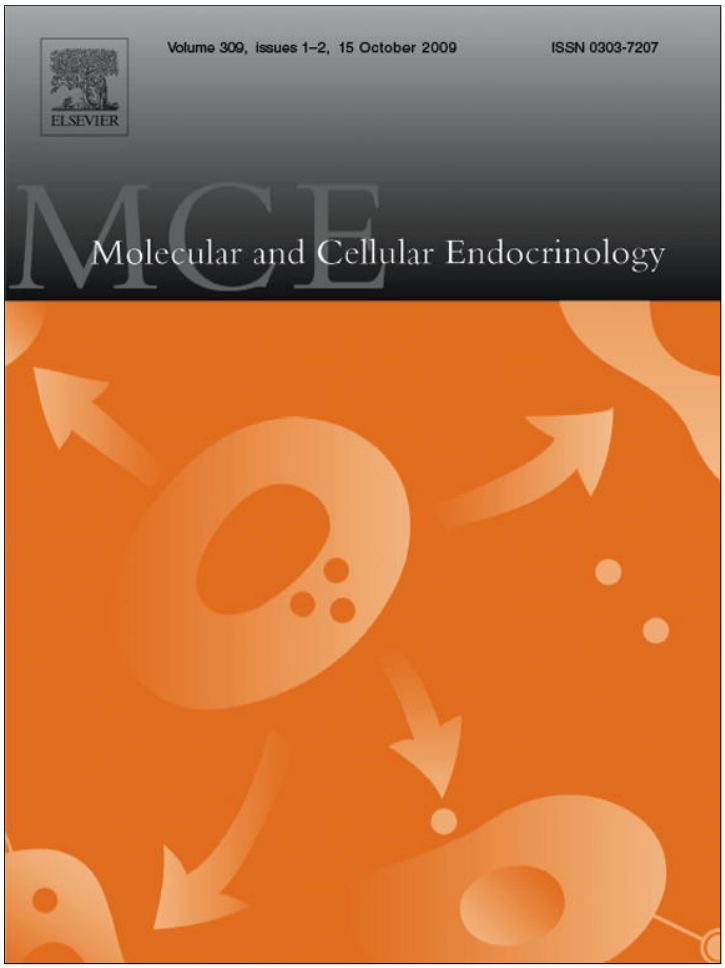

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Regulation of inhibin/activin expression in rat early antral follicles

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ABSTRACT

The aim of the present study was to determine the endocrine activity of cultured early antral follicles (EAF) isolated from prepubertal diethylstilbestrol-treated rats. The effect of steroidogenic substrates and FSH on steroid, inhibin A and B, Pro-αC and activin A production was evaluated. Androsterone was the predominant steroid produced by EAF. The addition of androstenedione, androstenedione + FSH and progesterone stimulated oestradiol production, whereas 25-hydroxycholesterol (25-OH-Chol) increased progesterone production. Inhibin A, B, Pro-αC, and activin A were produced under basal conditions. The predominance of inhibin B over inhibin A was not affected by the addition of androstenedione or progesterone. Inhibin A and activin A production was stimulated by FSH. 25-OH-Chol increased *Inha*, *Inhba* and *Inhbb* mRNA expression and the production of the three molecular forms of inhibins but decreased activin A production. These results show that FSH and the steroid follicular microenvironment differentially modulate the gene expression of inhibin/activin subunits, their assembly and secretion.

1. Introduction

The ovarian follicles play a central role in female reproductive physiology; they are the functional units involved in the production of a mature oocyte and endocrine homeostasis.

Folliculogenesis is a complex and dynamic process requiring the coordinate interactions of multiple intragonadal and extragonadal factors acting on different cell types (McGee and Hsueh, 2000). The early stages of follicular development up to the formation of early antral follicles (EAF) are independent of the pituitary gonadotrophins stimuli; in rodents, follicles at the early antral stage become highly responsive and dependent on follicle-stimulating hormone (FSH) stimulus which is modulated by a number of intraovarian factors (Knight and Glister, 2006; Hsueh et al., 2000). In the progressive development of follicles, most of them undergo atretic degeneration whereas only a few reach the preovulatory stage. Early antral is the stage when follicles become most susceptible to atresia and FSH is the major survival factor (Chun et al., 1996). The magnitude of the FSH stimulus, the variation in the cellular capacity to respond to gonadotrophins and the follicular steroid microenvironment appear to dictate the fate of a given follicle (Billig et al., 1993). The endocrine activity of developing ovarian follicles is crucial for the process of follicular selection.

Dimeric inhibins are glycoproteins predominantly produced in the gonads (Robertson et al., 1988) and their primary role is the selective inhibition of the FSH β-subunit mRNA expression (Attardi et al., 1989; Carroll et al., 1989). They are composed of α- and β-subunits; heterodimerization of α-subunit with either form of the β-subunit, βA and βB, generates dimeric inhibin A and inhibin B, respectively. Homo- or heterodimerization of β-subunits gives rise to the formation of activins; these peptides potently stimulate FSH secretion from the pituitary (Woodruff, 1998; Pangas and Woodruff, 2000).

Both inhibins and activins, like other members of the transforming growth factor-β (TGF-β) superfamily, have been implicated as autocrine and paracrine regulators of ovarian follicle development and survival (Lin et al., 2003; Knight and Glister, 2006). They are produced in a stage-specific pattern suggesting a differential regulation across folliculogenesis. Inhibin B is the predominant inhibin observed in serum during metoestrus in the cycling rat (Woodruff et al., 1996; Arai et al., 2002). In previous studies we have reported that cultured granulosa cells isolated from immature estrogen-treated rats produce predominantly inhibin A over inhibin B (Lanuza et al., 1999); conversely, inhibin B was the predominant dimeric inhibin detected in serum in these animals. It has been proposed that

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different intraovarian factors, such as TGF-β, activin A and oocyte-derived factor(s), might be responsible for the shift toward the predominance of inhibin B (Lanuza et al., 1999).

A variety of culture systems have been used in rodent species to explore the ovarian follicular physiology (Nayudu and Osborn, 1992; Eppig and Wigglesworth, 1995; Cortvrindt et al., 1996; Xu et al., 2006). The stimulation of ovarian follicle development produced by DES-treatment in immature rats allows the isolation of follicles that exhibit characteristics of EAF including their size, small antral cavity, and a thin theca layer (Chun et al., 1996). Several studies have used this model to examine the regulation of apoptosis in EAF (Chun et al., 1996; Kaipia et al., 1996; Li et al., 1998; Parborell et al., 2001; Vitale et al., 2002). This experimental model allows the study of the endocrine follicular activity in a system that maintains the structure of the follicle and the interaction between the different follicular cell types. In addition, in a recent study we have demonstrated that EAF obtained from DES-treated rats are physiologically similar to EAF from cycling rats (Abramovich et al., 2009). The steroidogenic capacity in response to different exogenous substrates, the inhibin and activin production and the hormonal regulation of isolated EAF in culture has not yet been fully examined.

The hypothesis addressed in the present study is that EAF endocrine activity may be regulated by the follicular steroid microenvironment and their major survival factor, FSH. Consequently, this study was aimed at assessing the effect of different steroidogenic substrates and FSH, on steroid, inhibin A and B, free inhibin α-subunit (Pro-αC) and activin A (βA–βA dimer) production in cultured early antral follicles. In addition, for comparative purposes, selected experiments in granulosa cell cultures were performed.

2. Materials and methods

2.1. Reagents

Recombinant human FSH (rhFSH) was purchased from the National Hormone & Peptide Program of the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK)-NIH (Torrance, CA, USA). Progesterone (P), androstenedione (Δ_4A), 25-hydroxycholesterol (25-OH-Chol), BSA, insulin, transferrin, ascorbic acid, aminoglutethimide (AG) and diethylstilbestrol (DES) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM, 4.5 g glucose/l), Ham F12 nutrient mixture (F12), fungizone (250 μg/ml), and gentamicin (10 mg/ml) were obtained from Invitrogen (Carlsbad, CA, USA). Oestradiol [2,4,6,7-^{3}H(N)] (^{3}H-E_2), androsterone [9,11-^{3}H(N)] (^{3}H-A) and progesterone [1,2-^{3}H(N)] (^{3}H-P), were obtained from New England Nuclear (Boston, MA, USA). All other chemicals were of reagent grade from standard commercial sources.

2.2. Animals

General care and housing of rats was carried out at the Instituto de Biología y Medicina Experimental (IBYME) in Buenos Aires. Immature female Sprague–Dawley rats, 21–23-day-old, were allowed food and water ad libitum and kept at room temperature (21–23 °C) on a 12L:12D cycle. Rats were injected s.c. with 1 mg DES (dissolved in corn oil) daily for 3 days; this treatment stimulates follicular development until an early antral stage. Animals were killed by cervical dislocation, and the ovaries were removed for granulosa cell isolation or follicle dissection. All experimental procedures involving rats were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the local Institutional Ethic Committee (IBYME-CONICET).

2.3. Early antral follicles culture

The isolation and culture of early antral follicles (EAF) were carried out as previously described (Parborell et al., 2001). Briefly, EAF (~350 μm in diameter) were dissected from ovaries collected following DES-treatment and cleansed of adhering tissue in culture medium. Ovarian follicles from various animals were pooled for different treatments, and cultures were initiated within 1 h of ovary removal at 37 °C. Sixty follicles per culture plate well were incubated under serum-free conditions in 350 μl DMEM:F12 (1:1, vol/vol)–bicarbonate (2.2 g/l) supplemented with fungizone (250 μg/ml), gentamicin (10 mg/ml), BSA (3 mg/ml), insulin (5 μg/ml), transferrin (10 μg/ml) and ascorbic acid (50 μg/ml) in the absence or presence of Δ_4A (25 μM), P (30 μM), 25-OH-Chol (25 μM), AG (50 μg/ml) or rhFSH (50 ng/ml). Steroids were dissolved in absolute ethanol before addition to the culture medium (final ethanol concentration <0.1%). Follicles were maintained at 37 °C in an incubator with 95% O_2 and 5% CO_2. Follicles were cultured for 6 h to assess inhibin subunits gene expression and for 24 h to determine hormone production. Following incubation, follicles were stored at −80 °C until RNA extraction and conditioned media were stored at −20 °C until hormone measurements were carried out.

2.4. Granulosa cell isolation and culture

Granulosa cells from DES-treated rats were isolated by follicular puncture, as previously described (Bley et al., 1992). As a result of DES-treatment, this cell preparation was enriched in granulosa cells derived from EAF. Cells were seeded onto plastic 24-well plates (Nunc, Roskilde, Denmark) precoated with rat-tail collagen. Initial plating density was 3.5×10^5 viable cells/well and cells were maintained at 37 °C with 5% CO_2. After 3 h, medium was changed to remove nonattached cells and replaced with 350 μl fresh DMEM:F12 (1:1, vol/vol)–bicarbonate (2.2 g/l) supplemented with fungizone, gentamicin and Δ_4A (0.25 μM), in the absence or presence of rhFSH (5 ng/ml).

2.5. Hormone measurements

Oestradiol was determined by RIA using a specific antibody which cross-reacts less than 1% with oestrone and oestriol as previously described (Escobar et al., 1976). Intra- and interassay coefficients of variation were 8% and 15%, respectively.

Androsterone and progesterone levels were determined by RIA, in suitable dilutions of the culture media, as previously described (Irusta et al., 2003). A specific antibody supplied by Dr. G. Barbe (Department of Physiology, University of Western Ontario, London, Canada) was used for androsterone determination; intra- and interassay coefficients of variation were 8.1% and 14.5%, respectively. A specific antibody supplied by Dr. G.D. Niswender (Animal Reproduction and Biotechnology Lab, Department of Physiology, Colorado State University, Fort Collins, CO, USA) was used for progesterone determination; intra- and interassay coefficients of variation were 8.0% and 14.2%, respectively.

Dimeric inhibins A and B, free inhibin α-subunit (Pro-αC) and total activin A levels in the culture media were measured using specific two-site enzyme-linked immunoabsorbent assays (ELISA) (Oxford Bio-Innovation Ltd., Oxon, UK) for each peptide, as previously described (Groome et al., 1994, 1995, 1996; Knight et al., 1996). Recombinant human inhibin A and B and activin A (Genentech, San Francisco, CA, USA), and a partially purified (>75% purity) Pro-αC preparation were used as standards. The assay sensitivity was 7 pg/ml for inhibin A, 15 pg/ml for inhibin B, 2 pg/ml for Pro-αC and 78 pg/ml for activin A. Activin A, activin B, and follistatin had less than 0.1% cross-reaction in dimeric inhibin assays. Inhibin A had less than 0.5% cross-reaction in the inhibin B assay whereas inhibin B had less than 0.1% cross-reaction in the inhibin A assay. The Pro-αC assay has less than 0.1% cross-reactivity with inhibin A, B, activin A, B and follistatin. The total activin A assay had less than 0.5% cross-reaction with inhibin A, inhibin B or activin B, less than 0.1% cross-reaction with bovine Pro-αC or follistatin and only a small (1–5%) cross-reaction with activin AB. Intra- and interassay coefficients of variation were less than 10% for all four assays.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA from frozen follicles (60 follicles per treatment) was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. All samples were subjected to DNA-free™ (Ambion, Austin, TX, USA) treatment according to manufacturer protocol to remove possible residual contaminating genomic DNA. The purified RNA was stored at −80 °C until further analysis. Complementary DNA templates for PCR amplification were synthesized from 1 μg of total RNA using oligo-dT primers and SuperScript II Reverse Transcriptase (Invitrogen) in a total volume of 20 μl. Two microlitres of the single stranded cDNA obtained from the RT reaction was used for PCR. For amplification of the rat inhibin α-subunit (*Inha*) and inhibin/activin βA-subunit (*Inhba*) cDNA, the primers were designed to span intron regions. A forward primer (5′-GCTGCCTCGAAGACATGC-3′) and a reversed primer (5′-GGGCCTAGAGCTATTGGA-3′) common to rat inhibin α-subunit cDNA were used for amplification of a 277-bp fragment. *Inhba* cDNA was amplified with a forward primer (5′-AGATGGTAGAGGCTGTCAAG-3′) and a reversed primer (5′-TGAAACAGACGGATGGTG-3′) bearing a 373-bp length fragment. Inhibin/activin βB-subunit (*Inhbb*) cDNA was amplified with a forward primer (5′-TCTTCATCGACTTTCGGCTCAT-3′) and a reverse primer (5′-TGTCAGGCGCAGCCACACTCCT-3′) as previously described by Albano et al. (1993), yielding a 304-bp length cDNA fragment. A 289-bp fragment of the housekeeping gene β-actin (*Actb*) was amplified in each sample for normalization of the results using a forward primer (5′-CGGAACCGCTCATTGCC-3′) and a reverse primer (5′-ACCCACACTGTGCCCATCTA-3′) purchased from Biodynamics (Buenos Aires, Argentina). The PCR reaction mixture included 2 μl cDNA of RT reaction, Cl_2Mg 1.5 mM, 0.8 mM dNTP mix, 0.3 μM of forward and reverse primers, 2.5 U Platinum Taq DNA Polymerase (Invitrogen) and 1× polymerase buffer in a total volume of 50 μl. Amplification was carried out for an optimal annealing temperature in a T-gradient thermocycler (Biometra, Goettingen, Germany). A variable amount of cDNA and number of cycles in the PCR amplification were assessed to verify that PCR products amplify linearly and to determine the optimal number of cycles allowing their detection without saturation of the signal. PCR products and a 100-bp

DNA ladder were separated by size on 1.5% agarose gels containing 0.15 μg/ml ethidium bromide (Invitrogen) and documented under UV trans-illuminator. The net intensity of each band was quantitated using Gel-Pro analysis software (Media Cybernetics Inc., Bethesda, MD, USA) and expressed as integrated optical density (IOD). The relative inhibin subunit gene expression was measured by determining the ratio between the target gene and *Actb* IODs.

2.7. Statistical analysis

Data are expressed as mean ± SEM. Comparisons between treatments were carried out using parametric or non-parametric unpaired *t*-test and one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison test, as appropriate. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

3.1. Steroid production

The endocrine activity of EAF was first assessed in terms of the steroidogenic capacity. Cultured EAF produced oestradiol (60 ± 7 pg/ml), progesterone (8.7 ± 2.3 ng/ml) and androsterone (21 ± 2 ng/ml) under basal conditions. The addition of 25-OH-Chol, previously identified as the best steroidogenic substrate for pregnenolone and progesterone production in ovarian cells, induced a 2.5-fold increment in progesterone ($P < 0.05$) whereas it had no effect on oestradiol levels. Although a trend to decrease was observed in androsterone production when 25-OH-Chol was present in the culture, this difference was not statistically significant ($P = 0.09$) (Fig. 1, Panel A).

When EAF were cultured in the presence of androstenedione or progesterone, oestradiol production increased significantly (6.8- and 2.7-fold increase over basal, respectively, $P < 0.01$). A further increase was observed when rhFSH was added to the culture in the presence of androstenedione (1.5-fold, $P < 0.05$). However, rhFSH did not increase oestradiol production when progesterone or 25-OH-Chol was used as steroidogenic substrate (Fig. 1, Panel B).

Cultured granulosa cells isolated from EAF produced oestradiol in the presence of androstenedione: 548 ± 68 pg/10^6 cells; the addition of rhFSH induced an 8.4-fold increase over basal oestradiol production (4619 ± 221 pg/10^6 cells, $P < 0.01$).

3.2. Inhibins and activin A production

We then analysed EAF endocrine activity in terms of inhibins and activin A production. Early antral follicles produced inhibin A (690 ± 21 pg/ml), inhibin B (3644 ± 329 pg/ml), Pro-αC (3513 ± 465 pg/ml) and activin A (1324 ± 222 pg/ml) under basal conditions. The inh A/inh B apparent ratio was 0.19. Conversely, cultured granulosa cells isolated from EAF predominantly produced inhibin A (286 ± 23 pg/10^6 cells); inhibin B and Pro-αC were also secreted (67 ± 7 and 438 ± 35 pg/10^6 cells, respectively). The inh A/inh B ratio was 4.3 (Fig. 2).

No differences in inhibin A, B and Pro-αC production were observed when EAF were cultured in the presence of androstenedione or progesterone when compared to basal conditions. Conversely, a significant increment in inhibin A, B and Pro-αC production was observed in the presence of 25-OH-Chol (1.8-, 2.0- and 2.1-fold increase over control, respectively, $P < 0.05$). The inh A/inh B ratio in the presence of 25-OH-Chol was 0.17 (Fig. 3, Panels A–C).

A significant increase in inhibin A and activin A production was observed when rhFSH was added to the culture in the absence of steroidogenic substrate (5.2- and 2.8-fold increase over basal, respectively, $P < 0.05$); no effect was observed on inhibin B and Pro-αC (Fig. 3, Panels A and D, B and C). Under this experimental condition the inh A/inh B apparent ratio was 0.47. The addition of rhFSH in the presence of androstenedione or progesterone stim-

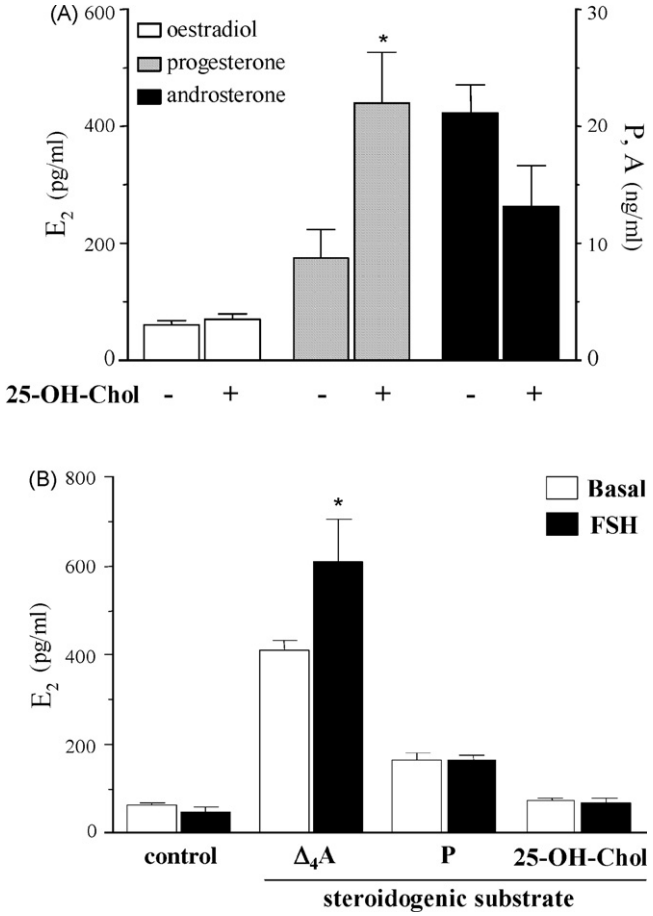

Fig. 1. Steroid production by cultured EAF. Panel A: Early antral follicles were cultured for 24 h in the absence or presence of 25-OH-Chol (25-hydroxycholesterol, 25 μM) as described in Section 2. Oestradiol (E_2), progesterone (P) and androsterone (A) levels were determined in the conditioned media. Panel B: Follicles were cultured for 24 h with or without rhFSH (50 ng/ml) in the absence (control) or presence of Δ_4A (androstenedione, 25 μM), P (progesterone, 30 μM) or 25-OH-Chol (25-hydroxycholesterol, 25 μM) as described in Section 2. Oestradiol (E_2) levels were determined in the conditioned media. Data are presented as mean ± SEM of three pools of follicles obtained from different ovaries. These experiments were repeated at least three times. *$P < 0.05$ when compared to basal.

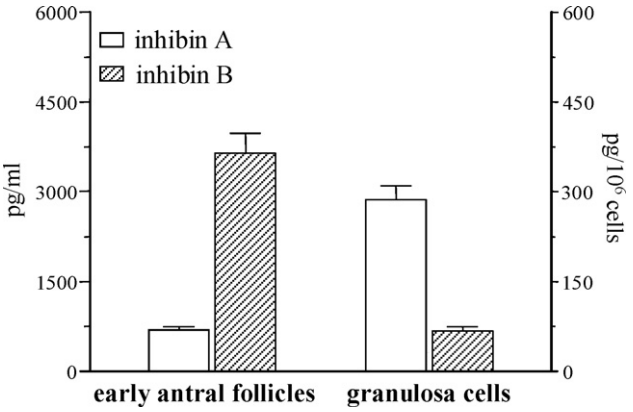

Fig. 2. Inhibin A and B production in early antral follicles (EAF) and granulosa cells (GC) under basal conditions. EAF and GC, isolated from EAF, were incubated for 24 h as described in Section 2. Inhibin A and B were determined in the conditioned media. EAF data are presented as mean ± SEM of at least three pools of follicles obtained from different ovaries (left axis; pg/ml); GC data are presented as mean ± SEM of triplicate cultures (right axis; pg/10^6 cells). These experiments were repeated at least three times.

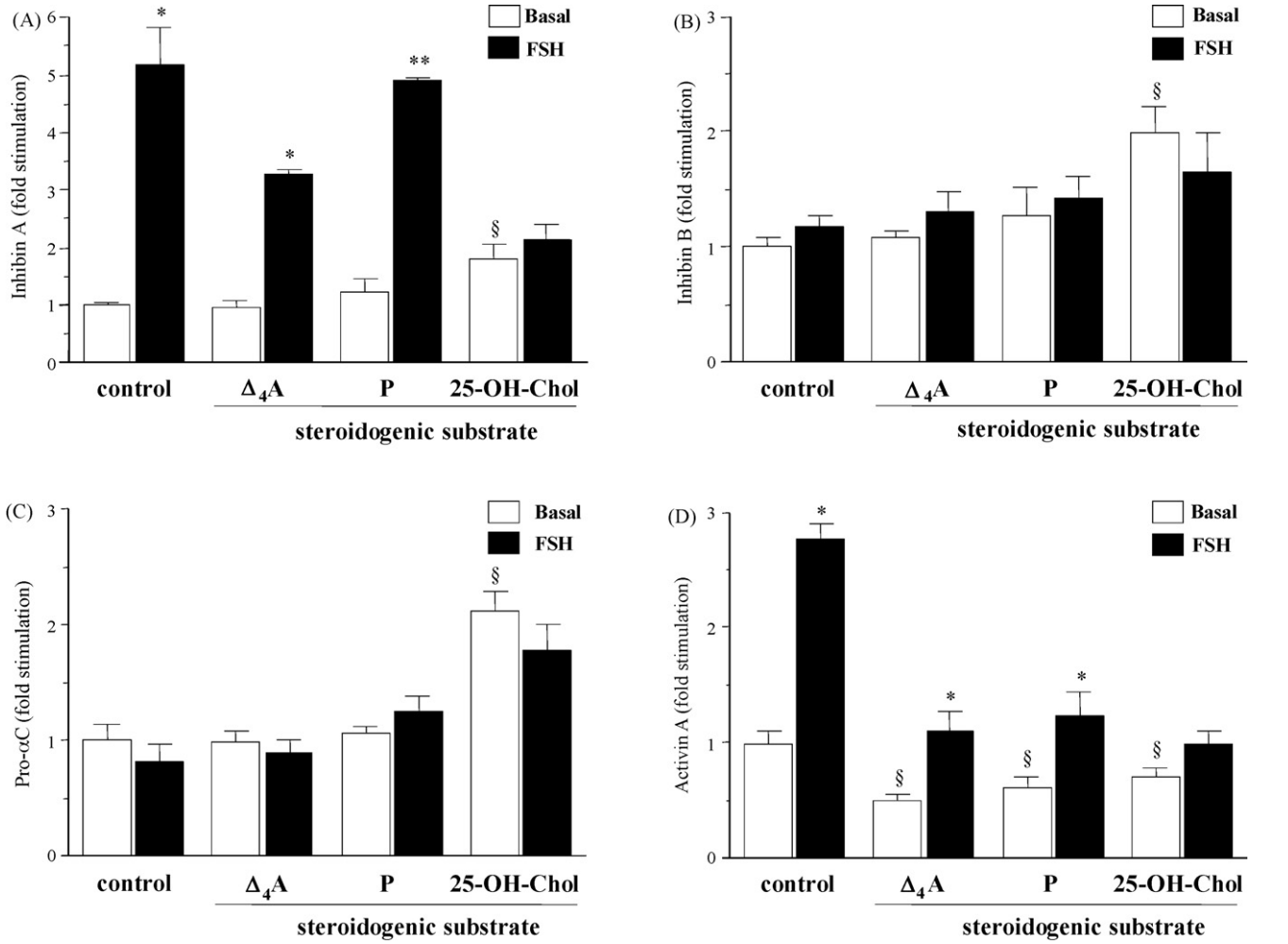

Fig. 3. Effect of rhFSH and esteroidogenic substrates on inhibin A (Panel A), inhibin B (Panel B), Pro-αC (Panel C) and activin A (Panel D) production by cultured EAF. Follicles were cultured for 24 h with or without rhFSH (50 ng/ml) in the absence (control) or presence of Δ_4A (androstenedione, 25 μM), P (progesterone, 30 μM) or 25-OH-Chol (25-hydroxycholesterol, 25 μM) as described in Section 2. Inhibin A, B, Pro-αC and activin A levels were determined in the conditioned media. Results are presented as fold stimulation over respective control production (without stimulus). Values are mean ± SEM of three pools of follicles obtained from different ovaries. These experiments were repeated at least three times. *$P < 0.05$; **$P < 0.01$ when compared to respective basal (absence of rhFSH). §$P < 0.05$ when compared to control.

ulated inhibin A production (3.3- and 3.9-fold increase over basal, respectively, $P < 0.05$) and it had no effect on inhibin B and Pro-αC. The addition of rhFSH in the presence of 25-OH-Chol did not further enhance inhibin A, B and Pro-αC production (Fig. 3, Panels A–C).

A significant decrease in activin A production was observed in the presence of androstenedione (50%), progesterone (39%) or 25-OH-Chol (30%) when compared to control (absence of steroidogenic substrate) ($P < 0.05$). The addition of rhFSH induced an increase in activin A production when androstenedione and progesterone were present in the culture (2.1- and 2.0-fold increase over basal, respectively; $P < 0.05$); the apparent increase in activin A production observed in the presence of 25-OH-Chol did not reach statistical significance ($P = 0.09$) (Fig. 3, Panel D). The stimulatory effect of rhFSH on activin A production in the presence of androstenedione and progesterone did not reach the magnitude of the response observed in the absence of steroidogenic substrate.

3.3. Effect of 25-OH-Chol on inhibin A, B, Pro-αC and activin A production in the presence of aminoglutethimide

To cast some light on the understanding of the mechanism of action of 25-OH-Chol on inhibin/activin production by EAF, the effect of this oxysterol was then evaluated in the presence of aminoglutethimide (AG, 50 μg/ml), an inhibitor of the cholesterol side-chain cleavage (CYP11A1) enzyme activity which prevents the metabolism of cholesterol to steroid hormones. The addition of AG to the culture media abolished basal and 25-OH-

Table 1
Effect of 25-hydroxycholesterol (25-OH-Chol) and aminoglutethimide (AG) on progesterone, inhibin A, inhibin B, Pro-αC and activin A production by cultured EAF.

Treatment	Progesterone	Inhibin A	Inhibin B	Pro-αC	Activin A
Control	1.00 ± 0.24[a]	1.00 ± 0.03[a]	1.01 ± 0.07[a]	1.00 ± 0.13[a]	1.00 ± 0.11[a]
25-OH-Chol	2.51 ± 0.49[b]	1.81 ± 0.25[b]	1.98 ± 0.23[b]	2.12 ± 0.18[b]	0.70 ± 0.07[b]
AG	0.10 ± 0.01[c]	0.76 ± 0.10[a]	1.11 ± 0.20[a]	1.12 ± 0.01[a]	0.82 ± 0.10[a]
25-OH-Chol + AG	0.02 ± 0.00[c]	2.25 ± 0.13[b]	2.49 ± 0.21[b]	2.17 ± 0.41[b]	0.43 ± 0.06[b]

Results are expressed as fold stimulation over respective control production (without stimulus). Values are expressed as mean ± SEM of three pools of follicles obtained from different ovaries. These experiments were repeated at least three times. Different superscripts indicate significant differences between treatments ($P < 0.05$).

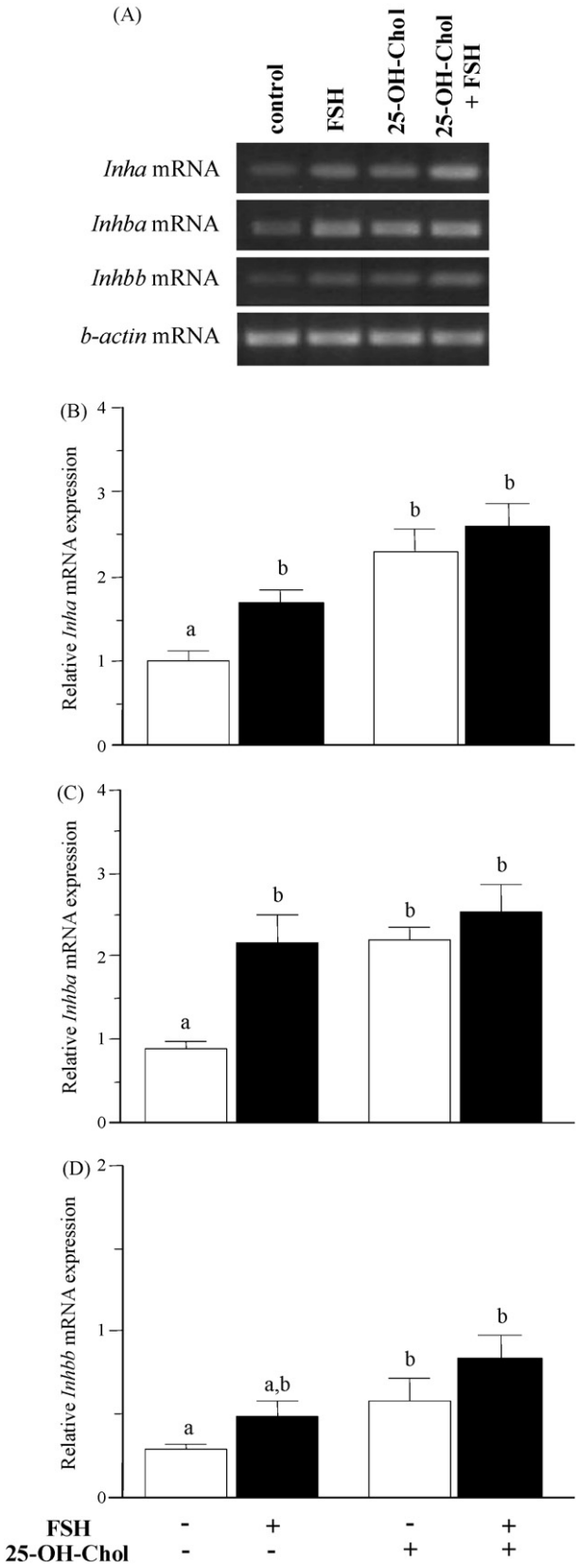

Fig. 4. Effect of 25-OH-Chol and rhFSH on inhibin/activin α-subunit (*Inha*, Panel B), βA-subunit (*Inhba*, Panel C) and βB-subunit (*Inhbb*, Panel D) mRNA expression by cultured EAF. Follicles were cultured for 6 h with or without rhFSH (50 ng/ml) in the absence (control) or presence of 25-OH-Chol (25-hydroxycholesterol, 25 μM). Inhibin/activin subunits mRNA expression was evaluated by RT-PCR as described in Section 2. Panel A: Representative gel illustrating the relative inhibin/activin subunits mRNA expression with different treatments. Panels B–D: The bands for inhibin/activin subunits were quantitated and normalized with the corresponding β-actin (*b-actin*) bands. Data are presented as mean ± SEM of at least three pools of follicles obtained from different ovaries. These experiments were repeated at least three times. Different superscripts indicate significant differences between treatments ($P < 0.05$).

Chol stimulated progesterone production by EAF (91% and 99% decrease, respectively; $P < 0.05$); however, it did not affect either basal or 25-OH-Chol stimulated inhibin A, B and Pro-αC production ($P > 0.05$). The inhibitory effect of 25-OH-Chol observed on activin A production was unaffected in the presence of AG ($P > 0.05$) (Table 1).

3.4. Effect of 25-OH-Chol on inhibin/activin subunits mRNA expression

To further examine the stimulatory effect of 25-OH-Chol on monomeric and dimeric inhibin production, the inhibin/activin subunits mRNA expression was evaluated.

Early antral follicles expressed *Inha*, *Inhba* and *Inhbb* mRNA under basal conditions; *Inhbb* mRNA expression was lower than that observed for *Inha* and *Inhba*.

The addition of rhFSH significantly stimulated *Inha* and *Inhba* mRNA expression (1.7- and 2.2-fold increase over basal, respectively; $P < 0.05$) (Fig. 4, Panels B and C); the apparent increase observed in *Inhbb* mRNA expression did not reach statistical significance ($P = 0.12$) (Fig. 4, Panel D).

The expression of *Inha*, *Inhba* and *Inhbb* mRNA increased when 25-OH-Chol was added to the culture (2.0-, 2.5- and 2.0-fold increase over basal, respectively, $P < 0.05$). The addition of rhFSH in the presence of 25-OH-Chol did not further enhance this expression (Fig. 4).

4. Discussion

The follicle culture technique used herein provides an important model for understanding the follicular physiology due to the maintenance of the follicular architecture and interaction between the different cell types and surrounding follicles. The present study shows, for the first time, that the follicular steroid microenvironment and an oxysterol differentially modulate the production of inhibins and activin A in EAF.

Early antral follicles in culture had a full capacity to produce steroids, inhibins and activin A. Isolated granulosa cells and follicles in culture were both able to convert an exogenous aromatizable steroidogenic substrate into estrogens and this capacity was enhanced by FSH. The marked response to the addition of androstenedione and FSH to the culture showed that aromatase activity was already present at this stage of follicular development. It is possible that the well-characterized stimulatory effect of this gonadotrophin and the possible influence of androstenedione on granulosa cell expression and synthesis of P450 aromatase may have contributed to sustained oestradiol production (Fitzpatrick and Richards, 1991; Hamel et al., 2005). The clear predominance of androsterone and the scant oestradiol production observed under basal conditions suggest that aromatizable steroids are not available to granulosa cells in EAF. A very high 5α-reductase activity has been previously described in the immature rat ovary (Erickson et al., 1985). Consequently, endogenous oestrogen precursors synthesized within the follicle may be easily converted to a non-aromatizable 4-ene-5α-reduced product and rendered androsterone as the principal steroid produced at this stage of follicular development.

The steroidogenic capacity of EAF was also evaluated in the presence of a more soluble sterol substrate. 25-OH-Chol, that bypasses

lipoprotein receptors and StAR, favoured progesterone production as previously reported by Toaff et al. (1982). However, this increase did not induce a consequent increment of androsterone or oestradiol production. Active 5α-reductase and 20-hydroxysteroid dehydrogenase may jeopardize steroid biosynthesis beyond C-20 precursors, possibly due to the rapid conversion of progesterone to its inactive metabolites (Goldring and Orly, 1985).

There was a clear predominance of inhibin A production over that of inhibin B when isolated granulosa cells were cultured under basal conditions. Conversely, the inhibin profile determined under identical experimental conditions in cultured EAF showed an inverse relationship and confirmed the predominance of inhibin B over inhibin A that had been previously described in rat serum during metoestrus and diestrus (Woodruff et al., 1996). A similar relationship between inhibin A and B was reported by Smitz and Cortvrindt (1998) in cultured single preantral mouse follicles. We have previously shown that activin A, TGF-β and oocyte-derived factor(s) selectively stimulated inhibin B production in cultured rat granulosa cells (Lanuza et al., 1999). This effect may be exerted on βB-subunit expression without affecting α- or βA-subunit mRNAs, as reported by Erämaa et al. (1995) and Erämaa and Ritvos (1996). Likewise, additional factors present in cultured EAF, such as growth differentiation factor-9 (GDF-9) and bone morphogenetic proteins (BMPs) produced by the oocyte and theca cells, may lead to the predominance of inhibin B over inhibin A under basal conditions (Kaivo-Oja et al., 2003; Roh et al., 2003; Jaatinen et al., 2002).

The addition of steroidogenic substrates to EAF, as androstenedione and progesterone, or the presence of aminoglutethimide did not affect inhibin production and the predominance of inhibin B. These results suggest that the follicular steroid microenvironment did not affect the action of diverse factors locally produced in the gonad which favoured the synthesis of the α–βB heterodimer. This was not the case for activin A production which was affected by the hormonal intrafollicular milieu, particularly in the presence of a marked estrogenic tone. The inhibitory effect of estrogens on activin subunit gene expression and activin A signaling in the mouse ovary has been previously reported (Kipp et al., 2007).

FSH was a potent stimulus for inhibin A production, independently of the hormonal changes induced by the metabolism of steroidogenic substrates. On the other hand, the magnitude of the stimulatory effect of FSH on activin A production was more marked in the absence of a steroidogenic substrate, confirming the dependence of activin A production on the follicular steroid milieu.

The mechanisms involved in the differential regulation of inhibin/activin subunit dimerization are not fully understood. N-linked glycosylation of inhibin α-subunit has been reported to influence dimer assembly: the loss of N-glycan sites reduces heterodimer formation and favours homodimerization of βA-subunits (Antenos et al., 2007). More recently, hydrophobic residues in the prodomain of the inhibin α-subunit have been proposed as additional determinants of heterodimer assembly and secretion (Walton et al., 2009). No information is available on the existence of regulatory mechanisms governing α-/βB-subunit dimerization. In the present study, the oxysterol 25-OH-Chol had a modulatory effect on inhibin and activin A production by cultured EAF, suggesting a different function to its classic role as a steroidogenic substrate in ovarian cells. 25-OH-Chol had opposite effects, stimulating both dimeric and monomeric inhibins and diminishing activin A. Furthermore, we showed that when the steroidogenic pathway was fully blocked by aminoglutethimide, 25-OH-Chol was able to exert identical actions on inhibin/activin production by EAF, indicating that the effect of this oxysterol was not mediated by a steroidogenic metabolite. This novel modulatory action of 25-OH-Chol was further explored. In coincidence with the increment of the inhibin dimers, 25-OH-Chol stimulated the mRNA expression of all inhibin/activin subunits (*Inha*, *Inhba* and *Inhbb*). The opposite effect observed on inhibin A and activin A dimers suggested that 25-OH-Chol may act at a post-transcriptional level favouring heterodimerization of inhibin α- and βA-subunits. These observations reinforce the hypothesis that different post-transcriptional events govern the assembly and secretion of inhibin/activin subunits in the ovary.

Oxysterols are known to be potent regulators of genes involved in sterol and fatty acid metabolism (Schroepfer, 2000). In particular, 25-OH-Chol has also been identified as a regulatory factor of StAR and LHR mRNA expression, in gonadal cells (Christenson et al., 1998; Wang et al., 2007). The stimulatory effect of 25-OH-Chol on inhibin α-subunit mRNA expression could be explained on the basis of interactions already described between this oxysterol, SF-1 and the classic cAMP transduction pathway (Lala et al., 1997; Ito et al., 2000; Weck and Mayo, 2006). In addition, it can be speculated that 25-OH-Chol may down-regulate the gene expression of gonadal cytokines that affect inhibin production (Englund et al., 2001; Morales et al., 2006). All these considerations support the hypothesis of a critical role for the oxysterols on the endocrine follicular activity. It has been reported that testicular macrophages are able to produce 25-OH-Chol, which in turn stimulates testosterone production (Lukyanenko et al., 2001). Likewise, macrophages have been implicated as important regulators of ovarian function (Wu et al., 2004). There is no available information supporting that macrophages may be a potential source of ovarian 25-OH-Chol. Then, the presence of this oxysterol under physiological conditions in the ovarian follicle and its mechanism of action on inhibin/activin production deserve further investigation.

In summary, the results showed herein demonstrate that EAF have full potential to produce steroids, inhibins and activin A. However, their endocrine activity may be hierarchically determined by the complex interplay of FSH and paracrine factors acting on steroidogenic enzyme activity, the gene expression of inhibin/activin subunits, their assembly and secretion. In addition, a novel modulatory action of 25-OH-Chol on inhibin/activin follicular expression was described. Further experiments will be carried out in order to determine whether the effect of 25-OH-Chol on the ovary could be extended to other oxysterols.

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