

# Inhibitory effect of valproic acid on ovarian androgen biosynthesis in rat theca-interstitial cells

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**Abstract** The objective of the study was to evaluate the effect of valproic acid (VPA) on ovarian androgen biosynthesis in primary cultures of theca-interstitial (T-I) cells isolated from rat ovaries. Ovarian T-I cells were cultured with VPA in the presence or absence of hCG. VPA did not increase basal or hCG-stimulated androgen synthesis when added to primary cultures of T-I cells. However, the addition of VPA caused a marked concentration-dependent inhibitory effect on hCG-stimulated androstenedione synthesis. Treatment of T-I cells with 8-Bromo-cAMP resulted in a marked increase in the production of androstenedione, and VPA inhibited this stimulatory effect, suggesting that the mechanism of VPA's inhibitory effect on androstenedione production occurs at a step after second messenger activation. Treatment of T-I cells with hCG resulted in a significant increase in the mRNA expression of steroidogenic enzymes CYP17A1 and 17 $\beta$ -hydroxysteroid dehydrogenase. Addition of VPA sharply blunted the stimulatory effect of hCG, reducing the mRNA expression of the steroidogenic enzymes to basal levels. In conclusion, VPA exerts an

inhibitory effect on hCG-stimulated androgen synthesis in rat T-I cells.

**Keywords** Theca-interstitial cells · Androgen synthesis · hCG · Steroidogenesis · Androstenedione · Valproic acid · PCOS

## Introduction

Valproic acid (2-*n*-propylpentanoic acid, VPA) is a short-chain fatty acid that is widely used as a mood-stabilizer for bipolar disorder, in addition to its well established use for the treatment of epilepsy and other neurological disorders such as chronic neuralgia and migraine headache [1]. Because of its off-label use for various conditions, it is one of the most commonly prescribed medications in the United States [2]. A higher incidence of menstrual and reproductive disturbances including menstrual irregularities, weight gain, and hyperandrogenism has been reported in patients with epilepsy [3, 4]. Treatment of epileptic female patients with VPA has also been associated with endocrine side effects, including many of the characteristics associated with some women with polycystic ovary syndrome (PCOS), like weight gain, oligomenorrhea, hyperinsulinemia, and hyperandrogenism [5]. Whether this increase is secondary to the underlying neuropathy of seizure disorder or the treatment with anti-epileptic drugs (AED) remains controversial. According to a recent review [6], the risk of developing PCOS during VPA treatment seems to be higher in women with epilepsy than in those with bipolar disorders. This indicates that the underlying neuroendocrine dysfunction may be related to the seizure disorder in altering the hypothalamic-pituitary-ovarian axis, although the role of VPA can not be excluded.

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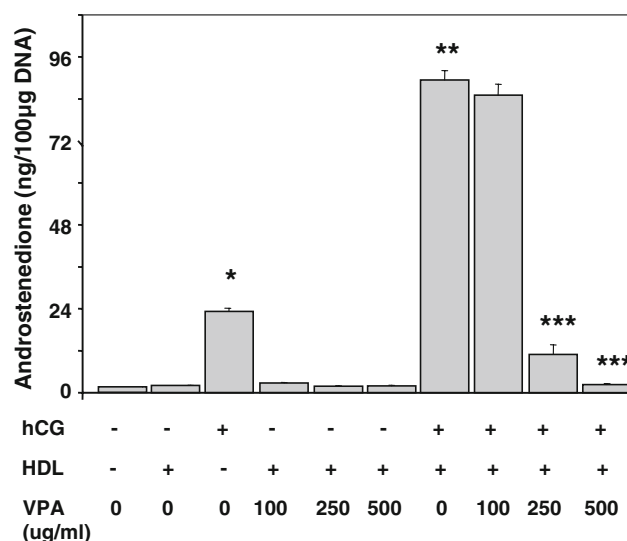
A number of studies in the literature suggest an association between AED therapy and the development of PCOS-like symptoms [5, 7–10] while other studies, both in humans and animals, have found little or no association between VPA use and PCOS [2, 11, 12]. Despite the widespread use of VPA for non-epileptic conditions, all the available clinical studies examining the effect of VPA on endocrine side effects in women of reproductive age are comprised of female patients with epilepsy or bipolar disorder, making it difficult to sort out whether the underlying disease condition or VPA plays the primary role in the induction of the PCOS-like symptoms. Interestingly, there are no studies to date that have examined PCOS incidence rates in patients treated with VPA for off-label indications such as migraines or neuralgia [2]. Although many of the patients taking VPA for migraines, bipolar disorder, and epilepsy are women of reproductive age [1], there is conflicting clinical information available regarding its implication in reproductive disruption. As for hyperandrogenism, one of the diagnostic criteria of PCOS, normal gonadotropin levels in VPA-treated hyperandrogenic patients suggested a direct effect of VPA on peripheral sex hormone production in the ovary rather than an effect of VPA-induced increase in  $\gamma$ -hydroxybutyric acid [5]. Similar conclusions have been drawn from studies using a rat model [13].

In this context, the present studies focused on determining the effect of VPA on androgen production and on key steroidogenic enzymes using cultured rat theca-interstitial cells, to test whether VPA use might contribute to PCOS-like phenotypes. The present results clearly show that VPA exerts an inhibitory effect on androgen production by theca-interstitial cells by reducing the expression of key steroidogenic enzymes, acting at a step after the formation of cyclic AMP.

## Results

### Dose-dependent effect of VPA on hCG-stimulated androstenedione synthesis

Treatment of T-I cells with hCG resulted in a 14-fold increase in androstenedione production from basal level,  $1.6 \pm 0.1$  ng/100  $\mu$ g DNA to  $23.9 \pm 0.9$  ng/ $\mu$ g DNA (Fig. 1). Treatment with hCG and HDL increased androstenedione production further to  $89.6 \pm 0.3$  ng/ $\mu$ g DNA, which is a 56-fold increase from baseline. The purpose of HDL was to provide cholesterol, the precursor of androgens. Addition of various doses of VPA to cultures treated with HDL alone did not show any increase in androgen biosynthesis over basal levels. VPA, when added in the presence of hCG plus HDL, resulted in a concentration-dependent reduction of the stimulatory effect of hCG and HDL. At a

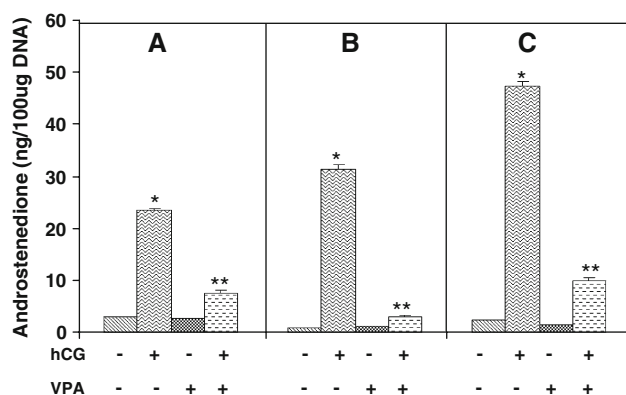


**Fig. 1** Effect of VPA on androstenedione production by T-I cells: dose-response. T-I cells were treated with different concentrations of VPA (0, 100, 250, and 500  $\mu$ g/ml) and HDL (50  $\mu$ g/ml), as a provider of cholesterol for androgen production, in the presence or absence of hCG (100 ng/ml) for 36 h. After incubation, cells and media were collected and androstenedione was measured by RIA. Values are mean  $\pm$  SEM of triplicate cultures. \*  $P < 0.01$  vs. control, \*\*  $P < 0.01$  vs. hCG alone, \*\*\*  $P < 0.001$  vs. hCG plus HDL

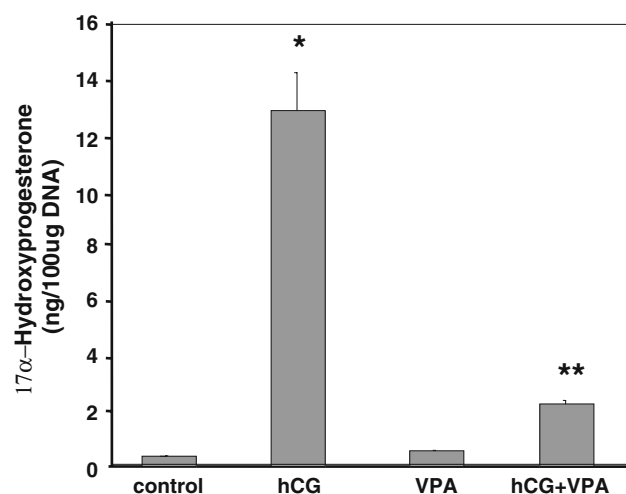
dose of 250  $\mu$ g/ml, there was a decrease from  $89.6 \pm 0.3$  ng/100  $\mu$ g DNA to  $11.5 \pm 2.0$  ng/100  $\mu$ g DNA, and at a dose of 500  $\mu$ g/ml, androstenedione production dropped to  $2.6 \pm 0.1$  ng/100  $\mu$ g DNA, essentially reducing androstenedione production to near basal levels. These results show that the addition of VPA to primary cultures of rat T-I cells caused a marked concentration-dependent inhibitory effect on hCG-stimulated androstenedione synthesis. Addition of VPA to cells produced no inhibitory effect on cell number since DNA content in control and VPA-treated cultures was essentially similar (7.65  $\mu$ g of DNA/ $1 \times 10^6$  cells). As shown in Fig. 2a–c, the inhibitory effect of VPA on the hCG-stimulated androgen production was observed as late as 72 h after the addition of VPA. Treatment of T-I cells with hCG, as expected, resulted in a marked increase in the production of  $17\alpha$ -OHP, the precursor of androstenedione. Addition of VPA to these cultures produced inhibition of the stimulatory effect of hCG on  $17\alpha$ -OHP (Fig. 3). VPA addition also inhibited testosterone production (data not presented) suggesting that the inhibition of hCG-stimulated androstenedione production by VPA was not a result of its increased conversion to testosterone.

### Effect of VPA on 8-bromo-cAMP-stimulated androstenedione production

Since the cellular effects of LH/hCG are exerted after its interaction with the receptor followed by the activation of

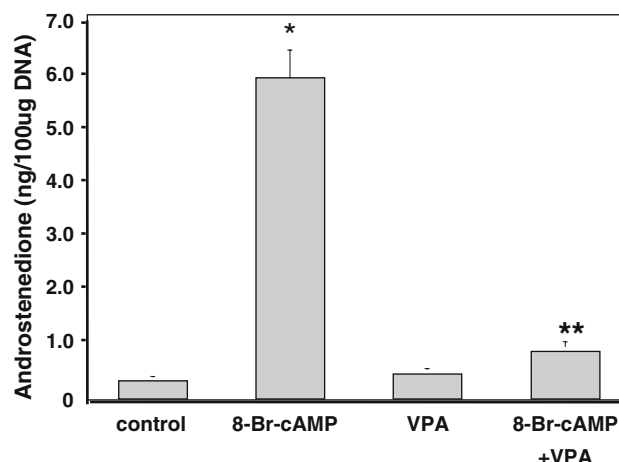


**Fig. 2** Duration of VPA's effect on androstenedione production by T-I cells. T-I cells were treated with VPA (500  $\mu$ g/ml) for 36 h (a), 48 h (b), and 72 h (c) in the presence or absence of hCG (100 ng/ml). After incubation, cells and media were collected and androstenedione was measured by RIA. Values are mean  $\pm$  SEM of triplicate cultures. For (a), (b), and (c), \*  $P < 0.001$  vs. control, \*\*  $P < 0.001$  vs. hCG alone



**Fig. 3** Effect of VPA on the production of 17 OHP by T-I cells. T-I cells were treated with VPA (500  $\mu$ g/ml) for 36 h in the presence or absence of hCG (100 ng/ml). After incubation, cells and media were collected and 17 OHP was measured by RIA. Values are mean  $\pm$  SEM of triplicate cultures. \*  $P < 0.001$  vs. control, \*\*  $P < 0.001$  vs. hCG alone

cyclic AMP production [14, 15], the present studies also examined whether the effects of VPA occur prior to or after cyclic AMP production. As expected, treatment of T-I cells with 8-Bromo-cAMP resulted in a marked increase in the production of androstenedione, and VPA caused a marked inhibition of this stimulatory effect (Fig. 4), suggesting that the site of VPA's inhibitory effect on androstenedione production lies at a step after cyclic AMP formation. However, an additional site of action of VPA on steroidogenesis prior to cAMP production can not be ruled out.



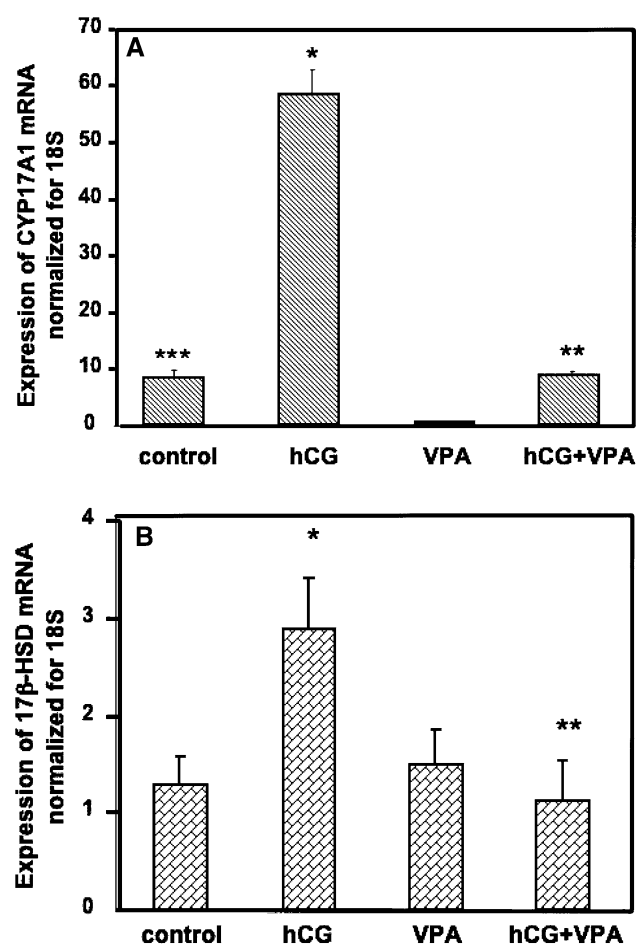
**Fig. 4** The effect of VPA on 8-Br-cAMP-induced androstenedione production. T-I cells were treated with VPA (250  $\mu$ g/ml) for 36 h in the presence or absence of 8-Br-cAMP (1 mM). After incubation, cells and media were collected and androstenedione was measured by RIA. Values are mean  $\pm$  SEM of triplicate cultures. \*  $P < 0.001$  vs. control, \*\*  $P < 0.001$  vs. 8Br-cAMP alone

#### Effect of VPA on steroidogenic enzymes

The direct effect of VPA on the expression of key enzymes involved in androgen production was then examined. Treatment with hCG resulted in a sevenfold increase in the expression of CYP17A1 mRNA from a basal level of  $8.4 \pm 0.1$  to  $58.4 \pm 4.7$ . The addition of VPA completely inhibited the stimulatory effect of hCG, reducing the expression of CYP17A1 mRNA to basal level of  $8.8 \pm 0.7$  (Fig. 5a). Expression of 17 $\beta$ -HSD mRNA was also affected in a similar manner, although the change was not as pronounced. HCG treatment resulted in a twofold increase in the expression of 17 $\beta$ -HSD mRNA, from basal level of  $1.3 \pm 0.2$  to  $2.9 \pm 0.5$ . Addition of VPA again eliminated hCG's stimulatory effect, reducing the expression of 17 $\beta$ -HSD mRNA to basal level of  $1.13 \pm 0.4$  (Fig. 5b). These results suggest that VPA inhibited the ability of hCG to stimulate the expression of key enzymes in androgen production.

#### Discussion

Although overrepresentation of PCOS in women with epilepsy [3, 4] and a higher prevalence of PCOS in those treated with VPA has been reported [5, 16], whether the increased risk for this PCOS-like state in women with epilepsy is due to the underlying disease or due to the antiepileptic treatment continues to be controversial. The present study shows that the addition of VPA to primary cultures of T-I cells does not increase basal or hCG-stimulated androgen biosynthesis. Instead, VPA produced a marked concentration-related inhibitory effect on androgen



**Fig. 5** Effect of VPA on CYP17A1 and 17 $\beta$  HSD mRNA expression in T-I cells. T-I cells were treated with VPA (500  $\mu$ g/ml) for 36 h in the presence or absence of hCG (100 ng/ml). After incubation, total RNA was isolated and reverse transcribed. After the reverse transcription, CYP17A1 (A) and 17 $\beta$ HSD (B) mRNA expression was measured by real time PCR. The mRNA expression was normalized for 18S rRNA. Values are mean  $\pm$  SEM of triplicate cultures. **a** \*  $P < 0.001$  vs. control, \*\*  $P < 0.001$  vs. hCG alone; \*\*\*  $P < 0.01$  vs. VPA alone. **b** \*  $P < 0.01$  vs. control, \*\*  $P < 0.01$  vs. hCG alone

biosynthesis. Measurement of various steroids, including 17 $\alpha$ -OHP, androstenedione, and testosterone as well as mRNA expression of steroidogenic enzymes shows that VPA exerts a marked inhibitory effect on hCG-stimulated androgen synthesis in rat T-I cells. The study also demonstrates that the site of VPA's inhibitory effect on androgen synthesis likely occurs at a step after cyclic AMP production although a second site of action prior to cyclic AMP formation can not be ruled out.

Previously published studies examining the effect of VPA on androgen production using different in vivo and in vitro models have yielded conflicting results. Several in vivo studies failed to induce PCOS-related alterations in non-epileptic animal models; for instance, treatment of female rats in vivo with VPA failed to significantly modify

their estrous cycle [17]. VPA treatment in rats resulted only in the formation of cystic appearing ovaries and weight gain with normal androgen production [2]. Similarly, long-term VPA treatment of normally cycling Rhesus monkeys had no effect on androgen synthesis, glucose tolerance, lipid profile, menstrual cycles, or ovarian morphology [12]. Furthermore, AED, including VPA, did not influence human 3 $\beta$ -HSD or CYP17A1 activities at concentrations normally used in AED therapy [11].

In contrast to the above studies, VPA treatment has been reported to induce alterations in human theca cell gene expression in vitro [7] and to potentiate androgen biosynthesis in human ovarian theca cells by enhancing CYP11A and CYP17A1 gene transcription [8]. The stimulatory effect of VPA on androgen production and steroidogenic enzymes reported in these two studies and the inhibitory effects seen in our present studies could perhaps be explained on the basis of the differences in the model systems used. While our studies employed primary cultures of rat T-I cells, the stimulatory effects were seen in propagated human T-I cells [7, 8] although the precise mechanistic basis for this difference is not known at the present time. VPA treatment has been reported to increase progesterone and testosterone production, and decreased estradiol production has been reported in porcine follicular cells in vitro [18]. In an in vivo model, rats with long-term VPA treatment have been reported to produce a pronounced reduction in estrogen levels and a marked increase in the testosterone to estrogen ratio [13].

It is possible that the discrepancies between the inhibitory effects of VPA on androgen production, described in this study and other previous reports [18, 19] indicating a stimulatory effect of VPA on androgen production are due to differences between in vitro and in vivo systems. The in vitro data are obtained in precisely defined conditions whereas the in vivo results are likely to be compounded by extraneous factors. For instance, it has been shown that pilocarpine induction of epilepsy in rats leads to specific increases of testosterone, among other ovarian steroids that remain unaffected [20], and that the frequency of LH pulses is increased in epilepsy [21]. Additionally, it has been reported that VPA treatment of epileptic patients results in increased blood levels of insulin, possibly due to lower metabolism of insulin [22]. Thus, it is possible that in patients or in whole animal systems, epilepsy can trigger an increase in testosterone production by the combined actions of LH and insulin-stimulated androgen production. This effect may in turn, obscure the direct inhibitory effects of VPA on T-I cell production of androgens. Our study was not carried out using a PCOS model, but rather it merely shows that in isolated theca-interstitial cells VPA exerts an inhibitory effect on androgen production and on the expression of selected steroidogenic enzymes.

Another possible explanations for the differences in the effect of VPA on androgen biosynthesis in different study paradigms may be due to the differences in human and rodent steroid biosynthesis, as adult rodents do not express CYP17A1 in their adrenals [23, 24]. However, the absence of CYP17A1 in the adrenal does not fully explain the discrepancy noted in ovarian androgen synthesis in the two species as well as other non human primates such as the Rhesus monkey. Another possible explanation may be the difference between *in vivo* and *in vitro* conditions. For example, insulin resistance, one of the main features of PCOS patients, is described only in obese but not in lean VPA-treated epileptic females [25], suggesting that the reason for the higher incidence of PCOS in VPA users may be complicated by the involvement of several factors acting simultaneously to cause this reproductive-endocrine disorder, including the underlying disease itself. The present study does not support a role for VPA in induction of hyperandrogenism, at least in the rat model.

## Materials and methods

### Reagents

Purified hCG (CR-127) was purchased from National Hormone and Pituitary Agency (Torrance, CA). TRIzol reagent was obtained from Life Technologies, Inc. (Gaithersburg, MD). Collagenase (CLS I; 260 U/mg) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Reagents for androstenedione and 17-OH progesterone RIA were obtained from Diagnostic Systems Laboratories, Inc. (Webster, TX). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Medium 199, McCoy's 5A medium, L-glutamine, HEPES buffer, Nystatin and Gentamycin were purchased from Invitrogen/GIBCO (Carlsbad, CA). Penicillin–Streptomycin was purchased from Roche Diagnostics (Indianapolis, IN). Valproic acid sodium salt and 8-Bromoadenosine-3', 5'-cyclic monophosphate, sodium salt (8-Br-cAMP) were obtained from Sigma (St. Louis, MO). ADVIA Centaur Testosterone assay reagent pack was obtained from Siemens Medical Solutions Diagnosis (Los Angeles, CA). TaqMan Reverse Transcription Reagents, Universal PCR Master Mix Reagents, and TaqMan primers and probes were obtained from Applied Biosystems (Foster City, CA). All other reagents used were of analytical grade.

### Animals

Sprague–Dawley female rats (25-day old) were purchased from Harlan, Inc. (Indianapolis, IN) and were used without

further treatment. The animals were killed by CO<sub>2</sub> asphyxiation. The ovaries were removed under sterile conditions and were processed immediately for the isolation of theca-interstitial cells. These procedures were approved by the institutional animal care and use committee at the University of Michigan (UCUCA).

### Isolation and culture of theca-interstitial (T-I) cells

The T-I cells were isolated, dispersed and cultured following a previously published protocol [15, 26]. Briefly, freshly collected ovaries were placed in 100 mm plates containing warm (37°C) Medium 199 containing 25 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mg/ml BSA, 100 U/ml penicillin, and 100 µg/ml streptomycin. A dissecting microscope was used to observe ovaries as they were cleaned of adhering fat and actively punctured with a 27G needle to release granulosa cells. The ovaries then were washed three times with medium to release remaining granulosa cells. The tissues then were minced and incubated for 30 min at 37°C in the same medium, supplemented with 0.65 mg/ml collagenase type 1 plus 10 µg/ml deoxyribonuclease. The dispersion was encouraged by mechanically pipetting the ovarian tissue suspension with a 10 ml pipette. The T-I cells released by this digestion were centrifuged at 250×g for 5 min and washed in collagenase-free medium two times to eliminate remaining collagenase. The dispersed cells were then resuspended in McCoy's 5A medium containing 2 mM L-glutamine, 1 mg/ml BSA, 100 U/ml penicillin, and 100 µg/ml streptomycin and subjected to a 5-min unit gravity sedimentation to eliminate small fragments of undispersed ovarian tissue. Cell viability was assessed by trypan blue exclusion and was always above 90%. The cells were plated at a density of  $1 \times 10^6$  in 6-well plates for radioimmunoassay studies and  $3 \times 10^6$  in 6-cm dishes for real time PCR. The plated cells were maintained overnight in McCoy's 5A medium containing 2 mM L-glutamine, 1 mg/ml BSA, 100 U/ml penicillin, and 100 µg/ml streptomycin, in a humidified atmosphere of 95% air-5% CO<sub>2</sub> at 37°C. T-I cell purity, averaging 95%, was determined by histochemical staining for 3-beta-hydroxysteroid dehydrogenase [27].

### Treatment of T-I cells

In order to determine the effect of VPA, cells were cultured for different time periods with different doses of VPA in the absence or presence of hCG (100 ng/ml) or 8-Bromo-cAMP (1 mM) as indicated under each experiment. All additions were made simultaneously. At the end of the treatment periods, the cells were processed as follows.



### Measurement of androgen production by radioimmunoassay (RIA)

After incubation, the cells and media were collected from each dish, kept in a water bath at 75°C for 20 min to lyse the cells. Trace amounts of [<sup>3</sup>H] androstendione were added to the lysate to monitor recovery and samples were extracted with five volumes of ether two times. The extracts were dried down with N<sub>2</sub>, and the residues were reconstituted in 0.1% gelatin/PBS. Androstendione and 17 $\alpha$ -hydroxyprogesterone (17 $\alpha$ -OHP) were measured by radioimmunoassay using the manufacturer's protocols (Diagnostic System Laboratories, Webster, Texas). The results were normalized to the cellular DNA content in each well and expressed as ng/100  $\mu$ g DNA.

### Measurement of testosterone production

After incubation, the cells and media were collected from each dish, kept in a water bath at 75°C for 20 min to lyse the cells. After centrifugation, testosterone in the supernatant was measured, using ADVIA Centaur Assay system (Siemens Medical Solutions Diagnostics).

### RNA isolation and real time PCR for the expression of CYP17A1 and 17 $\beta$ -hydroxysteroid dehydrogenase 1 (17 $\beta$ -HSD)

Total RNA was isolated using the TRIzol reagent, following the manufacturer's protocol (Applied Biosystems). Total RNA (250 ng) was reverse transcribed in a volume of 50  $\mu$ l using 125 pmol random hexamer, 500  $\mu$ M deoxynucleoside triphosphate mix, and 62.5 U Multiscribe reverse transcriptase. After the reverse transcription, the real time PCR reaction was carried out using pre-designed primers and probes for CYP17 and 17 $\beta$ -HSD. The cDNAs were diluted to 250  $\mu$ l with ribonuclease free water. Each real time PCR consisted of 5  $\mu$ l cDNA template, 12.5  $\mu$ l 2 $\times$  Taqman Universal PCR Master Mix, and 10 pmol forward and reverse primers in a final volume of 25  $\mu$ l. Reactions were carried out on an ABI PRISM 7300 sequence detection system (Applied Biosystems) for 40 cycles (95°C for 15 s, 60°C for 1 min) after initial 10 min incubation at 95°C. We have followed the protocol provided in the Applied Biosystems 7300 Real Time PCR System Manual for absolute quantitation using the standard curve method. Briefly, known amounts of cDNA were used to amplify a standard curve for CYP17A1 and one for 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) as well as for the internal control (18 S). After analyzing the data, the quantity value of each steroidogenic gene obtained from its standard curve was normalized with the quantity value of

the 18 S for the same sample, obtained from the 18 S standard curve.

### Miscellaneous techniques

Cellular DNA content was determined colorimetrically [28].

### Statistical analysis

Values represent means  $\pm$  SEM. Statistical analysis was carried out using ANOVA. If ANOVA indicated significant differences within the data sets, comparisons were made using unpaired Student's *t* test. Each experiment was repeated at least three times, with comparable results. Significance was accepted as *P* < 0.05.

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