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RESEARCH

A link between Notch and progesterone maintains the functionality of the rat corpus luteum

P Accialini[1], S F Hernández[1], D Bas[1], M C Pazos[1], G Irusta[1], D Abramovich[1] and M Tesone[1,2]

[1]*Instituto de Biología y Medicina Experimental (IByME-CONICET), Vuelta de Obligado 2490, Buenos Aires 1428, Argentina and* [2]*Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*

Correspondence should be addressed to M Tesone; Email: martatesone.uba@gmail.com

Abstract

In this study, we investigated the interaction between the Notch pathway and progesterone to maintain the functionality of the corpus luteum (CL). When Notch signaling is activated, the γ-secretase complex releases the active intracellular domains (NICD) of their receptors, which exert survival effects. We designed studies to analyze whether the *in vitro* inhibition of Notch affects progesterone production, steroidogenic regulators, apoptotic parameters, and signaling transduction pathways in the cultures of CL isolated from pregnant and superovulated rats. We detected a decrease in progesterone production when corpora lutea (CL) were incubated with *N*-(*N*-(3,5-difluorophenacetyl-L-alanyl))-S-phenylglycine t-butyl ester (DAPT), a γ-secretase inhibitor. This effect could be in part due to the decrease detected in the CL protein levels of P450scc because STAR and 3β-hydroxysteroid dehydrogenase were not affected by Notch inhibition. Besides, the addition of aminoglutethimide to the CL culture medium decreased NICD of NOTCH1. We observed an increase in the expression of active CASPASE3 (CASP3) after inhibition by Notch, which was reversed by the presence of progesterone. The BAX:BCLX$_L$ ratio was increased in CL treated with DAPT and the presence of progesterone reversed this effect. In addition, phosphorylation of AKT was inhibited in CL treated with DAPT, but had no effect on ERK activation. To demonstrate that the action of DAPT is specifically related with the inhibition of Notch, CLs were incubated with DLL4 antibody and a decrease in progesterone production was detected. These results suggest the existence of a novel link between progesterone and the Notch signaling pathway to maintain the functionality of the CL.

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Introduction

The corpus luteum (CL) is a transient endocrine gland whose main function is to secrete the steroid hormone progesterone essential for implantation of the blastocyst and maintenance of pregnancy in mammals (Bowen-Shauver & Gibori 2004, Stouffer 2004, Stocco *et al.* 2007). One important step in luteal steroidogenic biosynthesis is the enzymatic conversion of cholesterol to pregnenolone catalyzed by the cytochrome P450scc enzyme; however, before this conversion, the active delivery of cholesterol to the inner mithocondrial membrane is carried out by the protein STAR (reviewed in Stocco *et al.* (2007)). Although the development and differentiation of a structurally and functionally mature CL depend mainly on the activation of the luteinizing hormone (LH) receptor, they also depend on other factors such as receptors, transcription factors, and signaling proteins (Stouffer 2004, Stocco *et al.* 2007). In addition, if pregnancy does not occur or when the CL is no longer required for the maintenance of pregnancy, the CL ceases to produce progesterone and regresses in a process called luteolysis (Bowen-Shauver & Gibori 2004, Stouffer 2004, Stocco *et al.* 2007).

The Notch system is an evolutionary conserved pathway involved in cell fate decisions, including proliferation, differentiation, and apoptosis. In mammals, the Notch family of proteins consists of four receptors (NOTCH1–4) and five ligands (JAGGED1–2, DELTA-like 1, 3, and 4 (DLL4)) expressed on the cell surface. When Notch signaling is initiated, the receptors expose a cleavage site in the extracellular domain to the metalloproteinase TACE and, following this cleavage, Notch undergoes another cleavage mediated by the presenilin–γ-secretase complex. This results in the release of the active intracellular domain (NICD), which translocates to the nucleus (Kopan & Ilagan 2009) and exerts pleiotropic effects by initiating a transcriptional cascade (Ranganathan *et al.* 2011).

Notch proteins and ligands have been localized in granulosa, luteal, and vascular cells of the rodent ovary (Johnson *et al.* 2001, Vorontchikhina *et al.* 2005, Jovanovic *et al.* 2013). In addition, corpora lutea (CL) from marmosets treated with a neutralizing antibody

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against DLL4 during the periovulatory period exhibit increased vascular density, but smaller size, with involution of luteal cells, increased cell death, and suppressed plasma progesterone concentrations (Fraser *et al*. 2012). Recently, Jovanovic *et al*. (2013) have demonstrated that the *in vivo* inhibition of the Notch signaling pathway in mice impairs folliculogenesis and induces the disruption of gonadotropin-stimulated angiogenesis. We have previously demonstrated that Notch1, Notch4 and DLL4 are expressed in small and large luteal cells of CL from pregnant rats and have shown evidences that Notch signaling promotes both luteal cell viability and steroidogenesis. In addition, our results suggest that the luteolytic hormone PGF2α might act, in part, by reducing the expression of some components of the Notch system (Hernandez *et al*. 2011).

On the other hand, several studies have demonstrated that progesterone can locally stimulate its own secretion and protect the CL from cell death (Stocco *et al*. 2007). In rat granulosa and luteal cells, Progesterone has an autocrine action on steroidogenesis and protects cells from apoptosis. In addition, it has been suggested that the effects are exerted through a progesterone receptor localized on the luteal cell membrane (Telleria *et al*. 1999).

The phosphotidylinositol-3 kinase (PI3K)/AKT/ERK1/2 interaction mediates relevant pathways involved in the promotion of cell survival or apoptosis inhibition (Gerber *et al*. 1998, Thakker *et al*. 1999), whereas the protein products of the *Bcl2* family genes have also been described as apoptotic regulatory factors (Korsmeyer 1992, Boise *et al*. 1993). Follicle-stimulating hormone (FSH) and LH mediate luteinization by inducing a complex pattern of gene expression in ovarian cells that is regulated by the coordinate input from different signaling cascades such as the cAMP/protein kinase A, PI3K/AKT, and ERK1/2 cascades (Hunzicker-Dunn & Maizels 2006, Fan *et al*. 2008). In luteal cells, the luteolytic hormone PGF2α also regulates the ERK1/2 pathway (Chen *et al*. 1998). Moreover, depending on the balance between pro- and antiapoptotic proteins, the initiator CASPASE9 is activated and several effector caspases such as CASPASE3 (CASP3) or CASPASE7 are sequentially activated and promote apoptosis in various systems (Budihardjo *et al*. 1999). In previous studies, we reported the expression and activation of proapoptotic caspase-mediated pathways during both spontaneous luteolysis in pregnancy and natural cycles, and PGF2α-induced luteolysis (Peluffo *et al*. 2006, Hernandez *et al*. 2009). In addition, different reports have demonstrated the interaction between the Notch signaling and PI3K/AKT pathways in cancer cells (Meurette *et al*. 2009, Wang *et al*. 2011). In this context, we have recently demonstrated that Notch induces granulosa cell tumor proliferation, decreases apoptosis-mediated cell death, and might be interacting with the PI3K/AKT signaling pathway (Irusta *et al*. 2013). Yet, the cellular mechanisms by which progesterone exerts its local effects and whether progesterone interacts with the Notch system in CL survival remain incompletely understood. We thus hypothesized that there is a link between the Notch signaling pathway and progesterone, which exerts a regulatory mechanism to maintain the functionality of the CL. Therefore, we designed studies to analyze whether the *in vitro* inhibition of Notch signaling affects progesterone production, steroidogenic regulators, apoptotic parameters, and signaling transduction pathways in cultures of CL isolated from pregnant and superovulated rats.

Materials and methods

Animal models

Pregnant rats

Adult female Sprague–Dawley rats (body weight 200–250 g, 8-weeks-old) were housed at room temperature (21–23 °C) with a 12 h light:12 h darkness photo period in an air-conditioned environment. The rats had *ad libitum* access to food and water. The animals showing three consecutive 4-day cycles were used for the experiment and cycling stages were determined daily by vaginal cytology. Proestrous females were caged with fertile males overnight, separated the next morning, and vaginal smears analyzed for the presence of spermatozoa. The day spermatozoa were detected was considered day 1 of pregnancy. The animals were killed by CO_2 aspiration and ovaries were collected on day 16 of pregnancy. In this experimental model (CL obtained from pregnant rats), key experiments were carried out: studies to elucidate the relationship between the Notch pathway and progesterone production in cultured CL and the role of AKT and ERK signaling pathways in Notch CL function.

Superovulated rats

Due to the limitations to obtain a large number of CL from pregnant rats, additional experiments were carried out in the CL of superovulated rats. For this purpose, 21–23-day-old immature female Sprague–Dawley rats housed in the same conditions as that of pregnant rats were injected subcutaneously with equine CG (eCG; 25 IU/rat) followed by human chorionic gonadotropin (hCG; 25 IU/rat) 48 h later. The treatment with eCG plus hCG causes a considerable (tenfold) increase in ovarian weight due to the stimulation and subsequent transformation of ovarian follicles into CL. The animals were killed by CO_2 aspiration 4 days after hCG administration. To validate this experimental model, similar experiments to those carried out in CL of pregnant rats were repeated in CL the of superovulated rats. In addition, steroidogenic regulator protein levels, apoptotic parameters, and specificity of Notch inhibition with DAPT using antibody against DLL4 were analyzed in CL cultures of superovulated rats. The experimental protocols were approved by the Animal Experimentation Committee of the Instituto de Biología y Medicina Experimental (IByME, Buenos Aires, Argentina) (PHS–NIH Approval Statement of Compliance no. A5072–01).

CL culture

The ovaries were removed and cleaned of adhering tissue in culture medium and CL were isolated by ovarian microdissection as previously described (Andreu *et al*. 1998, Hernandez *et al*. 2009).

For the inhibition of the Notch pathway, *N*-(*N*-(3,5-difluorophenacetyl-L-alanyl))-*S*-phenylglycine t-butyl ester (DAPT, Sigma–Aldrich), a chemical component that inhibits the activity of the γ-secretase complex, was used. This inhibitor has been extensively used for experimental studies of Notch signaling, both *in vitro* and *in vivo* (Gordon *et al*. 2008). Progesterone production was inhibited with aminoglutethimide (AG, Sigma–Aldrich), which blocks the conversion of cholesterol to pregnenolone by inhibiting the enzyme P450scc (Brueggemeier *et al*. 2005). When CLs were incubated in the absence of inhibitors, 0.05% of vehicle solution (DMSO) was added to the medium.

Previous experiments of the time course and DAPT dose–response assessments were carried out to determine optimal incubation conditions (data not shown). Taking these results into account, four CL/well of superovulated rats were incubated for 1 or 4 h in 0.35 ml of DMEM:F12 containing bicarbonate and 0.5% BSA at 37 °C with 20 μM DAPT, 0.15 mM AG, 1.6 μM progesterone, or each inhibitor in the presence of progesterone. For each experiment, four ovaries from different rats were used to dissect CLs that were randomly distributed in the different wells (four wells/treatment). The four CLs from each well were pooled for western blotting assays. Each experiment was made in duplicate; in the case of pregnant rats, two CLs/well of the same animal were cultured in the presence or absence of the inhibitors and all the parameters measured were expressed as a fold-change relative to the control.

After incubations, the supernatants from each well were frozen for progesterone determination by RIA. The CLs were kept in dry ice and stored at −80 °C until processed for western blotting analysis or individually fixed in Bouin solution (Biopur, Waterlooville, Hampshire, United Kingdom) for 12 h and then processed for immunohistochemistry (IHC).

Incubation with neutralizing antibody against DLL4

Given that the γ-secretase complex has more than one substrate, Notch activation was also inhibited by incubating five CLs/0.35 ml with DLL4 neutralizing antibody (0.5, 2, and 5 μg/ml) and normal goat IgG as a control (0.5 μg/ml) for 4 h. After incubation, production of progesterone was measured in the culture medium by RIA.

RIA for progesterone

Progesterone was measured as described previously (Irusta *et al*. 2003) using specific antibodies supplied by Dr G D Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO, USA). Under these conditions, the intra-assay and inter-assay coefficients of variations were 8.0 and 14.2% respectively. The detection limit of the RIA was 25 pg.

Western blot analysis

For western blotting analysis, the CLs were resuspended in 250 μl of lysis buffer (20 mM Tris–HCl pH 8, 137 mM NaCl, 1% Nonidet P-40, and 10% glycerol) supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.025 mM *N*-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM *N*-p-tosyl-lysine chloromethyl ketone, and 0.025 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone), and phosphatase inhibitors (25 mM sodium fluoride, 0.2 mM sodium orthovanadate, and 10 mM b-glycerophosphate) and homogenized using an Ultra-Turrax (IKA-Werke GmbH & Co., Staufen, Germany) homogenizer. The samples were centrifuged at 4 °C for 10 min at 10 000 ***g*** and the resulting pellets were discarded. Protein concentration in the supernatant was measured by the Bradford assay. After boiling for 5 min, 20 μg protein were applied to a SDS–polyacrylamide gel (12–15%) and electrophoresis was carried out at 25 mA for 1.5 h. The resolved proteins were transferred onto nitrocellulose or PVDF membranes for 2 h. The blot was preincubated in a blocking

Table 1 Antibodies used for immunohistochemistry and western blotting analysis.

Peptide target	Manufacter, catalog no.	Species raised in	Dilution
NOTCH1	Santa Cruz Biotechnology, Inc., #sc-6014-R	Rabbit	1:400
BAX	Santa Cruz Biotechnology, Inc., #sc-493	Rabbit	1:250
BCLX	Santa Cruz Biotechnology, Inc., #sc-634	Rabbit	1:200
Phospho-AKT	Santa Cruz Biotechnology, Inc., #sc-7985-R	Rabbit	1:1000
ERK	Santa Cruz Biotechnology, Inc., #sc-154	Rabbit	1:2000
Phospho-ERK	Santa Cruz Biotechnology, Inc., #sc-7383	Mouse	1:1000
β-ACTIN	Santa Cruz Biotechnology, Inc., #sc-1616	Rabbit	1:3000
3β-HSD	Santa Cruz Biotechnology, Inc., (P-18) #sc-30820	Goat	1:200
STAR	Santa Cruz Biotechnology, Inc., (FL-285) #sc-25806	Rabbit	1:200
AKT	Cell Signaling, Danvers, MA, USA, #9272	Rabbit	1:8000
GAPDH	Cell Signaling, #2118	Rabbit	1:10 000
Cleaved CASPASE3	Biocare Medical (Concord. California, USA), #CP229C	Rabbit	1:100
P450scc	Dr Anita Payne. Stanford University Medical Center, Satanford, CA, USA	Rabbit	1:200
Anti rabbit HRP IgG	Sigma–Aldrich, #A-4914		1:1000
Anti mouse HRP IgG	R&D Systems (Minneapolis, Minnesota, USA), #HAF007		1:1000
Biotinylated anti-rabbit IgG	Vectastain ABC system; Vector Laboratories		1:400

buffer (5% nonfat milk, 0.05% Tween-20 in 20 mM TBS pH 8.0) for 1 h at room temperature, and then incubated with appropriate primary antibodies (Table 1) in 0.05% Tween-20 in 20 mM TBS of pH 8.0 overnight at 4 °C. The blots were then incubated with anti-rabbit or anti-mouse secondary antibodies conjugated with HRP (Table 1) and finally detected by chemiluminescence and autoradiography using X-ray film. Protein loading was normalized by reprobing the same blots with antibody against β-ACTIN or GAPDH (Table 1). Protein expression was quantified by densitometric analysis using Scion Image Software for Windows (Scion Corporation, Worman's Mill, CT, USA).

Immunohistochemistry

Corpora lutea were deparaffinized in xylene and rehydrated by graduated ethanol washes. Endogenous peroxidase activity was blocked with hydrogen peroxide in PBS, and nonspecific binding was blocked with 2% BSA for 20 min at room temperature. The sections were incubated with anti-cleaved CASP3 antibody overnight at 4 °C. After washing, the slides were incubated with biotinylated anti-rabbit IgG (Table 1) and after 30 min with avidin-biotinylated HRP complex (Vectastain ABC system; Vector Laboratories, Burlingame, CA, USA) for 30 min. Protein expression was visualized using diaminobenzidine staining. The negative controls were obtained in the absence of primary antibody. The reaction was stopped using distilled water, stained with hematoxylin, and dehydrated before mounting with mounting medium (Canada Balsam Synthetic; Biopack, Santa Barbara, CA, USA). To perform this study, four sections from each CL were analyzed (four CL/treatment) and stained cells were counted in the whole section.

The images were digitized using a camera (Nikon, Melville, NY, USA) mounted on a conventional light microscope (Nikon). Finally, the images were converted to a TIFF format (bilevel scale) for their analysis. The percentage of luteal cells were processed using Image J (Image Processing and Analysis in Java; National Institutes of Health, Bethesda, MD, USA; Rasband 2007). An apoptosis index (cleaved CASP3-positive cells expressed as a percentage of the total number of cells) was calculated for each section.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism Software, Inc. San Diego, CA, USA. All experiments were carried out at least three times and the conditions were performed in quadruplicates.

One-way ANOVA following Newman–Keuls as post-test was used to analyze the experiments carried out in superovulated rats. Repeated measures ANOVA followed by Newman–Keuls as a post-test was used to analyze the experiments carried out in pregnant rats. The experiments were analyzed with paired Student's *t*-test when two variables were involved.

Data are expressed as means ± S.E.M. of pooled results obtained from different independent experiments. The representative gels are shown in the figures. The values of $P<0.05$ were considered significant.

Results

Relationship between the Notch pathway and progesterone synthesis in cultured corpora lutea of pregnant rats

Treatment with DAPT caused a 52.0% decrease on the cleaved (active) form of NOTCH1 (NICD) when compared with vehicle treatment (control). Surprisingly, AG also was able to produce a 40.0% decrease on the active form of NOTCH1. However, the presence of both inhibitors did not significantly change the effect obtained using each inhibitor separately (35.0% decrease relative to control (Fig. 1A).

Regarding the effect of the inhibitors on progesterone production measured in the culture medium, AG caused a significant decrease in progesterone production (70.8%), as expected, whereas DAPT caused a lower but significant decrease (45.7%) (Fig. 1B). Nevertheless, the presence of both inhibitors in the culture medium caused a progesterone decrease similar to that obtained with AG alone (68.9%).

In vitro *effect of Notch inhibition on progesterone production and steroidogenic regulators in corpora lutea of superovulated rats*

Corpora lutea were isolated from the ovaries of rats treated with gonadotropins and incubated with AG or DAPT for 1 or 4 h. In a conditioned medium of CL cultured for 1 h, AG significantly decreased progesterone production, but DAPT showed only a tendency to decrease (control: 319.50 ± 49.01; AG: 51.75 ± 10.06; DAPT: 256.50 ± 17.53 ng/ml) (Fig. 2A). Similar to the

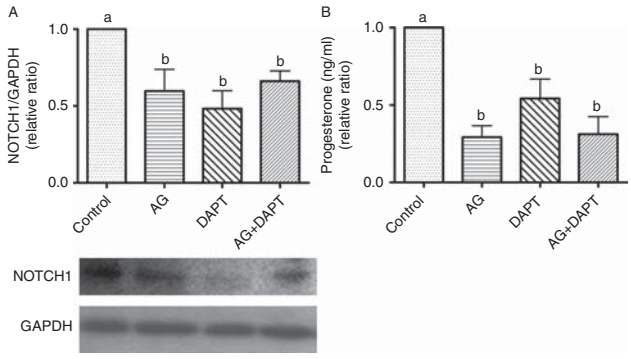

Figure 1 Effect of the incubation with aminoglutethimide (AG), DAPT, and AG + DAPT for 4 h on NOTCH1 protein content and progesterone production in corpora lutea (CL) of pregnant rats. (A) Densitometric quantification of NOTCH1. Optical density is expressed as arbitrary units ± S.E.M. normalized to GAPDH ($n=4$). Representative immunoblots of NOTCH1 content are shown in the lower panel. Groups with different letters over the error bars (a and b) represent significant differences ($P<0.05$). (B) The levels of progesterone in the culture medium of CL were determined by RIA. Values are shown as mean ± S.E.M. of ng/ml of progesterone in the culture medium ($n=4$). Groups with different letters over the error bars (a and b) represent significant differences ($P<0.001$).

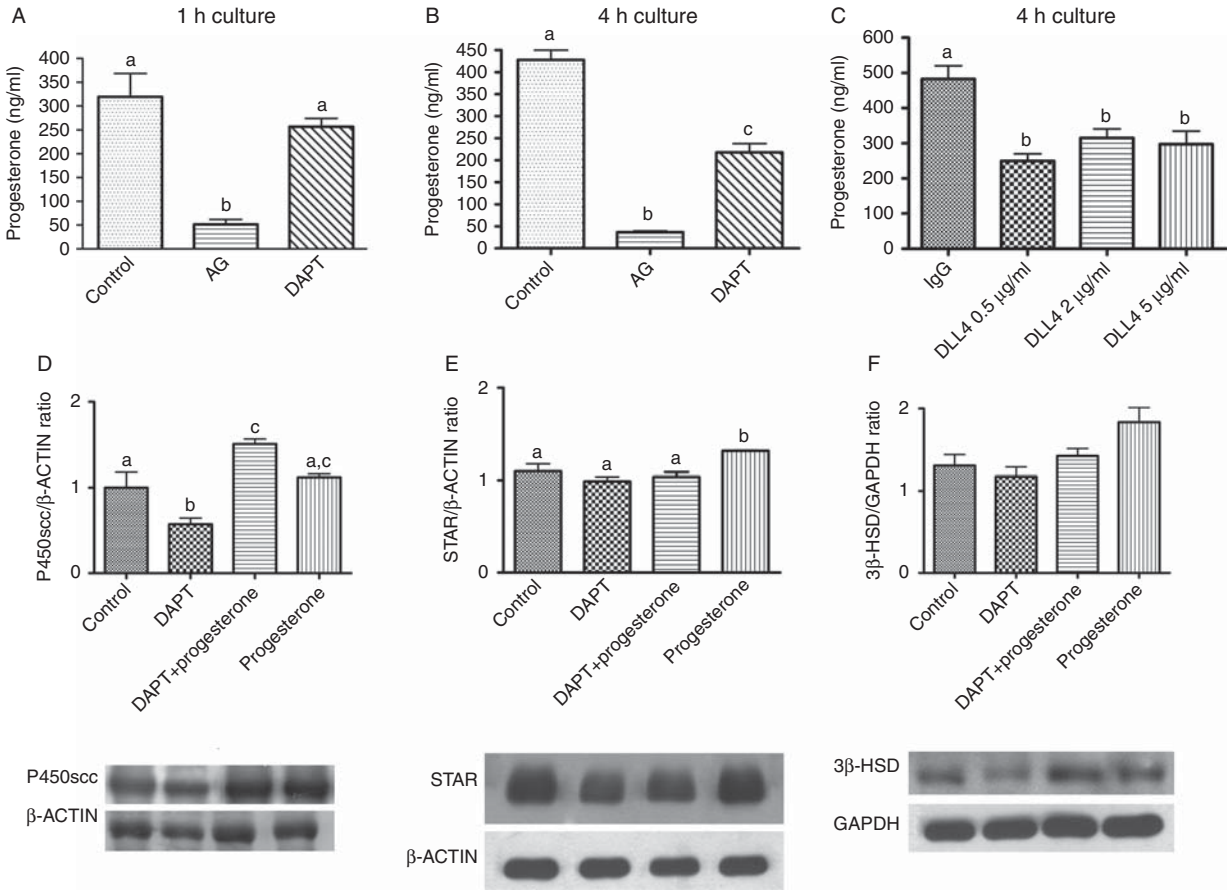

Figure 2 Levels of progesterone and steroidogenic enzymes in the culture medium and CL of superovulated rats respectively. (A) Progesterone in culture medium of CL cultured for 1 h with AG or DAPT (a vs b, $P<0.01$). (B) Progesterone in culture medium of CL cultured for 4 h with AG or DAPT (a vs b and c, $P<0.001$). (C) Progesterone in culture medium of CL cultured for 4 h with different concentrations of DLL4 Ab (a vs b, $P<0.01$). (D) P450scc protein content in the CL of superovulated rats cultured for 4 h (a vs b, $P<0.05$; a vs c, $P< 0.05$; b vs c, $P<0.001$). (E) STAR protein content in the CL of superovulated rats cultured for 4 h (a vs b, $P<0.01$). (F) 3β-HSD protein content in CL from superovulated rats cultured for 4 h. Values are expressed as mean ± S.E.M. of progesterone in the culture medium ($n=4$) or as arbitrary units ± S.E.M. normalized to β-ACTIN or GAPDH ($n=4$). Groups with different letters over the error bars (a, b, and c) represent significant differences.

results obtained in CLs of pregnant rats, both treatments significantly decreased progesterone production after 4 h of culture (control: 427.80 ± 21.98; AG: 36.67 ± 2.63; DAPT: 217.5 ± 20.03 ng/ml) (Fig. 2B).

Progesterone production was also inhibited on incubation of CL from superovulated rats with DLL4 antibody for 4 h. In this experiment, progesterone production was significantly decreased in the culture medium (IgG: 482.8 ± 37.5; DLL4 antibody (5 µg/ml): 297.1 ± 37.6 ng/ml). Similar results were observed with lower DLL4 antibody concentrations (Fig. 2C).

To determine whether some members of the progesterone synthesis pathway are regulated by Notch, the expression of P450scc, STAR and 3β-hydroxysteroid dehydrogenase (3β-HSD) was determined by western blotting analysis. We found a significant decrease in the CL protein levels of P450scc after 4 h culture with DAPT. The coincubation with progesterone was able to reverse the content of this enzyme even to levels higher than those of the control (Fig. 2D). In addition, only progesterone was able to change the levels of STAR. In fact, the incubation with progesterone for 4 h caused a 20% increase in the STAR CL protein levels with respect to control values. Interestingly, the presence of DAPT in the culture medium reversed this effect (Fig. 2E). In contrast, no changes were observed in the levels of 3β-HSD after 4 h culture with DAPT and/or progesterone (Fig. 2F). These experiments were repeated after 1 h of incubation and consistently with progesterone results at this time, no changes were observed in the levels of these proteins (data not shown).

Involvement of Notch signaling and progesterone in apoptotic parameters in the corpora lutea of superovulated rats

To identify whether the relationship between Notch and progesterone signaling influences the survival of luteal cells of superovulated rats, we cultured CLs with DAPT, DAPT in the presence of progesterone or progesterone

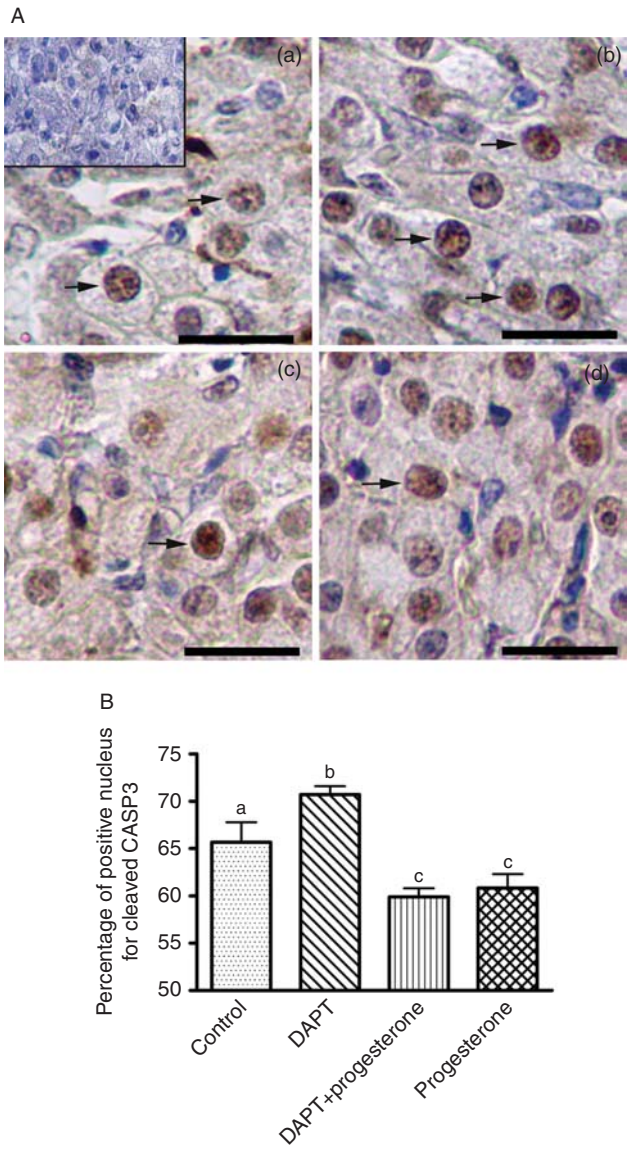

Figure 3 Immunohistochemistry for cleaved CASP3 in sections of CL from superovulated rats incubated with DAPT, DAPT+progesterone, and progesterone. (A) Panels show representative photomicrographs of CASP3 in the control group (a), DAPT (b), DAPT+progesterone (c), and progesterone (d). The inset shows the negative control. Arrows indicate nuclear staining of activated CASP3. Bar=20 μm. (B) Quantification of the percentage of positive immunostained cells for cleaved CASP3. Four sections from each CL were analyzed (four CL/treatment) and stained cells were counted in the whole section. Groups with different letters over the error bars (a, b, and c) represent significant differences ($P<0.05$).

alone. After 4 h of culture, we analyzed active CASP3 expression by IHC in CL sections incubated under the mentioned conditions. The luteal cells exhibited high staining for active CASP3 in the DAPT group (Fig. 3Ab). In contrast, the luteal cells of the control group (Fig. 3Aa) showed a low immunoreactivity for this protein. Active CASP3 staining was more evident in the nuclei of the CL sections, in coincidence with the fact that, once activated, active CASP3 translocate to the nucleus (Irusta *et al.* 2007, Parborell *et al.* 2008). Interestingly, the presence of progesterone in the culture medium not only reversed the increase in the immunoreactivity for active CASP3 observed with DAPT but also significantly decreased active CASP3 with respect to the control values (Fig. 3Ac and d). In addition, according to the antiapoptotic action described in rodent luteal cells, progesterone was able to decrease active CASP3 content in CLs cultured with this hormone (Fig. 3Ad). Figure 3B shows the quantification of these data obtained by IHC (control: 65.68±2.10%; DAPT: 70.69±0.90%; DAPT+progesterone: 59.88±0.90%; progesterone: 60.83±1.49%; $P<0.05$; $n=4$).

In light of these results, we examined the luteal content of the proapoptotic protein BAX and the antiapoptotic protein $BCLX_L$, two known regulators of ovarian apoptosis (Flaws *et al.* 1995, Tilly *et al.* 1995). Although DAPT treatment did not increase BAX protein content (Fig. 4A), it significantly decreased the levels of $BCLX_L$ relative to control levels (Fig. 4B). Consequently, the BAX:$BCLX_L$ ratio was significantly increased in CLs treated with DAPT compared with the control ratio (Fig. 4C). The coincubation with DAPT and progesterone reversed the values of $BCLX_L$ and BAX:$BCLX_L$ ratio to control levels (Fig. 4B and C). Incubation of CL with progesterone alone was not able to reverse the BAX or $BCLX_L$ levels to those obtained in the control group (Fig. 4A, B and C).

Participation of AKT and ERK signaling pathways in the survival role of Notch in the corpora lutea of superovulated rats

To determine whether Notch is involved in the phosphorylation of the AKT protein, we carried out a western blotting analysis of the phosphorylated form of AKT (pAKT) in CL. Preliminary results using 1 or 4 h of incubation showed significant differences only after 1 h of incubation with the different stimulus, suggesting that basal activation by phosphorylation is a rapid event probably independent of protein synthesis. For this reason, our next experiments were designed for 1 h incubation. In this condition, DAPT significantly decreased AKT phosphorylation. The coincubation of CL with DAPT and progesterone restored pAKT levels to the basal values (control), but no effect was observed in the presence of progesterone alone in the culture medium (control: 0.78±0.13; DAPT: 0.63±0.05; DAPT+progesterone: 0.56±0.04; progesterone: 0.58±0.03; control vs DAPT: $P<0.05$; $n=3$) (Fig. 5A).

In addition, ERK phosphorylation was studied by western blotting analysis of phosphoERK (pERK) from CL incubated in the same conditions described for the measurement of pAKT. No significant differences were observed in pERK levels among groups (Fig. 5B).

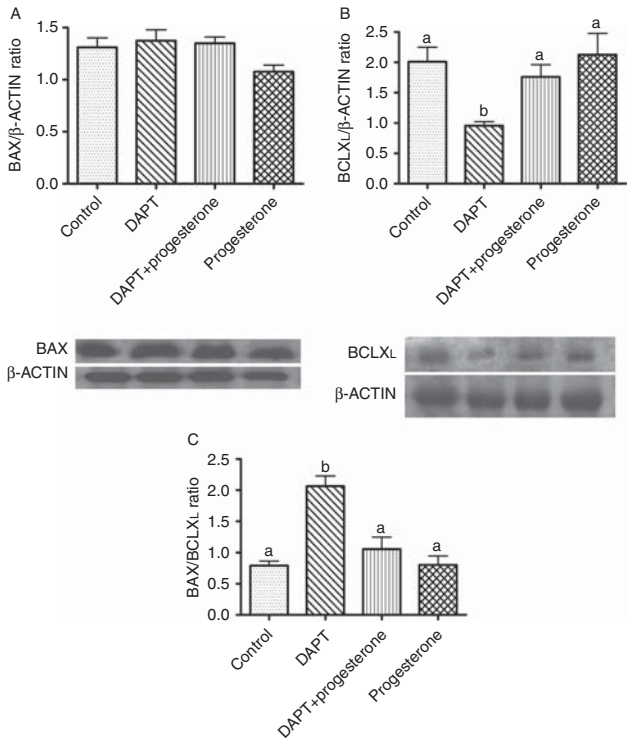

Figure 4 Effect of the incubation with DAPT, DAPT + progesterone, and progesterone for 4 h on BAX and $BCLX_L$ protein content in the CL of superovulated rats. (A) Densitometric quantification of BAX in CL. Optical density is expressed as arbitrary units ± S.E.M. normalized to β-ACTIN ($n=4$). Representative immunoblots of BAX content are shown in the lower panel. (B) Densitometric quantification of $BCLX_L$ in CL. Optical density is expressed as arbitrary units ± S.E.M. normalized to β-ACTIN ($n=4$). Representative immunoblots of $BCLX_L$ content are shown in the lower panel. (C) BAX:$BCLX_L$ ratio in the different groups. Groups with different letters over the error bars (a and b) represent significant differences ($P<0.05$).

Similar results were observed for pAKT and pERK in the cultures of CLs from pregnant rats in the presence of DAPT (pAKT decreased 1.7-fold; $P<0.001$; $n=3$, and no differences were observed in pERK levels) (Fig. 5C). These results suggest a common Notch signaling mechanism in both experimental models.

Discussion

This study was designed to determine a possible interaction between the Notch signaling pathway and progesterone to maintain the functionality of the CL. We describe for the first time that the inhibition of Notch signaling in rat CL cultures decreases progesterone production, in part through the regulation of the enzyme P450scc. In addition, Notch inhibition caused an increase in apoptotic parameters and a decrease in AKT phosphorylation, whereas progesterone inhibition decreased the NICD active Notch levels.

It is well known that LH is essential for the stimulation and maintenance of progesterone production by the CL; however, several autocrine and paracrine factors regulate the function of luteal cells (Stocco *et al.* 2007). Notch signaling plays a critical role in many developmental processes, influencing differentiation, proliferation and apoptosis, key mechanisms that regulate the dynamics and function of the CL (Artavanis-Tsakonas *et al.* 1999, Bolos *et al.* 2007). We have previously demonstrated both that notch pathway components are present in the CL of pregnant rats and that the mRNA expression of *Notch1*, *Notch4*, and *Dll4* decreases during PGF2α-induced luteolysis. In addition, the *in vivo* intrabursal administration of DAPT to pregnant rats decreases progesterone levels and increases luteal levels of active CASP3 and the proapoptotic/antiapoptotic protein ratio (Hernandez *et al.* 2011). In this context, Fraser *et al.* (2012) described that the administration of a DLL4 neutralizing antibody to marmosets affects early-luteal angiogenesis and subsequent luteal function. Other researchers also demonstrated that the blockage of the

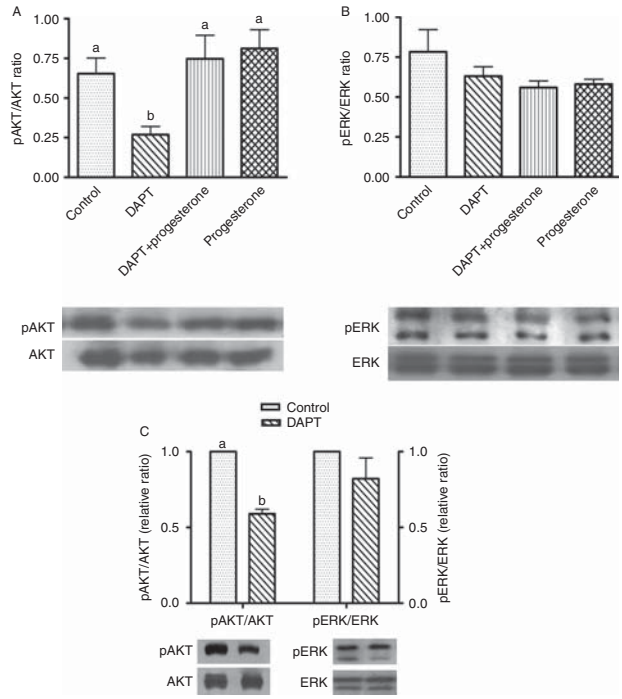

Figure 5 Effect of the incubation with DAPT, DAPT + progesterone, and progesterone for 1 h on the phosphorylation level of AKT (A) and ERK (B) in the CL of superovulated rats. Effect of the incubation with DAPT for 1 h on the phosphorylation level of AKT and ERK in the CL of pregnant rats (C). (A) The ratio between phosphorylated and total AKT is expressed as arbitrary units ± S.E.M. ($n=4$). Representative immunoblots of proteins content are shown in the lower panel. (B) The ratio between phosphorylated and total ERK is expressed as arbitrary units ± S.E.M. ($n=4$). Representative immunoblots of protein content are shown in the lower panel. (C) The ratio between phosphorylated and total AKT and ERK is expressed as arbitrary units ± S.E.M. ($n=4$). Representative immunoblots of protein content are shown in the lower panel. Groups with different letters over the error bars (a and b) represent significant differences ($P<0.05$).

Notch signaling pathway with the administration of an inhibitor of the γ-secretase complex or DLL4 blocking antibody alters follicular development and induces a disruption of the VEGF-dependent luteal angiogenesis (Garcia-Pascual *et al*. 2013, Jovanovic *et al*. 2013). The present data demonstrate a decrease in progesterone production when CLs obtained from pregnant or superovulated rats were incubated with DAPT, a γ-secretase inhibitor. This effect could be in part due to the decrease detected in the CL protein levels of P450scc, because the enzymatic conversion of cholesterol to pregnenolone, catalyzed by this enzyme, is considered as one of the rate-limiting steps in progesterone biosynthesis in steroidogenic tissues (Lieberman *et al*. 1984, Waterman & Simpson 1985). On the other hand, other steroidogenic regulators as the STAR, a protein able to regulate the delivery of cholesterol to the mitochondrion (Stocco & Clark 1996) and 3β-HSD, the enzyme located in the smooth endoplasmic reticulum involved in progesterone synthesis from pregnenolone (Peng *et al*. 2002), were not affected by Notch inhibition. However, changes in the expression of the enzyme 20α-HSD, that catabolizes progesterone into the inactive progestin and 20α-DHP cannot be rule out. Coincident with a previous report (Rekawiecki *et al*. 2005), the presence of progesterone in the culture medium caused an increase in CL STAR protein expression. It is worth noting that the inhibition of Notch overrides this effect, suggesting an influence of the Notch pathway on the progesterone autocrine CL regulation. Overall, our results suggest that Notch signaling is a novel mechanism that is able to regulate luteal steroidogenesis.

Interestingly, the addition of AG, a progesterone synthesis inhibitor, to the pregnant CL culture medium decreased the active NICD of NOTCH1. This result is in agreement to that obtained in primate endometrium, where progesterone increases the intracellular transcriptionally competent NOTCH1 (Afshar *et al*. 2012). In that work, the authors suggested that hCG and progesterone synergize to activate NOTCH1, which in turn induces alpha smooth muscle actin and inhibits stromal cell apoptosis. Further experiments are needed to elucidate the mechanism of action of progesterone on the Notch pathway in rat CL.

In addition, to demonstrate in our experimental model that the action of DAPT is specifically related with the inhibition of the Notch pathway, we blocked Notch action with an antibody against DLL4, the Notch ligand with a well-described luteotropic role (Hernandez *et al*. 2011, Fraser *et al*. 2012, Garcia-Pascual *et al*. 2013). This experiment showed a decrease in luteal progesterone production, confirming that the Notch system is involved in luteal function.

Progesterone plays a protective role against apoptosis in the CL of rodents (Stocco *et al*. 2007). In addition, this hormone is a potent survival factor for the pregnant rat CL, an effect that occurs in the absence of classic intracellular progesterone receptors (Goyeneche *et al*. 2003). In this context, Peluso *et al*. (2005) established that progesterone receptor membrane component 1 (PGRMC1) mediates the anti-apoptotic effects of progesterone observed in rat granulosa cells (Peluso *et al*. 2005), rat luteal cells (Peluso *et al*. 2005), human granulosa/luteal cells (Engmann *et al*. 2006), and spontaneously immortalized granulosa cells. In addition, progesterone activates a PGRMC1-dependent mechanism that promotes human granulosa/luteal cell survival but not progesterone secretion (Peluso *et al*. 2009). In the present work, we report an increase in the expression of active CASP3 and in the BAX:$BCLX_L$ ratio when Notch is inhibited in CL cultured with DAPT, an effect reversed by the presence of progesterone in the culture medium. To elucidate whether the cells are able to recover from apoptosis, a proliferation marker, proliferating cell nuclear antigen (PCNA), was measured by IHC in the CL cultured under the different conditions. DAPT treatment significantly decreased the percentage of positively stained nuclei and this effect was reversed by progesterone (data not shown). These results suggest that there is a direct link between the Notch system and progesterone, which upregulates the survival of luteal cells.

Different reports have demonstrated the interaction between Notch signaling and PI3K/AKT pathways. In breast epithelial cancer cells, Notch signaling induces an autocrine signaling loop that activates AKT and is necessary for Notch-induced protection against apoptosis (Meurette *et al*. 2009). More recently, Wang *et al*. (2011) have reported that in prostate cancer cells, the downregulation of NOTCH1 leads to the inhibition of cell growth mechanistically linked with the downregulation of AKT, suggesting that this protein is a downstream target of Notch1 signaling. In addition, in a previous work we have demonstrated that phosphorylation of AKT is repressed in a granulosa cell line (KGN) cultured with a Notch inhibitor (Irusta *et al*. 2013). In this study, we showed that phosphorylation of AKT was inhibited in CL treated with DAPT and that this effect was reversed with coincubation with progesterone. In this regard, it has been described that, in addition to transcriptional effects, progesterone activates the SRC/ERK1/2 and PI3K/AKT pathways in breast cancer and endometrial stroma cells (Ballare *et al*. 2006, Lee & Kim 2014). Our results suggest a similar mechanism in luteal cells, strengthening the hypothesis that an association exists between the antiapoptotic action of progesterone and Notch/PI3K/AKT signaling. Nevertheless, further experiments are needed to deeply investigate this relationship.

In summary, the results of this study provide the first evidence that there is a crosstalk between the Notch system and progesterone, which upregulates the survival of luteal cells. One mechanism of Notch action is the increase in P450scc synthesis and, in turn, progesterone

could regulate the NICD active Notch fragment. Our results demonstrate that Notch induces luteal progesterone *in vitro* production through P450scc activation and decreases apoptosis-mediated cell death. Also, the Notch/PI3K/AKT signaling pathway might be interacting with progesterone, intensifying the survival role of this hormone in luteal cells.

Declaration of interest

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

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