by transrectal ultrasonography and designated as day 1 of the estrous cycle, because ovulation occurs 24–32 h after the second injection of GnRH, as described by Pursley et al. (1995). Beginning on day 15 of a synchronized estrous cycle, five heifers received subcutaneous injections of tetracosactrin hexaacetate (1 mg/mL/animal; Synacthen Depot, Novartis, Basel, Switzerland), a synthetic polypeptide with ACTH activity, every 12 h for 7 d (Ortega et al., 2008). Five control animals received saline (1 mL) (Dobson and Smith, 2000; Ortega et al., 2008). Ultrasonographic ovarian examinations were performed in all animals, as previously described, using a real-time, B-mode scanner equipped with a 7.5 MHz, linear-array, transrectal transducer (Aloka, SSD 500; Wallingford, CT, USA) (Sirois and Fortune, 1988; Rodríguez et al., 2011). Daily ovarian ultrasonography was performed throughout one complete estrous cycle (21 d in the control group) and from day 14 (day 0 = day of estrus) until day 48 (treated group). Cysts detected by ultrasonography were defined as any follicular structure equal to or greater than 20 mm in diameter that was present for 10 d without forming a corpus luteum (Dobson and Smith, 2000; Silvia et al., 2002). All heifers used in this study developed follicular cysts. The first day of follicular cyst formation was the day on which a follicle attained 20 mm or more in diameter. The ovaries were removed 10 d later (approximately day 48) by ovariectomy, following surgical protocols described previously (Marelli et al., 2014). Control heifers were ovariectomized, to obtain normal growing follicles (approximately day 18), when the

dominant follicle reached a diameter greater than 10 mm. During dissection of the ovaries, the follicular diameter was measured using calipers and follicular fluid from each follicle was aspirated and stored separately at −20 °C. Tertiary follicles dissected from normally cycling ovaries were classified as small (<5 mm), medium-sized (5–10 mm) or large (>10 mm) (Parrott and Skinner, 1998).

Blood samples were obtained immediately before ovarian excision and centrifuged at 1000 × *g* for 30 min. The serum collected was stored at −20 °C until hormonal analysis (Ortega et al., 2008; Rey et al., 2010).

Follicular fluid was aspirated from both the preovulatory follicles of control animals and induced cystic follicles, and then frozen until analysis. The ovaries were fixed in 4% neutral buffered formaldehyde.

2.2. Spontaneous cystic ovaries

Multiparous (3.3 ± 1.5 lactations, range 2–7) Argentinean Holstein cows at least 45 d after calving (65.9 ± 27.8 d in milk), with high yield (mean 29.7 ± 6.2 kg of milk per day at diagnosis) with COD were selected for the study from dairy herds of the milk-producing region of Santa Fe, Argentina. Twenty-five cows were diagnosed with the disease during the periodic reproductive control by rectal palpation and ultrasonography (B-mode ultrasound equipped with a transrectal 5.0 MHz linear-array transducer, HS-101 V, Honda Co., Japan), considering previously described parameters (Dobson and Smith, 2000; Bartolome et al., 2005). The ovaries from 10 of these cows were removed by transvaginal ovariectomy following protocols previously described in detail (Marelli et al., 2014). Follicular fluid from these cysts was aspirated with a needle and syringe and was used to obtain granulosa cells, whereas a sample of the cystic follicular wall was used to obtain theca cells. Both samples were subjected to RT-PCR analysis. The rest of the ovary was fixed in 4% neutral buffered formaldehyde and subjected to immunohistochemistry (IHC) analysis. The ovaries from the remaining 15 cows were used to obtain follicular fluid, by follicular aspiration, and used to expand the number of samples of granulosa cells subjected to RT-PCR analysis.

Moreover, 10 cycling cows were synchronized by Ovsynch protocol and used as controls in proestrus. The follicular fluid of preovulatory follicles was obtained by follicular aspiration when the follicles attained more than 15 mm, and used to obtain granulosa cells for RT-PCR techniques.

Follicular fluid was aspirated 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, Brazil). The follicular fluid was transported to the laboratory at refrigeration temperature for processing and then stored at −20 °C until use. Blood samples were collected before the procedure, after feeding at the same intervals, processed and stored for hormone analysis.

After ovariectomy, samples were collected, refrigerated on ice and immediately transported to the laboratory. Follicular cysts were evaluated grossly, microscopically and by hormone analysis. Tissue fractions of ovaries from cystic follicles were immediately frozen at −80 °C until use in gene expression assays. Additional sections of ovarian tissue were fixed in 4% neutral buffered formaldehyde.

2.3. Sample preparation

For mRNA evaluation, follicular fluids containing granulosa cells from each follicle aspirated were centrifuged at 400 × *g* for 10 min. The granulosa cell pellets were resuspended in TRIzol LS reagent (Invitrogen, Life Technologies, CA, USA). The remaining follicular walls from each follicle previously aspirated were further washed several times with phosphate buffered saline (PBS) to remove residual granulosa cells. The theca cells of washed follicular walls from ovariectomized cows were excised from the surrounding stroma and washed several times with diethylpyrocarbonate (DEPC)-water (Salvetti et al., 2012; Amweg et al., 2013). All samples were aliquoted, snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

For light microscopy, 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 (Salvetti et al., 2004) or for use in IHC. Follicles were classified into primary, secondary, tertiary (Priedkalns, 1998) and cystic follicles (Silvia et al., 2002). Only cystic follicles with a complete granulosa cell layer and without signs of luteinization were analyzed.

2.4. Insulin and beta-hydroxybutyrate determination

Serum and follicular fluid concentration of insulin was assessed using RIA, as previously described (Lacau-Mengido et al., 2000; Becú-Villalobos et al., 2007). Insulin concentration was measured using anti-bovine insulin antibody (Sigma, St. Louis, Missouri, USA) and standard human insulin provided by Laboratorios Beta (Buenos Aires, Argentina); the minimum detectable concentration was 0.05 ng/mL. The intra-assay and interassay coefficients of variation were always lower than 8% and 11%, respectively. Beta-hydroxybutyrate (BHB) was assessed using reactive strips (FreeStyle Optium Xceed, Abbott Diabetes Care Ltd., Oxon, UK).

2.5. RNA extraction

Total RNA was isolated from granulosa and theca cells of different sized tertiary control follicles and cysts, after treatment with TRIzol LS reagent (Invitrogen, Life Technologies, CA, USA), according to the manufacturer's instructions but with slight modifications (Rodríguez et al., 2013).

2.6. Reverse transcription

To avoid putative genomic DNA contamination, RNA samples were treated with DNAse (Invitrogen) according to the manufacturer's instructions. First strand cDNA

Table 1
Primer sequences, regions of the target genes and product length, used for semi-quantitative RT-PCR of bovine tissues.

Primer	Sequence (5′ → 3′)	Gene accession no.	Length, bp
IR	For[a] ATCAACATTCGAGGGGGCAACA Rev[b] GGAAAGTGACACCAGGGCATAGGA	XM_002688832.2	117
IRS1	For[a] GGCGTCGTTCTGGAGGATG Rev[b] TGCGGTTGGGACTGAGGTTC	XM_002685642.1	178
PI3K	For[a] CCGAAAGGGTGCTAAAGAGGA Rev[b] GAGGTACTGGCCAAAGATTCAAAG	NM_174574.1	116
GAPDH	For[a] CACCCTCAAGATTGTCAGCA Rev[b] GGTCATAAGTCCCTCCACGA	BC102589	103
CYP17a1	For[a] GGAGGCGACCATCAGAGAAGTGC Rev[b] CAGCCGGGACATGAAGAGGAAGAG	NM_174304	319
CYP19a1	For[a] TAAAACAAAGCGCCAATCTCTACG Rev[b] GGAACCTGCAGTGGGAAATGA	BTCYP19	341

[a] Forward (For).
[b] Reverse (Rev).

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).

2.7. Real time PCR

An optimized real time PCR protocol was used to analyze the mRNA expression of *IR*, *IRS1* and *PI3K* using SYBR Green I (Invitrogen) technology in LightCycler (Pfaffl, 2001). The primer sequences for cytochrome P450 aromatase (CYP19a1) and cytochrome P450 17a-hydroxylase/17,20-lyase (CYP17a1) were used to confirm the mRNA purity of bovine granulosa and theca cells (no cross-contamination) (Lagaly et al., 2008; Marelli et al., 2014). Transcript levels were measured by relative quantitative real time PCR using a StepOne Real Time PCR System (Applied Biosystems, Life Technologies, CA, USA). An optimized protocol was used: initial denaturation at 98 °C for 3 min, 40 cycles of denaturation at 98 °C for 5 s and annealing at 60 °C (*IR*), 61 °C (*IRS1*), 57 °C (*PI3K*) or 52 °C (glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which was included as the housekeeping gene) for 20 s, extension at 72 °C for 10 s and fluorescence reading at 74 °C. All measurements for each sample were performed in duplicate and the CVs were calculated as described by Bustin et al. (2009). The efficiency of PCRs and relative quantities were determined from a seven-point standard curve. Standard curves were performed from a dilution series of pooled cDNAs (including seven dilutions from 1/2 to 1/128), and PCR efficiency was calculated using the StepOne software v2.3 (Applied Biosystems). In standard curves, R^2: 0.99. About 7 ng cDNA was used for all primers, except for *IRS1*, where 17 ng was used for each real time PCR reaction and was previously quantified by the Qubit method (Invitrogen). For amplification, 4 μL of cDNA was combined with a PerfeCta SYBR Green SuperMix, ROX (Quanta Biosciences, Inc., MD, USA) in a final volume of 20 μL. The primer sequences used are described in Table 1.

The mRNA expression levels of genes were recorded as cycle threshold (Ct) values that corresponded to the number of cycles at which the fluorescence signal can be detected above a threshold value. The Ct was calculated manually using StepOne v2.3 (Applied Biosystems). 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).

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. Immunohistochemistry

Protein expression of IR, IRS1 and the alpha p85 subunit of PI3K was detected using polyclonal antibodies (dilutions used: IR 1:100, IRS1, 1:750 and PI3K 1:150; antibody specifications are shown in Table 2). The extravidin biotin

Table 2
Antibodies, suppliers and dilutions used.

Antibodies	Type	Supplier	Dilution
Primary antibodies			
IR	Polyclonal. Sc-711	Santa Cruz Biotechnology.	1:100
IRS1	Polyclonal. Sc-559	Santa Cruz Biotechnology.	1:750
PI3K	Polyclonal. Sc-423	Santa Cruz Biotechnology.	1:150
Secondary antibody			
Anti-rabbit IgG	Polyclonal	CytoScan biotinilated link, CytoScan TM HRP detection system (Cell Marque, CA, USA)	Ready to use

immunoperoxidase method (CytoScan TM HRP detection system, Cell Marque, CA, USA) was carried out as described previously (Ortega et al., 2009; Salvetti et al., 2009). For antigen visualization, 3, 3′ diaminobenzidine (DAB) was used as the chromogen. Negative control sections in which the primary antibody was replaced by a non-immune rabbit serum were included (Ortega et al., 2009). Some sections were incubated with DAB alone to exclude the possibility that endogenous peroxidase activity had been unsuccessfully blocked.

The specificity of the primary antibodies used in this study (Table 2) was evaluated by western blot. Bovine ovarian sections were homogenized and then centrifuged to obtain the supernatants that were separated in SDS-PAGE. After blotting on nitrocellulose membranes (Hybond ECL nitrocellulose membrane; GE Healthcare, Buckinghamshire UK), the membranes were blocked to prevent unspecific protein binding and then incubated overnight at 4 °C with specific primary antibodies. Bound antibody was detected using anti-rabbit IgG peroxidase antibody (Cell Marque, CA, USA) and ECL plus western blotting detection reagents (GE Healthcare) (Ortega et al., 2010).

2.10. Image analysis

Images were analyzed using the Image Pro-Plus 3.0 system (Media Cybernetics, Silver Spring, MA, USA). Images were digitized using a color video camera (Motic 2,000, Motic China Group, China) mounted on the top of a conventional light microscope (Olympus BH-2, Olympus, Co., Japan) using an objective magnification of 40×, as described previously (Ortega et al., 2009; Ortega et al., 2010; Rodríguez et al., 2013).

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 in the follicular wall, at least three sections were evaluated for each specimen and antibody. The average density (% of immunopositive area) of the antibodies for IR, IRS1 and the alpha p85 subunit of PI3K 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 with the 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 interna). Sections were analyzed with the observer blinded to the experimental group. 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. Quantification of biological markers using this approach has been successfully applied to quantify immunoreactivity in different tissues (Ranefall et al., 1998; Ortega et al., 2009).

2.11. Statistical analysis

A statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used to perform the statistical test. The distribution of data was tested for normality using the Kolmogorov–Smirnov 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 a multiple comparison test. A value of $P < 0.05$ was considered significant. The mRNA expression from all follicular categories (small tertiary, medium tertiary, large tertiary and cystic follicles) was compared between them. Differences in protein expression were assessed separately in each follicular layer and the expression of markers of interest in the same follicular categories between groups was compared. Furthermore, analogous structures were compared with cysts, i.e. control tertiary follicles, spontaneous and induced cysts. The results are expressed as mean ± SEM.

3. Results

3.1. Insulin and BHB

Cows with spontaneous COD had significantly lower concentration of serum insulin than control animals (0.17 ± 0.08 ng/mL and 0.45 ± 0.12 ng/mL, respectively, $P < 0.001$), but similar levels of BHB (0.43 ± 0.35 mmol/L and 0.40 ± 0.23 mmol/L, respectively). Likewise, insulin concentration in follicular fluid was similar between groups (0.121 ± 0.033 ng/mL in cows with spontaneous COD and 0.120 ± 0.034 ng/mL in control animals) (Table 3).

3.2. IR, IRS1, PI3K 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.

Table 3
Concentration of insulin in serum and follicular fluid, and beta-hydroxybutyrate (BHB) in sera from control cows and cows with spontaneous COD.

	CONTROL Mean ± SD (n = 10)	COD Mean ± SD (n = 25)	P value
Serum insulin (ng/mL)	0.45 ± 0.12	0.17 ± 0.08	<0.001
Insulin in follicular fluid (ng/mL)	0.120 ± 0.034	0.121 ± 0.033	0.963
BHB (mmol/L)	0.40 ± 0.23	0.43 ± 0.35	0.570

The identity of the PCR products was confirmed by sequencing (100% homology with bovine sequences). All genes presented low overall variability (0.80–1.15% CV of Ct). *GAPDH* gene expression was similar between the different groups evaluated (P > 0.05) since no difference in average Ct of GAPDH between groups was obtained.

The mRNA expression levels of *IR*, *IRS1* and *PI3K* were analyzed in different follicular cell layers. The mRNA expression levels of *IR* and *IRS1* were lower in granulosa cells of follicular cysts than in the other follicular structures (P < 0.05) (Fig. 1A and B). In granulosa cells, the levels of *IR* and *IRS1* mRNA detected were similar between the control tertiary follicles of different size. However, the levels of *IR* were lower in cysts than in small and medium tertiary follicles (P < 0.05). The mRNA of *IRS1* was lower in cysts than in larger tertiary follicles. Moreover, in theca cells, mRNA expression levels of *IR* and *IRS1* were similar in the different follicular structures of both controls and cysts (Fig. 1A and B). No differences were detected in *PI3K* gene expression in granulosa cells between the different groups evaluated (P > 0.05) (Fig. 1C). Moreover, no differences were detected in the expression of all genes of theca cells from different follicles. It is interesting to note that the same pattern of PI3K mRNA expression was observed in both granulosa and theca cells.

3.3. Protein expression of IR, IRS1 and alpha p85 PI3K

Protein expressions of IR, IRS1 and alpha p85 PI3K were evaluated in follicles at different developmental stages. All insulin signaling components were detected both in granulosa and theca cells (Fig. 2). In control follicles, expression of IR was higher in granulosa cells than in theca cells and than in follicular cysts (Fig. 2B). IRS1 and alpha p85 PI3K protein expression showed a similar pattern in granulosa cells of both control and cystic structures. However, theca cells of control tertiary follicles showed more staining for alpha p85 PI3K than in follicular cysts (Fig. 2C–F).

IR protein expression was greater in primary and tertiary follicles of control ovaries than in the follicular cysts from cows with spontaneous COD and ACTH-induced COD (P < 0.05) (Fig. 3A).

IRS1 protein expression showed a similar pattern: it was greater in primary follicles than in follicular cysts from cows with spontaneous COD and ACTH-induced COD (P < 0.05) (Fig. 3B). However, no differences were detected between the different structures of the groups evaluated. Similar levels of IRS1 were obtained in secondary and

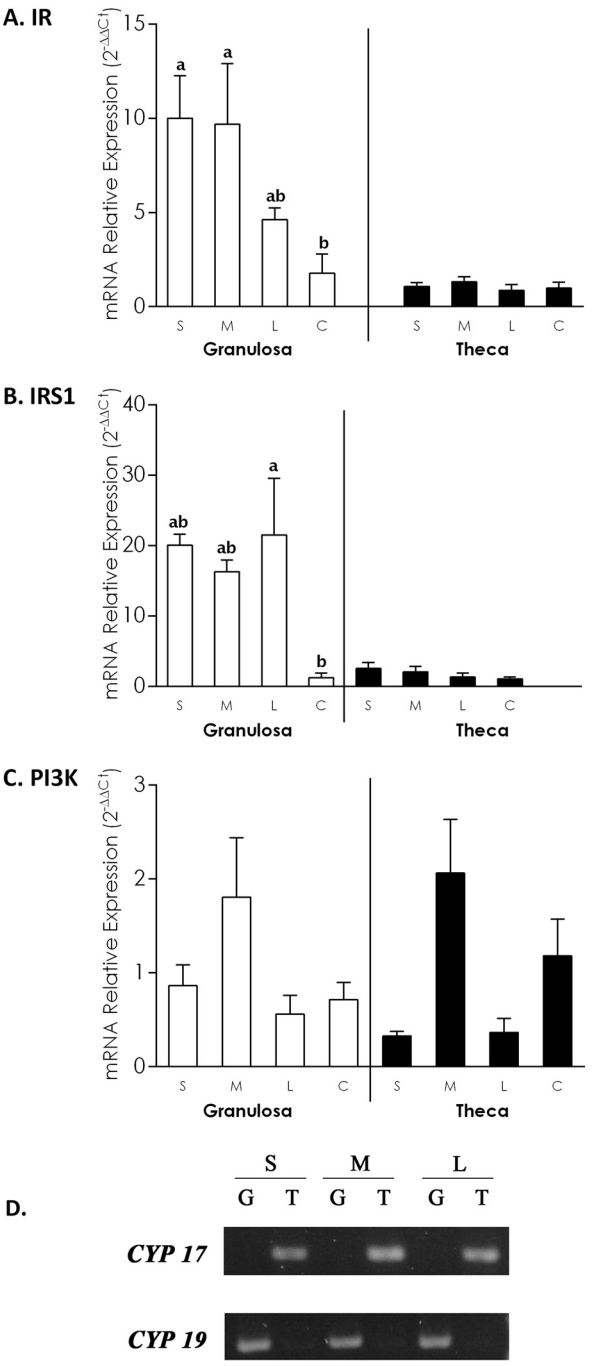

Fig. 1. mRNA relative expression ($2^{-\Delta\Delta Ct}$) of *IR* (A), *IRS1* (B) and *PI3K* (C) in granulosa and theca cells of small (S, <5 mm), medium (M, 5–10 mm) and large (L, 10 mm) tertiary follicles of controls and follicular cysts (C) of cows with spontaneous COD. Values represent mean ± SEM. Bars with different letters are different (P < 0.05). Gel electrophoresis of specific real time-PCR products (D). Specific primers expression in bovine ovarian components tissues showed absence of cross-contamination between follicular cell populations. *IR*, insulin receptor; *IRS1*, insulin receptor substrate-1; *PI3K*, phosphatidylinositol 3-kinase; COD, cystic ovarian disease; *G*, granulosa cells; *T*, theca cells.

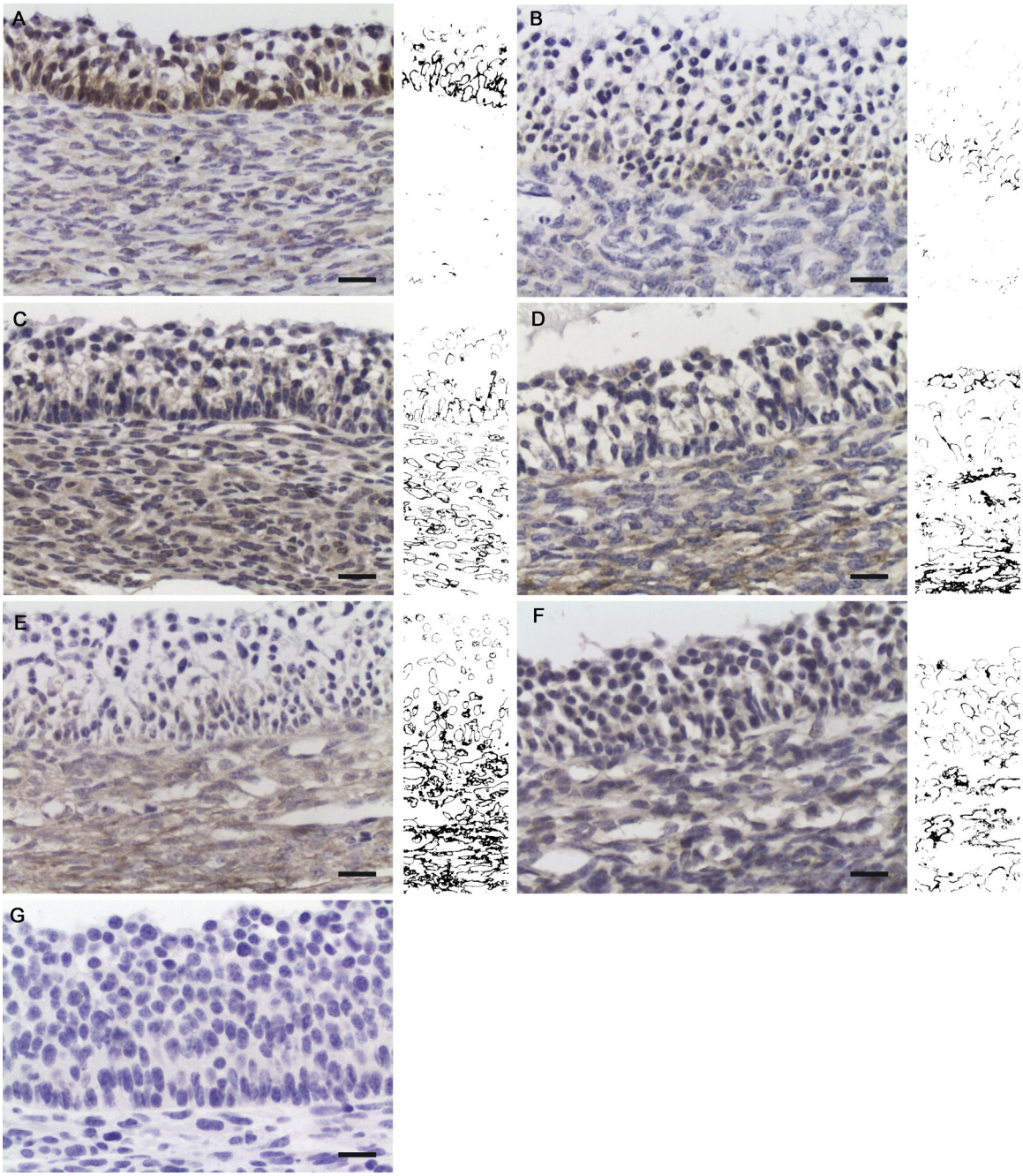

Fig. 2. Representative images of IR (A, B), IRS1 (C and D) and alpha p85 PI3K (E, F) protein localization by immunohistochemistry in bovine follicles. Images represent tertiary control follicles (A, C and E) and cystic follicles (B, D and F). In the right column of each image, the immunopositive areas were segmented by digital image analysis, showing in black the differential cytoplasmic immunostaining pattern. No staining was observed when the primary antibody was replaced with normal rabbit serum (negative control (G). Bars = 25 μm.

tertiary follicles of control and animals with COD (spontaneous and ACTH-induced) compared with cystic structures. Moreover, no differences in PI3K expression were observed in granulosa cell follicles of different stage from control and cystic animals (Fig. 3C) ($P > 0.05$).

IR and IRS1 of theca cells from tertiary and cystic follicular structures showed no differences between all groups evaluated (Fig. 3A and B). In contrast, alpha p85 PI3K protein expression was lower in cystic follicles from cows with spontaneous COD than in the control group ($P < 0.05$) (Fig. 3C).

4. Discussion

Our data indicate that the ACTH model to induce COD was adequate to study the molecules analyzed in

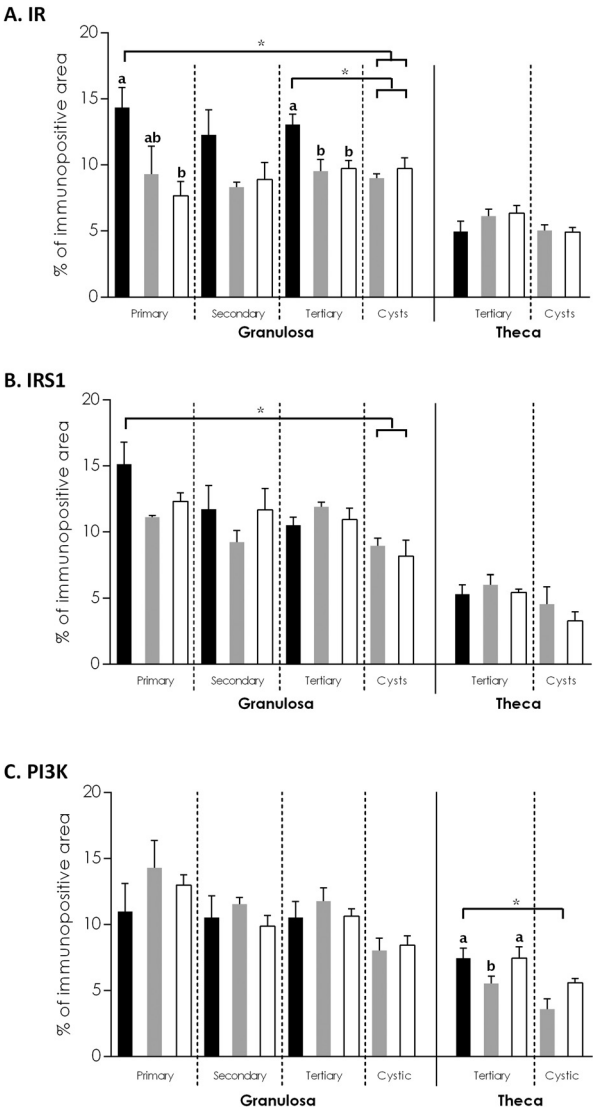

Fig. 3. Relative protein expression (measured as percentage of immunopositive area) of IR (A), IRS1 (B) and alpha p85 PI3K (C) in granulosa and theca cells of primary, secondary, tertiary and follicular cysts of controls, cows with spontaneous COD and cows with ACTH-induced COD. Values represent mean ± SEM. Bars with different letters within each follicle category are different ($P < 0.05$). Differences relative to the expression in follicular cysts in relation to control primary and tertiary follicles are also indicated: $^{*}P < 0.05$. IR, insulin receptor; IRS1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3-kinase; COD, cystic ovarian disease.

the insulin response since no differences were observed between induced and spontaneous COD. We determined that *IR* and *IRS1* mRNA levels were higher in granulosa cells and lower in spontaneous follicular cysts than in control tertiary follicles. However, *PI3K* mRNA levels were similar between the follicles evaluated. The protein expression of these signaling intermediates showed the same pattern as mRNA levels, considering tertiary and cystic follicles. However, alpha p85 PI3K protein expression was higher in theca cells from tertiary follicles than in both spontaneous and induced cystic follicles.

In the ovary, insulin modulates the gonadotropin response in granulosa cells (Willis and Franks, 1995; Shimizu et al., 2008) and has consequently been associated with steroidogenesis (Spicer and Chamberlain, 1998; Spicer et al., 2002; Silva et al., 2006). Willis et al. (1996) demonstrated that insulin enhances estradiol production in granulosa cells at physiological concentrations. On the other hand, Shimizu et al. (2008) determined greater levels of estradiol in follicular fluid of preovulatory follicles. This greater production of estradiol could be associated with an increase in IR expression in preovulatory follicles, which results essential to lead developing follicles to ovulation. In cystic follicles, we determined lower *IR* mRNA and protein expressions than in tertiary follicles. Moreover, the mRNA expression of *IRS1* was significantly lower in ovaries from animals with spontaneous COD. Considering the role of insulin in the ovulation process, such lower levels could participate in the insulin unresponsiveness in follicles of cows with COD, preventing follicle maturation and ovulation and favoring follicular persistence. In agreement with our results, Bossaert et al. (2010) reported higher protein expression of IR in granulosa cells than in theca cells. The high IR expression in granulosa cells suggests that the latter, rather than theca cells, are the main target cells for insulin action on follicular growth and function. The importance of insulin for the final development and ovulation of follicles (Gong, 2002; Kawashima et al., 2007), and for the stimulation of steroidogenesis (Spicer et al., 2002; Silva et al., 2006) is widely accepted. The lower IR presence in granulosa cells of cystic follicles is a novel finding and the reduced expression of the downstream intermediate PI3K in theca cells could indicate an altered insulin final response in these structures.

Insulin can stimulate cell division and survival by activating the IR, which in turn acts through two main signal transduction cascades: the Ras/MAP kinase (Lee and McCubrey, 2002) and the PI3K/Akt kinase pathways (Cantley, 2002). According to that, our results support the clear decrease in the proliferation index in all layers of cystic follicles in animals with COD (Isobe and Yoshimura, 2007; Salvetti et al., 2010), probably through an alteration of the intermediates of the signaling pathway.

The absence of changes in mRNA and protein expressions of PI3K at different stages of follicle development and cysts suggests that variations in PI3K could be exhibited more at the enzyme activity level than the absolute amount. Changes in PI3K downstream effectors, such as PPARG or mTOR, suggest an enzyme activity modification of this key regulator since no variations were detected in the amount of PI3K expression (Alam et al., 2004; Ortega et al., 2010). On the other hand, PI3K might be recruited and activated by different growth factors and hormones (Cantley, 2002), supporting the similar expression obtained in the present study. In our laboratory, increased levels of IGF receptor type 1 were detected in cystic ovaries, which are probably linked to the PI3K/Akt pathway to express the response (Rodríguez et al., 2014). A different feature was observed for alpha p85 PI3K protein expression in theca cells, where levels were higher for tertiary follicles than for cystic follicles. Palaniappan et al. (2013)

have recently shown a stimulatory effect of insulin on theca-interstitial cell proliferation and cell cycle regulatory proteins through the mammalian target of the rapamycin complex 1 (mTORC1)-dependent pathway, a direct downstream effector of PI3K. Probably, the decrease in alpha p85 PI3K protein expression in theca cells from cysts herein observed displays a more critical situation for the insulin functions. An alteration in advanced stages of insulin signaling could develop in theca cells and further compromise their proliferation as well as the apoptosis index.

Inhibition of PI3K function blocks the FSH-induced up-regulation/activation of several follicular differentiation markers and signaling proteins that control mRNA translation (Alam et al., 2004). Furthermore, IR expression is stimulated by FSH treatment in cultures of granulosa cells (Shimizu et al., 2008). Cows with COD have an altered function and regulation of the hypothalamus-pituitary-gonadal axis (Vanholder et al., 2006). Therefore, variations in gonadotropins could be participating in the altered gene and protein expressions of IR herein observed. In the present study, gonadotropins could be regulating the insulin response through modulation of the expression of intermediates in the signaling pathway here evaluated, beyond the similar levels of insulin found in follicular fluid of control and cystic cows. Apparently, these similar levels would not be associated with a potential different local response to insulin in cows with COD, being the variations in the intermediates of signaling pathway the ones that could modify the final response. Moreover, our results suggest a down-regulation of IR expression in cows with COD, mainly in tertiary follicles and at the first stages of folliculogenesis. Consequently, the final insulin response might be modified and ovarian functions, such as steroidogenesis, altered, as reported in previous studies (Ortega et al., 2008; Rey et al., 2010; Amweg et al., 2013).

Insulin is a metabolic hormone essential for follicular maturation and ovulation (Kawashima et al., 2012). During postpartum and early lactation, cows suffer from a negative energy balance because the nutrient requirements for maintenance and lactation exceed the ability of the cow to consume energy in the feed (Lucy, 2007). Cows in negative energy balance have lower blood concentrations of insulin and IGF1 (Kawashima et al., 2007; Lucy, 2007), which are in turn able to influence GnRH and LH secretion (Lucy, 2003). In the ovary, insulin and IGF1 may also increase the sensitivity to gonadotropins. Since the concentrations of the aforementioned hormones are low (Lucy, 2003), postpartum dairy cows are thought to be less sensitive to LH and FSH. This prevents the normal resumption of cyclicity and leads to follicle persistence and consequent cyst formation. Since our results showed similar insulin levels in follicular fluid in control and cystic cows, IGF1 could have an active role in this process since previous studies of our laboratory have demonstrated reduced IGF1 levels in COD cows (Ortega et al., 2008; Rodríguez et al., 2013). On the other hand, some authors have detected increased levels of IGF1 in cysts (Probo et al., 2011), whereas others have shown reduced levels of these metabolic hormones, which may reflect alterations in ovarian activity, thus favoring cyst formation (Braw-Tal et al., 2009).

5. Conclusions

Our results provide new insights into the insulin response in cows with COD. It is interesting to note that although we detected a similar concentration of insulin in follicular fluid of both cystic and control cows, we found alterations in the signaling cascade. 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 follicle persistence.

Conflict of interest

None.

Acknowledgments

This work was supported by grants (PICT 2012-2649) from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and CAI+D 2011 (501 201101 00394) from Universidad Nacional del Litoral (UNL).

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