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Apoptosis of bovine granulosa cells: Intracellular pathways and differentiation

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ABSTRACT

Follicular atresia in granulosa and theca cells occurs by apoptosis through weak hormonal stimulation. We have previously proposed an *in vitro* model to study this process by inducing apoptosis in BGC-1, a bovine granulosa cell line, and in primary cultures from ovaries with or without corpus luteum (CPGB+ and CPGB−, respectively), with different doses of gonadotropin releasing hormone (GnRH) analogs (leuprolide acetate (LA) as agonist and antide as antagonist). BGC-1 represent immature granulosa cells, whereas CPGB represent different degrees of luteinization.

Our aim was to evaluate the intracellular pathways involved in the GnRH regulation of apoptosis in BGC-1. Treatment with LA 100 nM but not with antide led to an increase in BAX over BCL-2 expression, showing antagonism of antide. All treatments inhibited phospholipase-D (PLD) activity compared to control, implying agonist behavior of antide. Progesterone *in vitro* production and 3β-hydroxysteroid dehydrogenase (3β-HSD) expression revealed different degrees of luteinization: BGC-1 were immature, whereas CPGB+ were less differentiated than CPGB−. We concluded that LA-induced apoptosis in BGC-1 occurs by activation of the mitochondrial pathway and by inhibition of PLD activity and that antide might work both as an antagonist of the intrinsic pathway and as an agonist of the extrinsic protection pathway by inhibiting PLD activity.

1. Introduction

Under normal physiological conditions, cells of a multicellular organism die predominantly through apoptosis, a process which is also crucial in development, normal cellular differentiation, and tissue homeostasis. Apoptosis consists of three successive stages: (1) commitment to death triggered by extracellular (Fas/FasL, caspase 8) or intracellular (Bcl-2, caspase 9) signals; (2) execution of cell killing by activation of intracellular proteases (caspases 3 and 7); and (3) removal of dead cells through engulfment of cell corpses or apoptotic bodies by other cells (efferocytosis), followed by degradation of the corpses (Vaux and Strasser, 1996). The study of the pathways that regulate cell death from outside the cell is complicated because the same pathways are often used to transmit signals required for growth and differentiation (Miyajima et al., 1999). External signals, such as trophic factors and hormones, can either activate or suppress death programs (Reed, 1989; Adams and Cory, 1998). A cascade of caspases plays the central executioner role by cleaving various mammalian death substrates, including cytosolic and nuclear proteins that have a role in DNA replication and repair, RNA splicing, cell division, and cytoskeletal structure. This biochemical process results in the morphological changes that are characteristic of apoptosis (Carou et al., 2015). The Bcl-2 family of genes associated with the mitochondrial intracellular pathway of apoptosis regulation includes, apoptosis-inhibiting (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Nr13, and A1/Bfl-1) and apoptosis-promoting (Bax, Bak, Bok, Diva, Bcl-xs, Bik, Bim, Hrk, Nip3, Nix, Bad, and Bid) members (Reed, 1989; Adams and Cory, 1998).

In the ovary, apoptosis is involved in several physiological processes such as recruitment of prenatal germ cells (oocyte death), follicular atresia (death of granulosa cells (GCs)), ovulation (death of follicular epithelial cells), and luteolysis (luteal cell death) (Tilly et al., 1991; Hussein, 2005). Folliculogenesis, atresia, ovulation and luteinization, which command ovarian physiology, are carefully regulated by hormones. Hormones such as gonadotropins, epidermal growth factor

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(EGF)/transforming growth factor-α (TGFα), basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), and estrogens (E_2) have been identified as follicle survival factors, whereas androgens, interleukin-6 (IL-6), and gonadotropin-releasing hormone (GnRH) are potential atretogenic factors (Hsueh et al., 2008). In all species studied, one of the first signs of follicular atresia is the initiation of apoptosis in GCs (Hughes and Grospe, 1991; Tilly and Hsueh, 1993; Palumbo and Yeh, 1994; Nahum et al., 1996; Yuan and Giudice, 1997; Manikkam and Rahamahendran, 1997; Saito et al., 2000; Yang and Rajamahendran, 2000). In addition, evidence suggests that GnRH plays a role in follicular atresia (Hsueh et al., 1984; Piquette et al., 1991; Billig et al., 1994). Various publications have reported GnRH receptor expression in GCs and luteal cells of a wide range of species (Clayton et al., 1979, 1992; Harwood et al., 1980; Jones et al., 1980; Reeves et al., 1980; Pieper et al., 1981; Latouche et al., 1989; Oikawa et al., 1990; Dong et al., 1993; Whitelaw et al., 1995), including cows (Ramakrishnappa et al., 2001; Carou et al., 2015). *In vitro* studies have shown that GnRH inhibits DNA synthesis (Saragueta et al., 1997) and induces apoptosis in cultures of rat (Billig et al., 1994) and human GCs (Zhao et al., 2000).

In previous studies, we found that treatment of a bovine granulosa established cell line (BGC-1) for 24 and 48 h with 100 nM leuprolide acetate (LA), a type I GnRH agonist, in the presence of 5% (v/v) fetal calf serum (FCS) led to significant induction of apoptosis (Carou et al., 2015). Apoptosis was evaluated morphologically by DAPI and hematoxylin staining and confirmed by TUNEL. These cells also showed phosphatidylserine exposure (a signal widely observed during apoptosis) when analyzed by flow cytometry (FACS). In the same study, we found that treatment with the GnRH antagonist antide partially inhibited the apoptosis induced by LA when tested by hematoxylin staining and FACS. In addition, in the absence of FCS, LA was not able to induce apoptosis (Carou et al., 2015). Our results in primary GC cultures obtained from ovaries with (+) or without (−) corpus luteum (CL) (CPGB+, luteal ovarian stage; CPGB−, follicular ovarian stage, respectively) suggest that the behavior of GCs treated with different doses of the GnRH agonist LA (1, 10 and 100 nM) depends on the estrous stage. There is only a single functional GnRH type I receptor in humans and cows (Morgan et al., 2006). Millar et al. (2008) proposed that the GnRH type I receptor can assume different conformations which have different selectivity for GnRH analogs and intracellular signaling protein complexes. This could explain the different responses of LA in GCs depending on the luteinization stage. In our *in vitro* model, BGC-1 represents immature GCs, whereas CPGB+ and CPGB− represent different degrees of luteinization (Carou et al., 2015).

Luteinization is a process of cell differentiation by which a follicle becomes a CL after ovulation. During luteinization, GCs gradually switch from producing E_2 to producing progesterone (P_4) and reach terminal differentiation. After ovulation, GCs undergo profound changes in their hormonal responsiveness and in their capacity to produce steroids. These luteinized GCs constitute a major component of the CL and are the main source of ovarian P_4. Both the morphology and the biochemical function of GCs are altered by luteinizing hormone (LH). Although GCs cultured in serum-containing medium spontaneously luteinize *in vitro*, prior exposure to LH *in vivo* seems to be an important factor for luteinization (Hsueh et al., 1984). Therefore, we believe that, for *in vitro* research, the classification of ovaries in terms of the presence or absence of CL is necessary to obtain GCs at different luteinization stages. Conely et al. (1995) indicated the importance of determining the degree of luteinization in GCs obtained from cows to study GC physiology *in vitro*. We believe that *in vitro* models are important to understand the GnRH local mechanism of action because, at the central level, the release of follicle stimulating hormone (FSH) and LH make *in vivo* models limited.

The current knowledge on the molecular signaling in bovine apoptotic GCs suggests that both the intrinsic (Choi et al., 2004) and extrinsic (Tilly, 1996; Glamočlija et al., 2005) pathways might be involved in regulating atresia by GnRH in mammalian follicles and that this regulation is likely dependent on the estrous stage (Quirk et al., 2005, 2006). *In vivo* and *in vitro* studies suggest that the regulation of apoptosis in rat (Zhao et al., 2000; Parborell et al., 2002; Peng et al., 2008) and human GCs (Tsai et al., 2005) treated with GnRH analogs occurs through the intrinsic pathway.

To completely understand the mechanisms involved in the hormonal regulation of atresia we propose BGC-1 as a suitable model to study this process *in vitro*. We believe it is necessary to first classify GCs according to their differentiation stages. For that purpose, it is also necessary to measure P_4 production *in vitro* and to determine apoptosis because P_4 production is due to luteinization but is also present in apoptotic GCs (Amsterdam et al., 2003) and follicular fluid from basal atretic follicles (Clark et al., 2004). Seeding conditions like the cell density should also be tested because high GC densities have been associated with improved amounts of P_4 *in vitro* production (Portella et al., 2010). Luteinized cells produce more P_4 than immature ones, so it is expected to find no P_4 production in BGC-1 and more P_4 production in CPGB− than in CPGB+ cultures. Expression of 3β-hydroxysteroid dehydrogenase/Δ5–4 isomerase (3β-HSD) in GCs and bovine ovaries classified as with (+) or without (−) CL should also be evaluated. 3β-HSD is a membrane-bound enzyme that catalyzes an essential step in the transformation of all 5-pregnen-3β-ol and 5-androsten-3β-ol steroids into the corresponding 3-keto-4-ene-steroids, namely P_4 as well as all the precursors of androgens, estrogens, glucocorticoids and mineralocorticoids, and is expressed in the ovary, adrenal gland, testis, placenta and some nonsteroidogenic tissues (liver, skin and kidney) (Labrie et al., 1992).

BGC-1 is an established cell line obtained by spontaneous immortalization of a primary culture of bovine GCs obtained from small follicles (2–5 mm). This cell line was obtained by continuous subculturing of primary cultures of high density after 75 passages. BGC-1 immature GC-like behavior has been well documented (Bernath et al., 1990; Lerner et al., 1995; Lanuza et al., 1998; Havelock et al., 2004; Fazzini et al., 2006). Here, we used BGC-1 as an immature GC model to study the effects of GnRH analogs on apoptosis regulation.

Steele and Leung (1993) found that GnRH stimulates phospholipase D (PLD) activity and associated this stimulation with P_4 synthesis. A very important signal transduction mechanism is initiated from phosphatidylcholine catabolism by PLD to give phosphatidic acid, which is then dephosphorylated to give diacylglycerol (Steele and Leung, 1993). Both phosphatidic acid and diacylglycerol can bind to molecules that modulate cellular events such as proliferation, apoptosis and cell differentiation (Taylor et al., 2005). *In vitro* assays with mouse and pig GCs describe this enzyme system as mediating regulation processes of steroidogenesis by GnRH at doses higher than 10 nM (Steele and Leung, 1993; Amsterdam et al., 1994), apoptosis and differentiation (Liscovitch and Amsterdan, 1989; Kim et al., 1999; Nakashima and Nozawa, 1999), and proliferation of GCs (Ming-Te, 2005). Kim et al. (1999) observed that GCs are protected from apoptosis by PLD activity through the extrinsic pathway (Kim et al., 1999). On the other hand, in previous studies, we found a dual behavior of the GnRH agonist LA, suggesting the presence of a protective alternative mechanism against high doses of LA (over 10 nM) in CPGB− but not in CPGB+, which behaved similarly to BGC-1 (Carou et al., 2015). We believe this dual behavior is dependent on the luteinization stage of GCs and expect that extrinsic pathway is more likely susceptible to degree of luteinization than intrinsic pathway.

Thus, in the present study; we aimed to explore the intracellular pathways regulating apoptosis in BGC-1 by treating cells with different doses of LA as well as with the GnRH antagonist antide. We also evaluate luteinization state of our three granulosa cell models, BGC-1, CPGB+ and CPGB−.

2. Materials and methods

2.1. BGC-1

A total of 310 cells/mm^2 (50,000 cells/mL) were seeded on 18 × 18 mm coverslips for immunocytochemistry (ICC) on 60-mm plates for WB and caspase 3 activity assays and on 100 mm plates for PLD activity determinations. A total of 155 cells/mm^2 (25,000 cells/mL) were seeded on 18 × 18 mm coverslips for P_4 determination by radioimmunoassay (RIA). Before hormonal treatment, cells were grown for 24 h in DMEM + F12 (Sigma D8900) supplemented with 2.4 g of sodium bicarbonate (pH 7.4), 5% FCS (of biotechnological quality; Internegocios SA), 2 mM glutamine (Sigma G8540), 50 mg/L gentamicin (Sigma G1264), 5% CO_2, saturated humidity and 38.5 °C until they reached confluence. Under these conditions, the mean doubling time was 17.5 h. The BGC-1 established cell line was gently provided by Lino Barañao, Instituto de Biología y Medicina Experimental (IByME), Buenos Aires, Argentina.

2.2. Primary culture of bovine granulosa cells (CPGB)

Follicular fluid was obtained by puncturing healthy medium-size (3–8 mm) ovarian follicles from cows and heifers after slaughter. Ovaries were classified as with (+) and without (−) CL. Cells were resuspended in DMEM + F12 supplemented with 2.4 g of sodium bicarbonate (pH 7.4), 5% FCS, 2 nM L-glutamine, 1,000,000 IU/L penicillin, 100 mg/L streptomycin (Penicillin-Streptomycin 100X, Gibco) or 50 mg/L gentamicin and 2.5 mg/L fungizone (amphotericin B, Sigma) and then plated in a manner similar to BGC-1. Regressing CL were classified as CPGB−.

2.3. Experimental design and treatment

All treatments were seeded in triplicate.

- a Apoptosis: Treatments performed with antide (A8802, Sigma) were incubated for 3 h before stimulation with LA (L0399, Sigma).Design 1: LA 0, 100 nM; LA 100 nM + antide 100 nM; antide 100 nM.Design 2: LA 0, 1, 10, and 100 nM.Design 3: LA 0, 1, 10, and 100 nM; LA 100 nM + antide 100 nM; antide 100 nM.For caspase 3 activity assays, two experiments for designs 1 and 2 and one for design 3 were performed. All of them were stopped at 24 and 48 h incubation.For WB and PLD assays, two experiments for design 3 were performed. WB was stopped at 48 h and PLD after 24 h incubation.

- a Luteinization stage: RIA for P_4 determination: in CPGB, four seeding densities (25,000; 12,500; 6250 and 3125 cells/mL) were evaluated at 4, and 8 days incubation (1 mL culture medium from each well was taken and kept at −20 °C until P_4 determination). In BGC-1, only a seeding density of 25,000 cells/mL was made due to the low expected secretion of P_4.

CPGB+ and CPGB− were seeded on 18 × 18 mm coverslips for morphological evaluation of apoptosis, mitosis and number of cells/coverslip (final density) by hematoxylin staining.

2.4. Expression of 3βHSD by ICC

To characterize the BGC-1 cell line more precisely and compare it with CPGB, ICC methods for detection of 3-βHSD were performed. Paraffin slides from bovine ovaries with and without CL were also evaluated by immunohistochemistry (IHC).

3β-HSD expression was assessed using the 3β-HSD polyclonal antibody (P-18) (sc-30820) (Santa Cruz Biotechnology), made in goat, at a dilution of 1:100, and incubated overnight (ON) at 4 °C. Biotinylated goat anti-IgG secondary antibody (Millipore, Tecnolab) was used at a 1:1000 dilution for 10 min at room temperature (RT). For the choice of antibody we rely on the work of Peralta et al. (2016) as well as the recommendation of Santa Cruz for the use of this antibody on bovine tissues.

A negative control was performed by incubating cells with secondary antibody alone. Images were captured using a microscope Leica DM4000B LED® with coupled camera Leica model DCC-380X®, and an image capture system supported by Leica LASZ software.

2.5. Hematoxylin staining

Cells were fixed with absolute methanol for 10 min and air dried after incubation. They were then stained for 15 min with Mayer's hematoxylin and toned with warm running water. Cells were air-dried for 1 h and mounted in DPX. Images of apoptosis were counted on a light microscope (60 fields at ×1000, approximately 500 cells per sample). Mitosis, necrosis, and phagocytosis were also recorded (data not shown). Images were captured using the image analysis system described above. The morphological evaluation of apoptosis in BGC-1 and CPGB has been described in detail in a previous publication (Carou et al., 2015).

2.6. Determination of P_4 by RIA

Samples were evaluated utilizing a commercial kit (IM118, Immunotech SA). Culture medium samples from BGC-1 (undiluted) and CPGB (diluted 1:3 with culture medium) were assessed (final volume: 100 µL). Antibody-coated tubes were successively added with 50 µL of standards (0, 0.12, 0.55, 2, 8.7 and 49 ng/mL), controls, or samples + 500 µL of tracer. Tubes were incubated for 1 h at 18 – 25 °C with shaking (350 rpm). The contents of the tubes were carefully aspirated. Two additional tubes were added with 500 µL of tracer to obtain total counts. Bound radioactivity and total counts were determined for 1 min (cpm). Non-specific binding was measured by addition of 300 µL of the calibrator (0 pg/mL). The measurement range (from analytical sensitivity to the highest calibrator) was 0.05 – 50 ng/mL. Intra assay CV was 0.06. Only one assay was performed.

2.7. Biochemical analysis of apoptosis by caspase 3 activity

BGC-1 were seeded in 60-mm plates and allowed to grow for 24 h until hormonal induction. After this, cells were incubated for 24 or 48 h and then scraped from culture dishes. The CASP-3-C kit (C8487, Sigma) was used for colorimetric detection of caspase 3 activity. The cell pellets were resuspended in lysis buffer for caspase in a ratio of 1 × 10^5 cells/µL at −80 °C until the enzymatic reaction. The cell lysate was centrifuged at 16,000 × *g* for 15 min at 4 °C. Samples were incubated with the substrate, acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA), in 96-multiwell ELISA plates in a moist chamber at 37 °C for 20 h. Absorbance was read in an ELISA reader at 405 nm. Positive control (purified caspase 3), nonspecific protease activity control (positive control or pool samples with caspase 3 inhibitor) and a calibration curve using p-nitroaniline (end product) were run at the same time. Time 0 of absorbance was used as blank.

Two ELISA determinations, one for 24 h and another one for 48 h incubation, each analyzing several experiments, were performed as follows: two experiments for design 1; two experiments for design 2 and one experiment for design3, leading to a sample size of 9 for 1 and 10 nM LA and 100 nM antide and a sample size of 15 for the control and 100 nM LA.

2.8. Analysis of the intracellular pathways that regulate apoptosis

2.8.1. Intrinsic pathway

The intrinsic pathway was analyzed by determining the protein

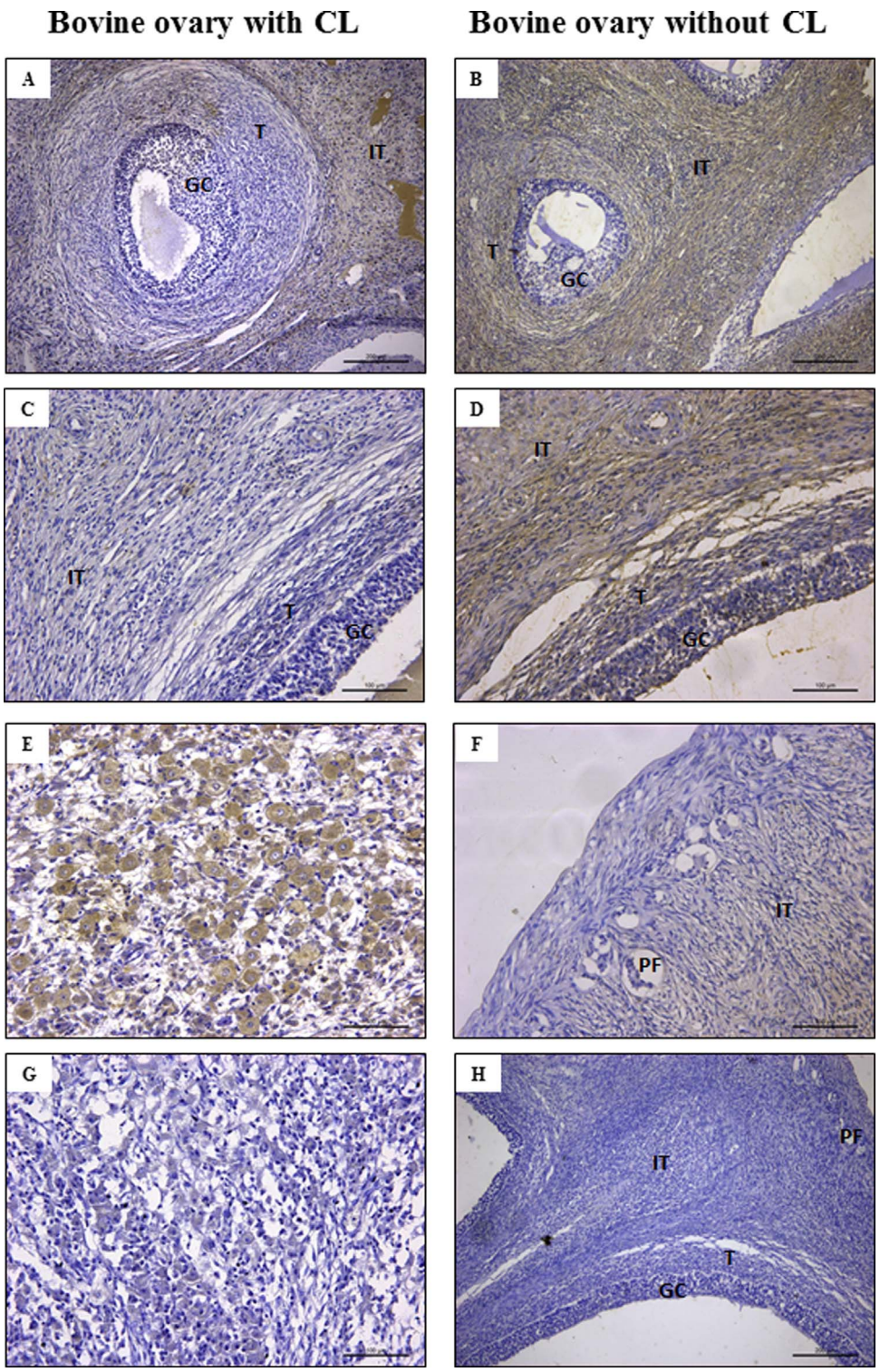

Fig. 1. Immunohistochemistry of the bovine ovary (with corpus luteum (CL): A, C, and E; without CL: B, D, and F) using 3β HSD antibody. A, B: small antral follicle (×100); C, D: large antral follicle (×200); E: CL (×200); F: primordial follicles (×200); G, H: negative control. G: ×200, CL; H: ×100 antral follicles. GC: granulosa cells; T: theca; IT: interstitial tissue; PF: primordial follicles.

expression of the BCL-2 family members (BAX and BCL-2) by WB. Representative samples of each treatment were seeded in a polyacrylamide gel 12% (40 μg of protein per lane). Samples were run for 2 h at 40 mA. The proteins were transferred to nitrocellulose membranes with 0.2 μm pore (Bio-Rad 162-0147) at 40 V, ON at 4 °C. After blocking with Nonfat-Dried Milk 5% (g/v) for 30 min, the membranes were incubated with the following primary antibodies: BAX: mouse monoclonal Anti-BAX from Sigma (B8449) 1:800 incubated ON at 4 °C (Nonfat-Dried Milk 1%); BCL-2: BCL-2 (C 21): sc-783, Santa Cruz, polyclonal rabbit, 1:200, incubated ON at 4 °C. The secondary antibodies against rabbit and mouse were used 1:10,000 and incubated for 2 h at RT with 2% Nonfat-Dried Milk (g/v). The membranes were treated with 2 N NaOH for 5 min before blocking antibodies and incubating them with anti-β-actin as the reference basal protein expression. The bands were identified by calculating "rf" and analyzed with the Image J program, expressed as relative band intensity against

β-actin.

2.8.2. Extrinsic pathway

The extrinsic pathway was analyzed by the PLD activity assay. The reaction was performed as in Yu et al. (2003) with some modifications. Samples were centrifuged in lysis buffer (the same as that used for the caspase 3 activity assay) at 4 °C (16,000 × *g*). Between 300 and 800 μg proteins per sample were used to perform the analysis. The reaction mix (360 μL per determination) was composed of: 25 mM HEPES, pH 7.2, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM phosphatidylcholine (P7081, Sigma), 6 mM oleic acid (O1008, Sigma), and 1.6 mM $(NH_4)_2SO_4$. Incubation was performed in a heat bath at 37 °C for 60 min. The tubes were placed in boiling water for 10 min, cooled to RT and 360 μL of chloroform was added. Samples were vortexed for 1 min at 2000 rpm and centrifuged at 4000 × *g* for 10 min, taking then 200 μL of the supernatant (aqueous phase) to test the concentration of choline released by PLD from phosphatidylcholine. For the determination of choline, 200 μL of standard of choline supernatant + 800 μL color reagent (45 mM TRIS-HCl (pH 8), 5 U peroxidase (P8375, Sigma), 0.3 mg 4-aminoantipyrine (A4382, Sigma), 0.2 mg phenol and 1 U choline oxidase (C5896, Sigma)) were incubated at 37 °C in a thermal bath in the dark for 90 min. The reaction was stopped with 1 mL 50 mM TRIS-HCl (pH: 8). Absorbance was measured in a spectrophotometer at 505 nm against choline standard curve (5–120 nmol choline chloride, C7527, Sigma) (Yu et al., 2003). One unit of PLD activity was defined as 1 nmol choline produced by 1 mg of BGC-1 lysate for 1 h at 37 °C (nmol choline/H/mg protein).

2.9. Protein samples

Treatments were stopped by scraping cells from the culture dishes by rake lifting into cold PBS and then centrifuged. The resulting pellet was resuspended in corresponding lysis buffer depending on the evaluation technique. For the WB assay, 50 μL of lysis buffer (1% (v/v) Triton X-100; 20 mM Tris base (pH 8); 137 nM NaCl and 10% (v/v) glycerol) was added. Then, 0.5 mM PMSF was added before freezing at −20 °C. To evaluate enzymatic activity, pellets were resuspended on caspase 3 lysis buffer (L2912, Sigma), as described in the caspase 3 protocol. In all cases, samples were centrifuged at high speed (16,000 × *g*) at 4 °C to eliminate membranes prior to protein dosage by the Bradford colorimetric assay.

2.10. Statistical analysis

Statistical comparisons were made by two-way ANOVA, using the Bonferroni test as a means comparison analysis. In some cases, we performed Dunnett's multiple comparison tests to compare all treatments against the control with a significance level $\alpha = 10\%$. For testing, we used Statistix 8.0 software.

3. Results

3.1. Expression of 3β-HSD by ICC and IHC

In bovine ovaries without CL, we observed high expression of 3β-HSD in the interstitial tissue, theca, and in granulosa of large antral follicles (Fig. 1B and D). Such expression was absent in ovaries with CL, where 3β-HSD expression was found only in the interstitial tissue and CL (Fig. 1A, C and E). Interstitial expression was higher in ovaries without CL (Fig. 1B and D) than in those with CL (Fig. 1A and C). No expression was observed in the subcortical zone or in primary and primordial follicles (Fig. 1F). The highest 3β-HSD expression was observed in CL (Fig. 1E).

Granulosa cell cultures showed dramatic differences in 3β-HSD expression between BGC-1 and primary cultures. No expression was observed in BGC-1 (Fig. 2A), whereas in CPGB the label was evidently bound to intracellular membranes, especially in the perinuclear area (Fig. 2B).

3.2. P_4 production by BGC-1 and CPGB

Total P_4 *in vitro* secretion was high in CPGB cultured for 4 days and even higher in those cultured for 8 days. P_4 secretion at 4 days incubation (30.7 ± 5.8 ng/mL) was 43.7% higher in CPGB− than in CPGB+ ($P = 0.0042$; $n = 12$), taking all seeding conditions together (Table 1). The final cell density (average number of cells/coverslip) was significantly influenced by the incubation time as well as by the seeding cell concentration, independently of the presence of CL ($P < 0.0001$; $n = 24$). So, we examined relative P_4 production (in relation to number of cells/coverslip). Relative P_4 secretion was 82% higher in CPGB obtained from ovaries without CL than in those from ovaries with CL (3.24 ± 0.3 ng/cells per field in CPGB−; $P = 0.0001$; $n = 12$) at 4 days incubation (Table 2). The increased production of P_4 at 4 days incubation in CPGB− was not due to the cell final density as the relative P_4 production remained high, but neither due to increased apoptosis because apoptosis was 4.89 times higher in CPGB+ with (3.63 ± 0.49%) than in CPGB− ($P = 0.03$; $n = 12$) (Table 3). The presence of CL was not decisive for P_4 production in long-term cultures (over 6 days of incubation). In contrast, in BGC-1, P_4 secretion was under detection levels even at 8 days incubation, where we found a small displacement (6.18 ± 2.19 fg/mL). Since the detection level is over 50 fg/mL, this small displacement should be interpreted as a displacement in relative but absolute terms.

3.3. Caspase 3 activity

Treatment with 100 nM LA led to an increase in caspase 3 activity ($P = 0.0156$; activity index respect to control = 1.5) at 24 h of incubation. The increase was not inhibited by the presence of antide 100 nM (Fig. 3).

Treatment with antide also led to an increase in caspase 3 activity compared to control ($P = 0.0497$) at 48 h incubation, indicating a possible *per se* agonist behavior of antide at 100 nM (Fig. 3).

3.4. Expression of BAX and BCL-2 evaluated by WB

Data from five membranes revealed for BAX and the proportion of BAX/BCL-2 yielded significant results ($P = 0.067$, $n = 5$; $P = 0.1$, $n = 5$, respectively). Treatment with 100 nM LA showed a higher level of BAX expression compared to control. The treatment showed no significant effect on the expression levels of BCL-2. The control and antide treatments showed a BAX/BCL-2 ratio near or below 1 (Fig. 4).

3.5. PLD activity

The protection pathway of apoptosis was analyzed through the extrinsic pathway by PLD activity determination. PLD activity was significantly inhibited ($P = 0.0079$, $n = 6$) after 24 h of incubation in all treatments against the control group in BGC-1 cells (Fig. 5). Therefore, no protective extrinsic pathway was seen in BGC-1 even at high doses of LA.

4. Discussion

The lack of 3βHSD expression found in BGC-1 is in concordance with the findings of Bernath et al. (1990) and Lerner et al. (1995), who proposed BGC-1 as a model for undifferentiated GC. The membrane bounded expression of 3β-HSD observed in CPGB clearly supports *in vitro* observations. In the rat ovary, 3β-HSD is more commonly found bound to the endoplasmic reticulum membrane as well as in crista and outer membranes of mitochondria (Pelletier et al., 2001). The differential behaviors of immature (BGC-1 and CPGB+) *versus* mature

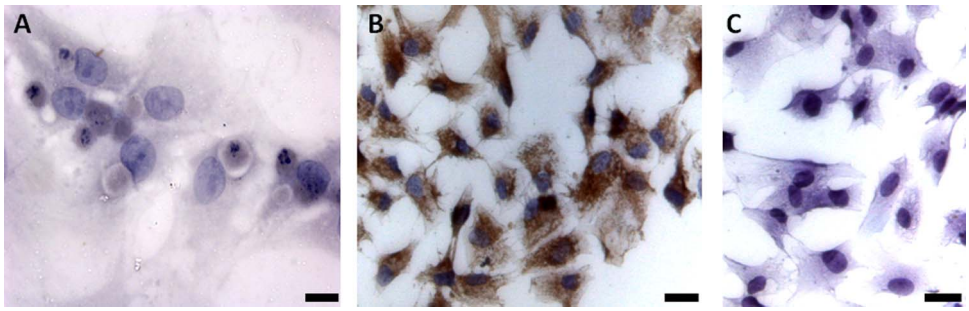

Fig. 2. Immunocytochemistry on BGC-1(A) shows no expression of 3βHSD in contrast to CPGB (B), which shows high expression of 3βHSD (×1000). C: negative control. Bars: 20 μm.

Table 1
Progesterone total production (ng P_4/mL ± s.e.m.; $n = 3$) by CPGB obtained from ovaries with (+) and without (−) CL in relation to seeding conditions and days in culture. P_4 total production in 4-day cultures was 43.7% higher in CPGB− than in CPGB + ($P = 0.0042$; $n = 12$) taking all seeding conditions together.

(cells/mL)	Day 4 (+)	Day 4 (−)	Day 8 (+)	Day 8 (−)
25,000	39.9 ± 6.4	60.78 ± 6.5	73.9 ± 6.4	116.9 ± 15.8
12,500	25.3 ± 3.3	64.65 ± 2.9	101 ± 13.9	105.8 ± 23.9
6250	15.8 ± 2.4	19.13 ± 2.4	107.1 ± 44.3	132.1 ± 40.6
3125	4.5 ± 0.1	12.01 ± 0.9	69.6 ± 19.9	28.5 ± 4.9
Mean	21.4 ± 4.2[b]	30.7 ± 5.8[a]		

Table 2
Progesterone relative production (ng P_4/mL/cells per field ± s.e.m.; $n = 3$) by CPGB obtained from ovaries with (+) and without (−) CL in relation to seeding conditions and days in culture. P_4 relative production in 4-day cultures was 82% higher in CPGB− than in CPGB+ ($P = 0.0001$; $n = 12$) taking all seeding conditions together.

(cells/mL)	Day 4 (+)	Day 4 (−)	Day 8 (+)	Day 8 (−)
25,000	1.6 ± 0.3	2.9 ± 0.4	1.9 ± 0.2	2.5 ± 0.5
12,500	1.8 ± 0.3	2.9 ± 0.5	2.4 ± 0.3	2.5 ± 0.5
6250	2.2 ± 0.1	3.1 ± 0.2	3.5 ± 1.4	5 ± 1.4
3125	1.5 ± 0.4	4.1 ± 0.7	2.3 ± 0.6	1.7 ± 0.2
Mean	1.8 ± 0.2[b]	3.2 ± 0.3[a]		

Table 3
Apoptosis % ± s.e.m.; $n = 3$, in CPGB obtained from ovaries with (+) and without (−) CL in relation to seeding conditions and days in culture. Apoptosis % was 4.89 times higher in CPGB+ than in CPGB− ($P = 0.03$, $n = 12$) taking all seeding conditions together.

(cells/mL)	Day 4 (+)	Day 4 (−)	Day 8 (+)	Day 8 (−)
25,000	2.08 ± 0.33	1.35 ± 0.32	4.94 ± 1.00	5.71 ± 0.73
12,500	4.16 ± 0.78	3.14 ± 1.03	5.25 ± 0.11	2.76 ± 0.25
6250	3.21 ± 0.77	2.44 ± 0.40	2.18 ± 0.60	2.57 ± 1.13
3125	5.05 ± 1.24	2.80 ± 0.71	1.77 ± 0.54	1.47 ± 0.31
Mean	3.62 ± 0.49[a]	2.44 ± 0.35[b]		

(CPGB−) granulosa models could be explained or at least correlate with 3βHSD expression. We observed CPGB− like behavior in porcine granulosa cells regardless of the presence of CL (data not published). In pigs, in contrast with that observed in cows, 3βHSD is highly expressed in granulosa and theca cells at all stages of development, consistent with the physiological constraints on substrate utilization by the Δ4 (P_4 to androstenedione) *versus* the Δ5 (dehydroepiandrosterone to androstenedione) pathway for E_2 synthesis in these species. The Δ5 pathway is obligatory for E_2 synthesis in cows, so the presence of high levels of 3βHSD in granulosa cells could inhibit E_2 synthesis by GC (Conely et al., 1995). Interstitial 3βHSD expression could provide the substrate for the Δ5 pathway for E_2 synthesis in cows. Interstitial expression of 3βHSD has been described in rat (Labrie et al., 1992) and human ovaries (Dupont et al., 1992) but not in ovaries of other species.

As P_4 production was associated with seeding conditions like cell density (Portella et al., 2010) as well as with the apoptotic process (Amsterdam et al., 2003), we believe that the low amounts of P_4 found at long-term incubation of BGC-1 could be due either to apoptosis or to the high cell density. In contrast, CPGB exhibited high production of P_4 even within 4 days of incubation. CPGB− showed higher production of P_4 than CPGB+. This higher production was likely due to the degree of luteinization of CPGB obtained from ovaries at different estrous stages and not to cell density or apoptosis. This is in concordance with that observed in the ovary evaluated for 3β-HSD expression by IHC. The presence of CL was not decisive for P_4 production in long-term cultures (over 6 days). We believe that long-term incubations allow immature cells from middle size follicles to luteinize *in vitro*, thus producing levels of P_4 similar to those of the already differentiated ones. So, we suggest that, in order to study GC behavior, both the estrous stage and the culture time should be taken into consideration. In addition, the *in vitro* model should be carefully established to obtain a stable behavior. Channing (1970) reported that the spontaneous luteinization of GC was only observed in cultures of large pre-ovulatory follicles, coincident with the peak of LH; the rest of the follicles did not suffer spontaneous luteinization, even when induced by LH and FSH. Calado et al. (2011) determined the presence of three types of GCs in the *cumulus* corona oocyte complexes (COCs) of cattle obtained from small (< 4 mm) and medium (4–8 mm) follicles from ovaries classified by having or not having CL. These authors observed that the luteal phase had high levels of P_4 production by CL (systemic P_4 or in follicular fluid) and contained a larger proportion of undifferentiated COCs. In COCs from ovaries at proestrus or luteal phase regression, where P_4 levels were low, the proportion of differentiated GCs was larger. Our observations in CPGB− correspond to those observed by Calado et al. (2011), who described COCs from pro-estrus or at follicular phase, whereas CPGB+ correspond to the less differentiated form of CPGB (at di-estrus). Besides, we have previously found that CPGB+ behave like BGC-1 when induced with 1, 10 and 100 nM LA respect to apoptosis and that CPGB− behave differently (Carou et al., 2015). Results in CPGB also agree with those of Quirk et al. (2005, 2006), who reported that GC susceptibility to *in vitro* FasL-induced apoptosis is dependent on E_2 concentration as well as on the LH surge and P_4 receptor, then linking the protective extrinsic pathway of apoptosis of GCs to the bovine estrous cycle. So there is a difference in comparing models of GC from big antral follicles associated with LH pike than models of GC from middle size antral follicles associated with presence of CL.

The fact that CPGB− behaved as more differentiated cells than CPGB+ is coherent with our previous results in which the growing curves of CPGB+ and BGC-1 were logarithmic (exponential or synchronized phase of cell growth), like established less differentiated cells do, and CPGB− were exponential (lag or asynchronous phase of cell growth), like differentiated cells do in primary cultures (Engelberg, 1961; Carou et al., 2015).

The increase in caspase 3 activity with 100 nM LA observed at 24 and 48 h confirmed our previous results in BGC-1 analyzed morphologically by DAPI and hematoxylin staining and biochemically by determining phosphatidylserine exposure by FACS (Carou et al.,

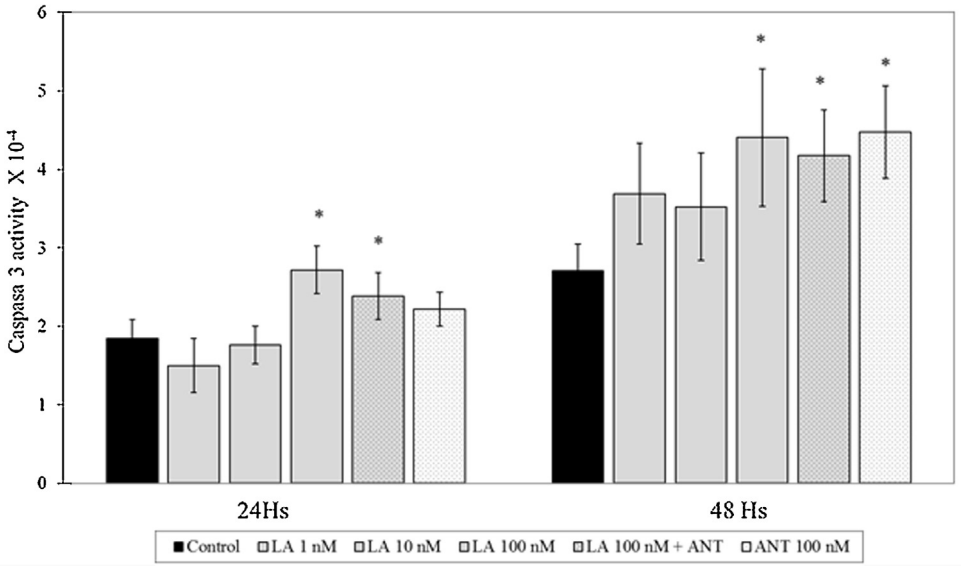

Fig. 3. Caspase 3 activity (μmol p-NA/well/μg prot/20 h) in BGC-1 treated for 24 and 48 h. Two-way ANOVA for 24 h ($P = 0.0156$) and for 48 h ($P = 0.0497$) of incubation. One-sided Dunnett's multiple comparisons with a control (FCS 5%). Within each panel, means (bars; mean ± s.e.m.) with no common superscripts are statistically different.

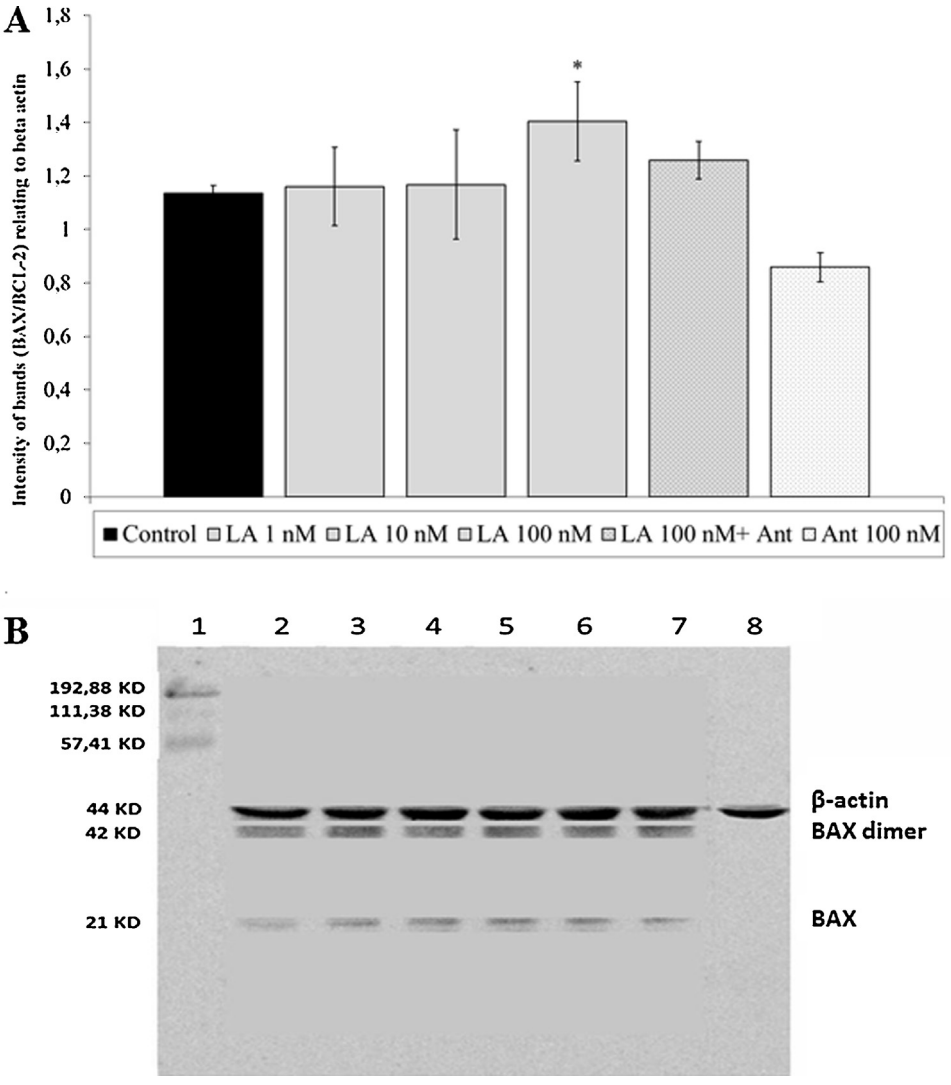

Fig. 4. Expression of BCL-2 family proteins (BAX and BCL-2) by Western blot in BGC-1 treated for 48 h. A: Two experiments were plated in triplicate ($n = 5$, $P_{BAX} = 0.065$, $P_{BAX/BCL-2} = 0.1$). One-sided Dunnett's multiple comparisons with a control (FCS 5%). B: 1, molecular weight marker; 2, control; 3, 1 nM LA; 4, 10 nM LA; 5, 100 nM LA; 6, 100 nM LA + 100 nM antide; 7, 100 nM antide; 8, Negative control. Within each panel, means (bars; mean ± s.e.m.) with no common superscripts are statistically different.

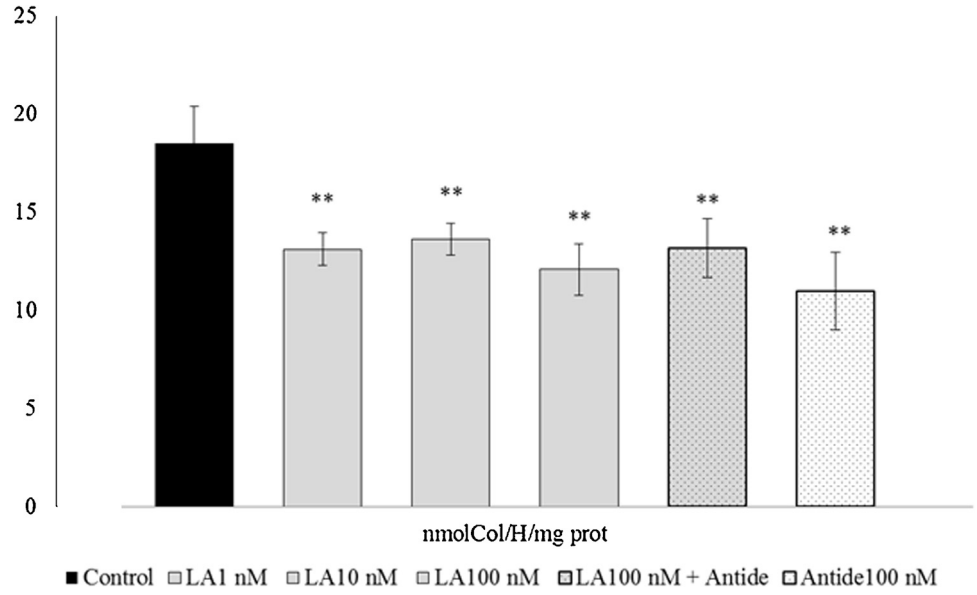

Fig. 5. PLD activity in BGC-1 treated for 24 h. Two experiments in triplicate were analyzed ($n = 6$, $P = 0.0079$). Two-way ANOVA and Bonferroni test as a means comparison analysis. Within each panel, means (bars; mean ± s.e.m.) with no common superscripts are statistically different.

2015). We propose a "*per se*" stimulatory effect by antide, in agreement with the results of Vitale et al. (2006), who found a stimulatory effect of antide on DNA synthesis. Caspase 3 activation by antide could be due to mitosis or differentiation rather than to apoptosis in a dose-dependent manner. Caspases have been suggested to contribute not only to apoptosis regulation but also to non-apoptotic cellular phenomena. Caspases have been reported to be involved in the cell cycle progression at mitotic phase (Toshiaki et al., 2011) as well as in skeletal muscle differentiation (Fernando et al., 2002), neurogenesis (D'Amelio et al., 2010), cancer cell differentiation (Arif et al., 2016) and erythropoiesis (Zhao et al., 2016), but nothing has yet been reported regarding the involvement of caspases in GC differentiation. We found no differences in the mitotic index when evaluated morphologically (Carou et al., 2015) or by flow cytometry (data not published), suggesting that caspase 3 activation by antide could be due to the luteinization process. This hypothesis should be analyzed in future projects.

The results obtained on PLD activity support the emerging hypothesis that antide is a weak agonist of GnRHr, not an antagonist. BAX expression was activated by 100 nM LA in agreement with other reports including *in vivo* studies in mice (Parborell et al., 2002; Peng et al., 2008) and *in vitro* studies in human GCs (Tsai et al., 2005).

No protective stimulation of PLD activity was seen in BGC-1; in contrast, PLD activity was inhibited even at high doses of LA. This is consistent with our previous results in CPGB+ and BGC-1, where no protective dual behavior of LA was seen at 100 nM (Carou et al., 2015). The activation of PLD by GnRH observed by Steele and Leung (1993) at doses higher than 10 nM in CGs from mouse and pig could be due to the luteinized stage of cells in those models.

We conclude that LA-induced apoptosis in BGC-1 occurred by activation of the mitochondrial pathway and by inhibition of PLD activity in a dose-dependent manner. We propose that antide might work both as an antagonist of the intrinsic pathway and as an agonist of the extrinsic protection pathway by inhibiting PLD activity. We also conclude that the degree of luteinization is an essential fact that modulates GC behavior and should be monitored when *in vitro* cell models are used to investigate GnRH regulation of GC physiology in cows. We purpose 3β-HSD expression is in direct relation to CG behavior *in vitro*. Future studies should deal with apoptosis protection mechanisms involving PLD activity and the extrinsic pathway in CPGB− as representative of a more differentiated form of bovine GCs.

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