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Regulation of Sertoli Cell Aromatase Activity by Cell Density and Prolonged Stimulation with FSH, EGF, Insulin and IGF-I at Different Moments of Pubertal Development

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Sertoli cell aromatase activity is high in very young animals and declines throughout pubertal development. Little is known about the regulatory factors which might be involved in the pronounced decline suffered by this enzymatic activity. In this paper we show that estradiol production in Sertoli cells is dependent on cell density in the culture and that chronic stimulation with hormones can decrease estradiol acute response to FSH. In 8-day-old Sertoli cells cultured at low density (LD: 7.1 ± 0.3 μg DNA), estradiol production was 151 ± 11 $pgE_2/\mu g$ DNA, while in those cultured at high density (HD: 30.3 ± 0.6 μg DNA), production was 30 ± 5 $pgE_2/\mu g$ DNA. Similar results were obtained in 20-day-old Sertoli cell cultures (LD: 57 ± 4 $pgE_2/\mu g$ DNA vs HD: 26.0 ± 0.6 $pgE_2/\mu g$ DNA). On the other hand, treatment of Sertoli cell cultures (8- and 20-day-old) for 96 h, with FSH (100 ng/ml), EGF (50 ng/ml), insulin (10 μg/ml) and IGF-I (50 ng/ml) at different densities resulted mostly in inhibition of aromatase activity. The effect caused by FSH was apparently not related to desensitization as aromatization with dbcAMP could not overcome the decreased ability of these cells to produce estradiol. The effect caused by EGF was observed in 8-day-old Sertoli cells cultured under high density conditions. Marked inhibition was observed with insulin and IGF-I in 8-day-old Sertoli cell cultures. Considering previous reports indicating a decrease in Sertoli cell aromatase activity with age, our results suggest a potential role for FSH, EGF, insulin and IGF-I on the Sertoli cell differentiation process which occurs throughout pubertal development.

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INTRODUCTION

Sertoli cells were identified as a source of estrogen in the immature rat testis many years ago [1]. Since estrogen production is maximum before the first wave of spermatogenesis is initiated, it has been suggested that it may play a role during the neonatal period. Particularly, it has been proposed that estradiol may be important for Sertoli cell mitosis and that it can be essential in reducing testosterone production by Leydig cells [2, 3]. Conversion of exogenous testosterone to estradiol by Sertoli cells is stimulated by FSH. However, the response to this hormone declines with age: at 30 days of age the Sertoli cells have lost the capacity to synthesize estrogens *in vitro* [4]. Similar observations have been made in *in vivo* studies [5].

Recently, Papadopoulos *et al.* have described changes in the ability of Sertoli cells to synthesize estradiol in response to FSH when cells were cultured in bicameral chambers coated with extracellular matrix [6]. The authors have suggested that the observed effects were related to a more differentiated state of Sertoli cells. The regulatory factors involved in Sertoli cell differentiation which lead to decreased estrogen synthesis are not known. Most of the studies on Sertoli cell aromatase activity have been done in primary cultures of these cells but, with few exceptions [6–8], little information is available on the influence of culture conditions and hormone treatments for prolonged

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periods of time. The current study was designed to examine the effect of culture conditions on Sertoli cell aromatase activity and to evaluate possible factors which might be involved in the complex process of Sertoli cell differentiation which has been poorly characterized. We have particularly focused on the effects caused by cell density and prolonged stimulation with FSH, EGF, insulin and IGF-I at two different moments of pubertal development.

MATERIALS AND METHODS

Materials

Ovine FSH (NIH-FSH-S-16) was obtained from the National Hormone and Pituitary Program, NIDDK. Human recombinant IGF-I was purchased from Gro Pep, Adelaide, Australia. Tissue culture media were purchased from Grand Island Biological Co. (Grand Island, NY, U.S.A.). All other drugs and reagents were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Sertoli cell isolation and culture

Sertoli cells from 8-day-old Sprague–Dawley rats were prepared based on the method described by Padmanabhan *et al.* [9]. Briefly, 8-day-old rats were decapitated, testes were removed, decapsulated and incubated in culture medium containing 0.03% collagenase, 0.003% soybean trypsin inhibitor, for 5 min at room temperature. Culture media consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle medium, supplemented with 100 IU/ml penicillin, 2.5 μg/ml amphotericin B and 1.2 mg/ml sodium bicarbonate. After the initial dispersal, seminiferous tubules were sedimented and supernatant discarded to remove interstitial cells. After several washes, a second collagenase treatment was performed. Tubules were treated for 10 min at room temperature with a solution of 0.03% collagenase, 0.003% trypsin inhibitor and 0.03% DNase. Cell preparation was resuspended in culture medium as described above with the following additions: 10 μg/ml transferrin, 5 μg/ml insulin, 5 μg/ml vitamin E and 4 ng/ml hydrocortisone. Sertoli cells were cultured at different densities in 24 multiwell plates at 34°C in a mixture of 5% CO_2: 95% air.

Sertoli cells from 20-day-old Sprague–Dawley rats were isolated as previously described [10]. Briefly, decapsulated testes were digested with 1% collagenase and 0.6% soybean trypsin inhibitor in Hanks' balanced salt solution (HBSS) for 5 min at room temperature. Seminiferous tubules were saved, cut and submitted to 1 M glycine-2 mM EDTA (pH 7.4) treatment to remove peritubular cells. The washed tubular pellet was then digested again with collagenase for 10 min at room temperature to remove germinal cells. The Sertoli cell suspension, collected by sedimentation, was resuspended and cultured in the same culture medium and in the same conditions described for 8-day-old Sertoli cells.

Culture conditions and aromatase activity

After the initial 24 h attachment period, parallel cultures were maintained for 4 days in the same culture medium described above but without insulin and with the addition of one of the following stimulants: 100 ng/ml FSH, 50 ng/ml EGF, 10 μg/ml insulin and 50 ng/ml IGF-I. Basal cultures were considered those not receiving any of these factors. Culture medium was changed 48 h later (day 3) and the same hormone additions were performed at this time. To evaluate aromatase activity, 100 ng/ml FSH and 3 μM testosterone as substrate for aromatization were added on the fourth day. No medium change was performed at this time. Media were collected 18 h later (day 5), centrifuged at 3000 rpm for 10 min and stored at -20°C until estradiol determinations were performed. The monolayers were washed with 0.9% NaCl solution and 0.5 ml of 2 mM EDTA (pH 7.4) was added. Cells were disrupted by ultrasonic irradiation and adequate aliquots for DNA determinations were saved.

Other assays

DNA was determined by the method of Labarca and Paigen [11]. Radioimmunoassay (RIA) of estradiol was performed according to the method of Escobar *et al.* [12], using a specific antibody which cross-reacts less than 1% with estrone and estriol. The RIA had a sensitivity of 6.25 pg/tube and intra and interassay coefficients of variation were 8 and 15%, respectively. Testosterone in conditioned medium was determined using a specific RIA as previously described [13].

Statistical analysis

Statistical analysis was performed by analysis of variance followed by Tukey's protected t-test for comparison of data from multiple groups using the GB-STAT version 4.0 statistical program (Dynamic Microsystems, Inc. Silver Spring, MD, U.S.A.).

RESULTS

Regulation of Sertoli cell aromatase activity by cell density

Figure 1 shows that estradiol production in Sertoli cell cultures obtained from 8-day-old animals is dependent on the cellular density in the culture. A marked decline with increasing cell densities was observed. Low density (LD) cultures, where DNA value was 7.1 ± 0.3 μg (mean $\pm$ SEM) produced 151 ± 11 $pgE_2/\mu g$ DNA, while high density (HD) cultures (DNA: 30.3 ± 0.6 μg) produced 30 ± 5 $pgE_2/\mu g$ DNA.

Figure 2 shows similar results obtained with Sertoli cell cultures from 20-day-old rats. A decrease in aromatase activity with increasing cellular densities

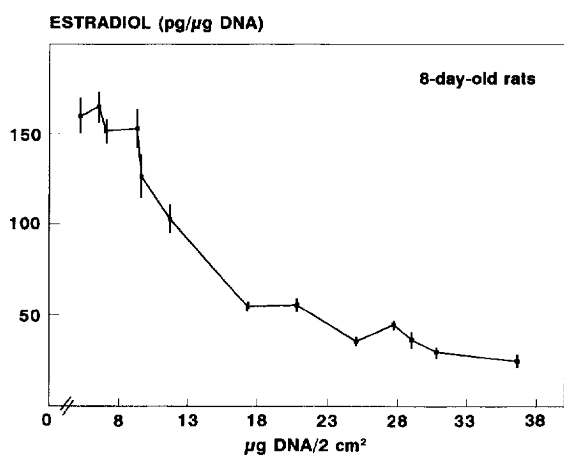

Fig. 1. Effect of cell density on estradiol production in Sertoli cells derived from 8-day-old animals. DNA content was determined at the end of the culture period. Results are expressed as mean ± SEM of triplicate incubations in a representative experiment out of three.

was also observed at this age (LD: $8.2 \pm 0.3\,\mu g$ DNA; $57 \pm 4\,pgE_2/\mu g$ DNA vs HD: $14.5 \pm 0.9\,\mu g$ DNA; $26.0 \pm 0.6\,pgE_2/\mu g$ DNA).

As decreased estradiol production might be caused by exhaustion of tissue culture medium components or presence of inhibitory factors in high density cultures, we designed the experiments shown in Fig. 3. This figure shows that exchanging tissue culture media from high to low density—and vice versa—prior to the addition of FSH and testosterone had no effect on aromatase activity.

Figure 4 shows the dose–response curves to FSH stimulation in 8-day-old Sertoli cells grown under low and high density conditions. In low density cultures maximum stimulating doses of FSH (100–500 ng/ml) increased the conversion of testosterone to estradiol 20-fold. A similar dose–response curve was obtained in

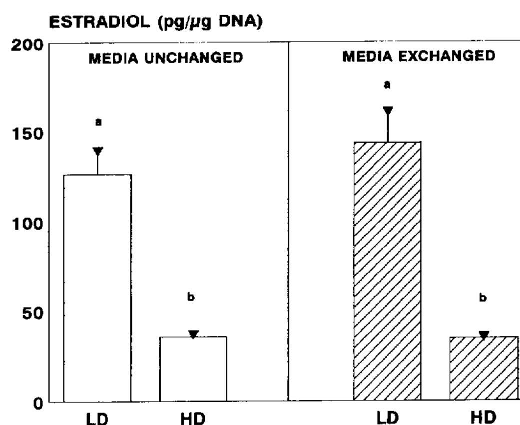

Fig. 3. Effect of exchanging Sertoli cell conditioned media from high to low—and vice versa—density cultures on aromatase activity in 8-day-old Sertoli cells. Sertoli cells were cultured at LD ($8.3 \pm 0.5\,\mu g\,DNA/2\,cm^2$) and at HD ($28 \pm 1\,\mu g\,DNA/2\,cm^2$) for 96 h. FSH and testosterone were then added to evaluate aromatase activity and culture was allowed to proceed for additional 18 h (left panel). In parallel cultures conditioned media from HD and LD cultures were exchanged just prior to the addition of FSH and testosterone (right panel). Results are expressed as mean ± SEM, $n = 4$. Different superscripts represent statistically significant differences ($P < 0.01$).

high density cultures but the maximal response was only 10-fold higher than basal.

Testosterone was determined by RIA in conditioned culture media after the aromatization period and a concentration of the steroid equivalent to $2.8 \pm 0.4\,\mu M$ was obtained.

Finally, to evaluate a potential inhibitory effect caused by estradiol, we added this steroid to the aromatization medium at a concentration similar to that produced by the cells when a saturating dose of FSH

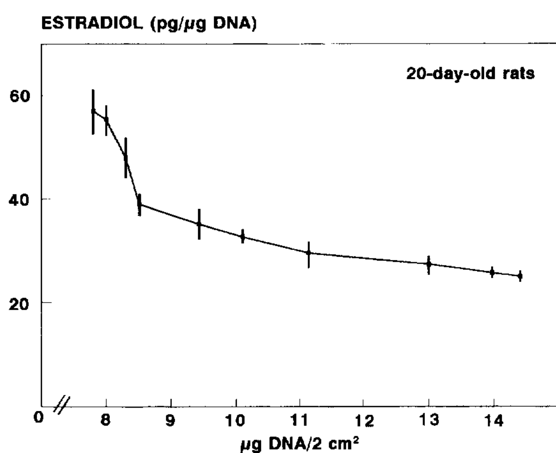

Fig. 2. Effect of cell density on estradiol production in Sertoli cells derived from 20-day-old animals. DNA content was determined at the end of the culture period. Results are expressed as mean ± SEM of triplicate incubations in a representative experiment out of three.

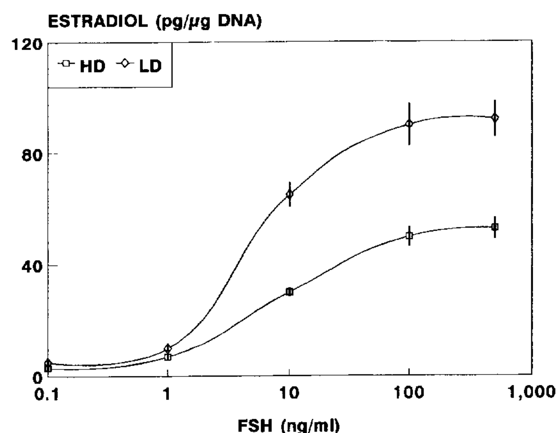

Fig. 4. Dose–response curve of aromatase activity to FSH stimulation in Sertoli cells obtained from 8-day-old rats. The upper curve was obtained with Sertoli cells cultured under low density conditions (LD: $9.3 \pm 0.6\,\mu g\,DNA/2\,cm^2$). Lower curve was obtained with high density cultures (HD: $26 \pm 1.3\,\mu g\,DNA/2\,cm^2$). Results are expressed as mean ± SEM ($n = 4$).

Table 1. Effect of estradiol added to aromatization medium on Sertoli cell aromatase activity

Culture conditions	Estradiol production (pg/μgDNA)
A	61 ± 7
B	66 ± 3
C	65 ± 11
D	71 ± 11

20-day-old Sertoli cells were cultured under low density conditions and with the addition of variable amounts of estradiol to the aromatization medium. (A) No estradiol; (B) 300 pgE_2/ml; (C) 450 pgE_2/ml; and (D) 600 pgE_2/ml. Reported values were calculated after subtracting appropriate blanks obtained in parallel cultures performed without FSH addition (mean ± SEM, $n = 4$).

was used. Once appropriate blanks were considered, calculated values for estradiol production were similar. These results are shown in Table 1.

Regulation of aromatase activity by prolonged treatment with FSH, EGF, insulin and IGF-I

Figures 5 and 6 show pooled data obtained in 7 independent experiments performed with 8- and 20-day-old Sertoli cells cultured at similar densities and under different hormone treatments. Cultures maintained with FSH, EGF and insulin for 4 days showed no change or lower aromatase activity in response to FSH stimulation. The decrease in estradiol production depended on hormone treatment, cellular density and animals' age.

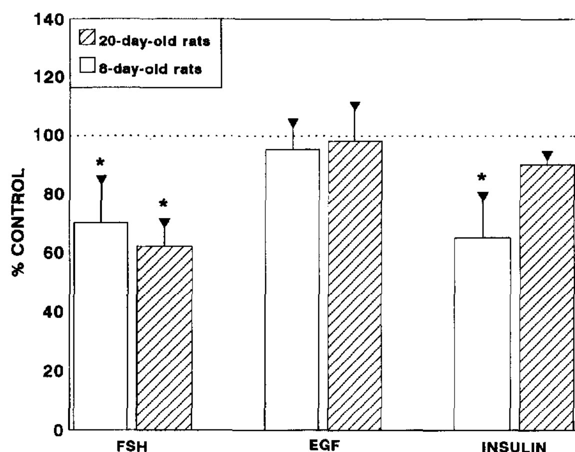

Fig. 5. Regulation of aromatase activity caused by prolonged treatment with FSH, EGF and insulin in 8- and 20-day old Sertoli cells cultured at low density. Cells were stimulated with FSH (100 ng/ml), EGF (50 ng/ml) and insulin (10 μg/ml) for 96 h. DNA ranged from 6.3 to 10.9 μg in 8-day-old and from 5.4 to 8.5 μg in 20-day-old Sertoli cells. Seven individual experiments were performed in triplicate. In each experiment mean estradiol production in control cultures (no hormone additions) was considered to be 100% and, in all other experimental conditions, estradiol production was expressed as percentage relative to this control value (% Control). Combined data from the seven experiments are shown as mean ± SEM. Asterisks indicate that the decrease in aromatase activity was statistically significant in all experiments performed ($P < 0.05$).

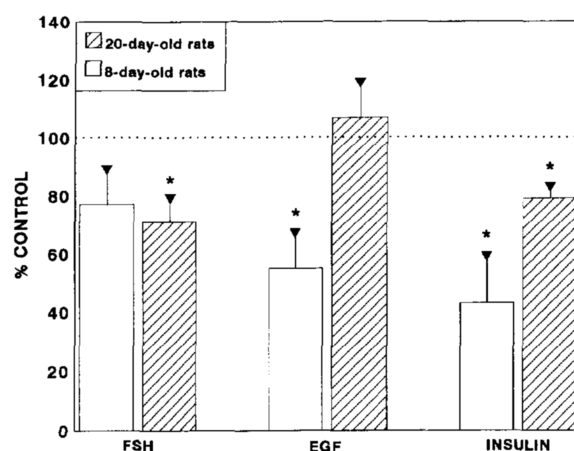

Fig. 6. Regulation of aromatase activity caused by prolonged treatment with FSH, EGF and insulin in 8- and 20-day old Sertoli cells cultured at high density. Cells were stimulated with FSH (100 ng/ml), EGF (50 ng/ml) and insulin (10 μg/ml) for 96 h. DNA ranged from 19.7 to 33.1 μg in 8-day-old and from 11.3 to 14.3 μg in 20-day-old Sertoli cells. Seven individual experiments were performed in triplicate. In each experiment mean estradiol production in control cultures (no hormone additions) was considered to be 100% and, in all other experimental conditions, estradiol production was expressed as percentage relative to this control value (% Control). Combined data from the seven experiments are shown as mean ± SEM. Asterisks indicate that the decrease in aromatase activity was statistically significant in all experiments performed ($P < 0.05$).

In cultures stimulated for 4 days with FSH, a decreased estradiol production at both densities and ages studied was observed (two columns on the left on Figs 5 and 6). However, results obtained with Sertoli cells from 8-day-old rats cultured at high density were not statistically significant in most of the experiments performed. The inhibition in aromatase activity by chronic treatment with FSH could not be overcome by using a dose of dbcAMP able to induce a maximal response in basal cultures (Table 2).

Table 2. Estradiol production in 20-day-old Sertoli cell cultures under FSH and dbcAMP stimulation

Culture conditions	Aromatization conditions: FSH	dbcAMP
	(Estradiol (pg/μg DNA))	
Basal	24.1 ± 1.0	21.3 ± 2.5
FSH	16.9 ± 3.0	16.1 ± 1.5

Cells were cultured for 96 h in basal medium or with the addition of 100 ng/ml FSH. During the last 18 h in culture aromatizable substrate and either FSH (100 ng/ml) or dbcAMP (0.2 mM) were added. DNA content of these cultures were as follows: basal, 9.1 ± 0.2; FSH, 9.5 ± 0.3 μg DNA (mean ± SEM, $n = 4$). Results from a single experiment repeated twice are shown.

Table 3. Regulation of aromatase activity by IGF-I

Cell density	Culture conditions	
	Basal	IGF-I
	(Estradiol (pg/μg DNA))	
LD	44.4 ± 1.5	19.1 ± 0.9*
HD	18.0 ± 1.2	10.2 ± 0.8*

8-day-old Sertoli cells were cultured under different density conditions (LD: 15.1 ± 0.5; HD: 25.0 ± 2.0 μg DNA) and with the addition or not of 50 ng/ml IGF-I for 96 h. Results are expressed as mean ± SEM, $n = 4$. *$P < 0.01$.

In high density cultures obtained from 8-day-old animals a modulatory effect of EGF was demonstrated. This regulatory effect of EGF was absent in 20-day-old Sertoli cells (Fig. 6, two columns in the middle).

Insulin treatment of the cultures also resulted in inhibition of aromatase activity (two columns on the right on Figs 5 and 6), except for Sertoli cells from 20-day-old rats cultured at low density, where the decrease was not statistically significant. Similar results were obtained when 50 ng/ml IGF-I was used to stimulate 8-day-old Sertoli cell cultures (Table 3). Insulin stimulation at 50 ng/ml had no effect on aromatase activity (data not shown).

DISCUSSION

Aromatase activity has been widely used as a marker of Sertoli cell function. Also, this enzyme activity has been very useful when analyzing signal transduction pathways [14–18], interactions of Sertoli cells with other cells in the testis [8, 10, 19–21] and more recently to analyze actions of extracellular matrix on differentiated functions of Sertoli cells [6]. Little information, however, is available on the effects of culture conditions on aromatase activity, particularly on the effects of chronic stimulation with hormones and growth factors [7, 8]. Over the last decade several reports have indicated that cell density is an important parameter to be considered in analyzing the hormonal regulation of Sertoli cell function [22–27]. In this paper we present evidence that shows that estradiol production in Sertoli cell cultures also varies with cell density. Similar results have been previously obtained for aromatase activity in other cells [28, 29]. In our experimental conditions aromatase activity showed a marked decline with increasing cell density in the cultures. This decline was demonstrated at both ages studied. We thought that these variations in estradiol production could be accounted for by at least one of the following reasons: (1) insufficient FSH and testosterone added in high density cultures; (2) presence of inhibitory factors or deficient levels of particular nutrient components in tissue culture media; or (3) inhibition of aromatase activity by the final product of the reaction, namely estradiol. In order to evaluate the first hypothesis, we compared the dose–response curves for FSH obtained with high and low density cultures and concluded that both were essentially identical in sensitivity. Even though maximal levels of estradiol in high density cultures were lower than those found in low density cultures, these maximal responses were reached at similar FSH concentrations. These results together with the fact that testosterone determination in conditioned media revealed a very low consumption of the substrate ruled out the first possibility. In order to evaluate the second possibility, we decided to exchange conditioned media from high to low density—and vice versa—cultures. No change in aromatase activity could be demonstrated under these experimental conditions. Also, we discarded the third possibility due to the fact that identical enzyme activities were determined when estradiol was included in the aromatization medium.

Our results seem to be in contrast with those recently published by Rosselli and Skinner [8] who showed that aromatase activity is independent of cellular density in the culture. However, three important experimental differences must be considered. In that report aromatase activity was assessed by the release of ^3H_2O from aromatization of radiolabeled testosterone and most importantly, the assay was performed under basal conditions and with freshly isolated cells. Also, Papadopoulos *et al.* [6] have reported no effect of cell density on aromatase activity when cells were cultured on bicameral chambers. The different substratum (membrane coated or not coated with Matrigel vs plastic) on which Sertoli cells were grown might be responsible for the discrepancy in the experimental results. On the other hand, these authors showed that Sertoli cells cultured on extracellular matrix exhibit a marked inhibition of estradiol production and they have suggested that these results might reflect a better differentiated state of the cells. Our results suggest that high density cultures are more differentiated than low density cultures at both ages studied. Density curves for 8- and 20-day-old Sertoli cells are not superimposable. In our experience, Sertoli cells from 20-day-old animals cannot be cultured at densities higher than 20 μg DNA/2 cm^2. A much higher cell density can be reached with Sertoli cells from 8-day-old animals. Comparing the results obtained with similar densities at both ages it becomes apparent that estradiol production is always higher in 8-day-old than in 20-day-old Sertoli cells confirming what has been previously published [4].

Kissinger *et al.* [30] have shown that the pattern of secreted polypeptides is different in 10- and in 20-day-old Sertoli cells. These results and the observed structural maturation of Sertoli cells [31] suggest an important maturative change occurring at this moment of pubertal development which might be involved in the onset of spermatogenesis. The mechanisms underlying these maturational changes are poorly

understood. Studying Sertoli cells at different ages using aromatase activity as a marker of cell differentiation may give important clues about hormones and other requirements associated with maturational changes. Considering that the synthesis of estradiol is higher in the immature rat, a decrease in the activity of this functional parameter after prolonged treatment with a hormone or growth factor *in vitro* may be considered as a reflection of a differentiating process.

Sertoli cells are particularly sensitive to FSH during pubertal development and in addition to its mitogenic effect [32] a differentiating action for this hormone has been postulated [33]. The inhibitory effects of chronic FSH treatment were statistically significant in all cases studied except in 8-day-old Sertoli cells cultured at high density. The most readily available explanation for the inhibition obtained with FSH is desensitization to the hormone. Considering that dbcAMP was not able to increase estradiol production up to the same value observed in basal cultures, it is possible that FSH differentiating effects are responsible for the decrease in aromatase activity. Verhoeven and Cailleau [7] have demonstrated that continuous exposure of Sertoli cells to androgens decreases aromatase activity. It seems likely that these *in vitro* findings have physiological relevance as the two hormones, which have been largely known to control Sertoli cell function and in this way spermatogenesis, regulate Sertoli cell aromatase activity in a similar fashion. Also, these results reinforce the idea that this enzyme activity can be regarded as a marker of Sertoli cell differentiation as has already been proposed by Papadopoulos *et al.* [6].

Inhibitory actions of EGF on Sertoli cell aromatase activity have been shown [15]. The experimental model used by Mallea *et al.* differs significantly from the one presented here. These authors have studied the effect of EGF for the last 24 h in culture, at the moment when aromatase activity is being determined. We, instead, have evaluated the consequences of a 4-day stimulation with EGF, and found inhibition of aromatase activity only in the case that 8-day-old Sertoli cells were cultured under high density conditions. We have no explanation for the lack of effect of EGF in all other situations. The observed modulatory action of EGF might be exerted through a Sertoli cell EGF receptor [34]. However, an indirect effect of this peptide involving peritubular cells which are present in Sertoli cell cultures from 8-day-old rats cannot be ruled out [35].

Actions of IGF-I related to differentiation in several testicular cells have been well documented [36]. Our results add a new parameter of Sertoli cell physiology which is under the control of insulin related peptides. Stimulation with insulin, at doses high enough to occupy IGF-I receptors, provoked a marked inhibition of aromatase activity in 8- and 20-day-old Sertoli cells. Similar results were obtained when cultures were treated with 50 ng/ml IGF-I. The inhibitory actions of insulin seem to be exclusively mediated through occupancy of IGF-I receptors as low doses of insulin were ineffective. Controversy exists about the existence of insulin receptors in Sertoli cells [37, 38]. Our results show that insulin by itself has no ability to regulate this Sertoli cell differentiated function unless it is used at concentrations high enough to occupy IGF-I receptors. These results do not preclude the existence of insulin receptors but, were they to be present, they would not be involved in regulating Sertoli cell aromatase activity.

In conclusion, considering that Sertoli cell aromatase activity *in vivo* and *in vitro* is inversely related to the differentiated state of the cell, our data demonstrate that Sertoli cells cultured at high density are more differentiated than Sertoli cells cultured at low density. On the other hand, the observed inhibitory effects of prolonged treatments with FSH, EGF, insulin and IGF-I point to a potential role of these hormones on Sertoli cell differentiation.

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