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Valproic acid alters mitochondrial cholesterol transport in Y1 adrenocortical cells

L. Brion, A. Gorostizaga, N.V. Gómez, E.J. Podestá, F. Cornejo Maciel, C. Paz *

IIHMNO – Dept. of Biochemistry, School of Medicine, University of Buenos Aires, Paraguay 2155, 5th C1121ABG, Buenos Aires, Argentina

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ABSTRACT

Several reports suggest putative interactions between valproic acid (VPA) treatment and the hypothalamus–pituitary–adrenal axis. Given that VPA alters mitochondrial functions, an action of this drug on a mitochondrial process such as steroid synthesis in adrenal cells should be expected. In order to disclose a putative action of VPA on the adrenocortical cell itself we evaluated VPA effects on regulatory steps of the acute stimulation of steroidogenesis in Y1 adrenocortical cells. This study demonstrates that VPA increases progesterone production in non-stimulated cells without inducing the levels of *St*eroidogenic *A*cute *R*egulatory (StAR) protein, which facilitates cholesterol transport. This result suggests that VPA increases mitochondrial cholesterol transport through a StAR-independent mechanism and is further supported by the fact that in isolated mitochondria VPA stimulates exogenous cholesterol metabolization to progesterone. VPA also reduces the cAMP-mediated increase of the StAR protein, mRNA levels, promoter activity and progesterone production. In summary, the present data show that VPA can alter steroid production in adrenal cells by a complex mechanism that mainly involves an action on cholesterol access to the inner mitochondrial membrane. The VPA-mediated increase of basal steroidogenesis could be linked to the increase of basal cortisolemia described in patients under VPA treatment.

1. Introduction

Valproic acid (VPA), a short chain branched fatty acid, is an antiepileptic drug frequently used in the treatment of focal and generalized seizures (Gurvich and Klein, 2002). Even though the molecular basis of its pharmacological action remains partially elusive, VPA is known to inhibit histone deacetylases (Gottlicher, 2004), promote ERK activation (Yuan et al., 2001) and induce cell membrane disorder (Perlman and Goldstein, 1984). One or more of these actions could be involved not only in its therapeutic properties but also in its side effects.

Several studies associate long-term treatment with VPA to metabolic syndrome (Verrotti et al., 2009) and other endocrine abnormalities (Bilo and Meo, 2008; Genton et al., 2001; Pylvanen et al., 2006; Rasgon et al., 2005; Roste et al., 2001; Sveberg Roste et al., 2001). *In vitro* studies using ovarian cells demonstrate that VPA potentiates androgen biosynthesis in porcine follicular and human ovarian theca cells (Nelson-DeGrave et al., 2004; Tauboll et al., 2002). Other reports also suggest the existence of interactions between VPA treatment and the hypothalamus–pituitary–adrenal axis. For example, a decrease of corticotropin-releasing factor was reported in specific areas of hypothalamus of rats under subchronic VPA treatment (Stout et al., 2001). Also, it was demonstrated that chronic administration of valproate blunts stimulated ACTH secretion in epileptic patients (Invitti et al., 1988) and that long-term VPA treatment increased basal serum cortisol in epileptic children (Aydin et al., 2005). Whether any of these results are due to a particular VPA action on adrenocortical cells deserves further investigation.

Since mitochondrial dysfunction has been associated with VPA treatment (Luís et al., 2007; Tong et al., 2005), all those cellular processes that require mitochondrial integrity could also be affected by this drug. In all steroidogenic systems, cholesterol transport across the mitochondrial membranes is the rate-limiting step in steroidogenesis (Crivello and Jefcoate, 1980). *St*eroidogenic *A*cute *R*egulatory protein (StAR) (Clark et al., 1994; Stocco and Clark, 1996) facilitates cholesterol access to the inner mitochondrial membrane where the lipid is metabolized to pregnenolone by the action of the cytochrome P450scc (cholesterol side-chain cleavage enzyme). Regarding StAR, steroidogenic hormones regulate steroid synthesis promoting the induction and activation of this protein (Bose et al., 2008; Miller, 2007; Stocco and Clark, 1996; Sugawara et al., 1997). StAR gene induction involves the hormone-dependent regulation of several transcription factors, as SF1 and Nur77 (Martin and Tremblay, 2009), many of which also participate in the expression of steroidogenic enzymes (Parker and Schimmer, 1997; Sugawara et al., 1997).

Several studies describe the effect of VPA on steroidogenesis in porcine and human ovarian cells (Nelson-DeGrave et al., 2004; Rattya et al., 2001; Tauboll et al., 2006, 2002). Regarding the action of this drug on adrenocortical cells, Gustavsen and col. recently

* Corresponding author. Tel.: +54 11 45083672x30; fax: +54 11 45083672x31.
E-mail address: crispaz@fmed.uba.ar (C. Paz).

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described that VPA exposure modifies both the steroid secretion profile and the expression of genes encoding for steroidogenic enzymes in H295R, a human carcinoma cell line (Gustavsen et al., 2009). In the present work, mitochondrial transport and metabolization of cholesterol as well as the expression of StAR were evaluated in Y1 adrenocortical cells exposed to VPA. Our findings indicate that VPA facilitates cholesterol transport into the mitochondria and reduces hormonal action on StAR protein induction. The resulting effect on steroidogenesis is an increase in basal production and a decrease under stimulated conditions.

2. Materials and methods

2.1. Cell cultures

Murine Y1 adrenocortical tumor cells are widely used to study the acute regulation of steroidogenesis and produce progesterone (P4) as major steroid. The cell line, generously provided by Dr. Bernard Schimmer (University of Toronto, Toronto, Canada) was maintained as indicated (Schimmer, 1979).

Cells were plated at a density of 450,000 cells/well in dishes of six wells. Seventy-two hours after plating, the medium was replaced by fresh serum-free medium and cell cultures were pre-incubated in the presence or absence of VPA at different concentrations in a final volume of 0.5 ml/well. After 2 h, the cells were treated with or without ACTH, 8Br-cAMP, pregnenolone or 22R(OH)-cholesterol for 6 h. Following treatments, the cells were processed in order to analyze cellular proteins and mRNA. The media were kept to quantitate P4 concentration by radioimmunoanalysis (Cornejo Maciel et al., 2005). The number of viable cells, determined by evaluation of trypan blue exclusion, remained unchanged after all treatments.

2.2. Western blot analysis

Western blot was performed in order to analyze the levels of StAR protein in Y1 cell lysates. For this purpose, the cultures were scrapped in buffer containing 30 mM Tris pH 7.4, 0.5% Triton X-100, 5 mM $MgCl_2$, 5 mM EGTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin and 2 mM sodium orthovanadate. The suspension was centrifuged at 10,000*g* at 4 °C for 10 min. Pellet was discarded and supernatant (total lysates) was subjected to Western blot analysis. Thirty micrograms of proteins of the total lysates were separated by SDS–PAGE (Laemmli, 1970) and electrotransferred to polyvinylidene difluoride membranes (Towbin et al., 1979). StAR protein was detected using a specific antibody (kindly provided by Dr. Douglas Stocco, Texas Tech University) and an enhanced chemiluminescence commercial kit. Detection of beta tubulin was used as loading control.

2.3. Northern blot

Total RNA was isolated from Y1 cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples (20 μg/lane) were analyzed by Northern blot, using StAR and 18S rRNA probes labeled by random priming. Other details are described in previous publications (Bey et al., 2003; Paz et al., 2002).

2.4. Transient transfection and luciferase assay

Y1 cells were transiently transfected using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions using 0.4 μg StAR promoter construct fused to the *Firefly* luciferase reporter gene and 40 ng pRL *Renilla* luciferase expression vector (control for transfection efficiency). The next day cells were washed and pre-incubated in the absence or presence of VPA for 2 h in serum-free medium, under basal conditions or under stimulation with 8Br-cAMP (0.3 mM) for 24 h. After treatment, luciferase activity in cell lysates was measured using the Dual Luciferase Assay System (Promega).

2.5. Progesterone production by isolated mitochondria

Mitochondrial fraction was obtained as previously described (Neher et al., 1982). A cell-free assay was performed mixing mitochondrial fraction with microsomal fraction and stimulation of mitochondrial steroidogenesis was performed as previously described (Neher et al., 1982). Mitochondria were incubated with or without VPA in the presence of 50 μM of cholesterol as substrate for 20 min at 30 °C (Poderoso et al., 2008). P4 concentration in the incubation media was determined by specific radioimmunoassay (Cornejo Maciel et al., 2005).

2.6. Statistics

Results are shown as the mean ± SD. Each experiment was performed at least three times under identical conditions. Unless otherwise indicated, results were analyzed separately and a representative experiment is shown in the figures. Statistical significance was evaluated using ANOVA followed by Tukey test. $P < 0.05$ was considered significant.

3. Results

In order to disclose a putative action of VPA on the adrenocortical cell itself we evaluated the effect of VPA on Y1 adrenocortical cell function. We evaluated the amount of steroids produced by Y1 adrenocortical cells exposed to 0.5 and 1 mM of VPA. These concentrations are similar to those that produce changes in ovarian steroidogenesis (Nelson-DeGrave et al., 2004; Tauboll et al., 2002). The incubation with the drug (8 h) significantly increased P4 synthesis by non-stimulated cells (Fig. 1). P4 production stimulated by a submaximal concentration of ACTH (5 mIU/ml) for 6 h

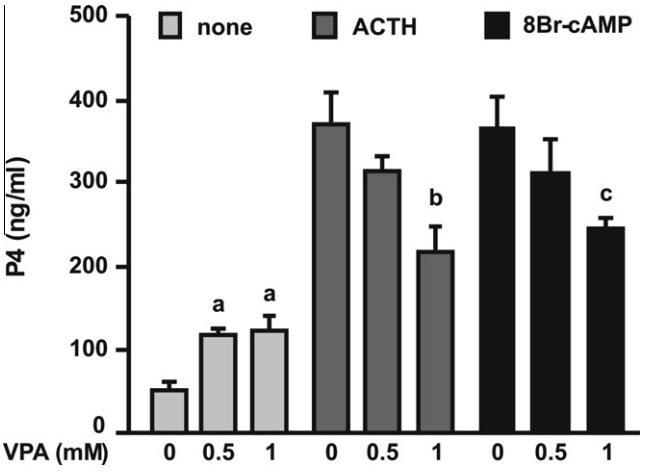

Fig. 1. Effect of VPA on steroid production by Y1 cells. Y1 cells were pre-incubated with or without 0.5 or 1 mM of VPA for 2 h and then incubated with or without ACTH (5 mIU/ml) or 8Br-cAMP (0.3 mM) for 6 h. Progesterone (P4) concentration was determined in the incubation media by radioimmunoassay and expressed in ng progesterone/ml incubation media. Each data point represents the means ± SD of a representative experiment, performed in triplicates and repeated three times. (a) $P < 0.001$ vs. non-stimulated cells incubated in the absence of VPA; (b) $P < 0.001$ vs. cell stimulated with ACTH in the absence of VPA; (c) $P < 0.001$ vs. cells stimulated with 8Br-cAMP in the absence of VPA.

was inhibited by 1 mM of VPA (added to the cell culture 2 h before the addition of the stimulus) but was not inhibited by 0.5 mM of VPA (Fig. 1). We also tested the effect of VPA on steroidogenesis in Y1 cells stimulated by an analogue of cAMP (8Br-cAMP). Again, 1 mM of VPA reduced the stimulatory effect of a submaximal concentration of 8Br-cAMP (0.3 mM) while a lower VPA concentration was unable to modify the cAMP-stimulated steroid synthesis (Fig. 1).

In order to analyze whether VPA-promoted increase of basal steroidogenesis could be due to an effect of the drug on mitochondria, we evaluated VPA effects on the capability of isolated mitochondria from non-stimulated cells to produce P4 from exogenously added cholesterol. VPA is able to induce the production of P4 in isolated mitochondria. Indeed, we detected a higher amount of P4 in those media corresponding to mitochondria incubated with VPA (Fig. 2), which evidences higher cholesterol access to the site of action of the P450scc complex.

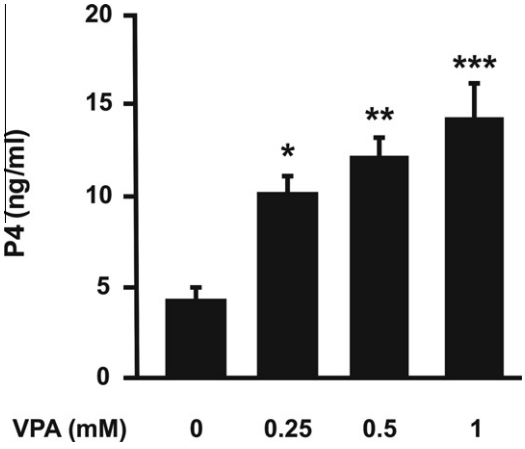

Fig. 2. Effect of VPA on P4 production by isolated mitochondria. Isolated mitochondria of Y1 adrenocortical cells were incubated in the presence of cholesterol (50 μM) together with the indicated concentrations of VPA for 20 min at 30 °C. Progesterone (P4) concentration was determined in the incubation media by radioimmunoassay and expressed in ng progesterone/ml incubation media. Each data point represents the mean ± SD of a representative experiment, performed in triplicates and repeated three times. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. mitochondria incubated in the absence of VPA.

The following experiments were performed to test whether VPA has also an effect on the conversion of cholesterol into pregnenolone (P5) and/or P5 into P4. For this, Y1 adrenocortical cells were incubated with a permeable analogue of cholesterol, 22R(OH)-cholesterol, or with P5, and with or without VPA. The results are shown in Fig. 3. VPA treatment did not modify the amount of P4 produced from 22R(OH)-cholesterol (Fig. 3, panel A) or P5 (Fig. 3, panel B). In contrast, well characterized inhibitors of P450scc or 3β-hydroxysteroid dehydrogenase (3β-HSD) such as hydrogen peroxide (H_2O_2) (Tsai et al., 2003) and estradiol (E2) (Byrne et al., 1986) respectively, were able to reduce P4 production (Fig. 3). Together, these results suggest that the activities of P450scc and/or 3β-HSD enzymes are not a target of VPA action in our experimental conditions. These results led us to conclude that VPA increases cholesterol transport across the mitochondrial membrane, leading to the increase in basal P4 production.

Next, we focused in the study of the site of action of VPA on the inhibition of ACTH- or 8Br-cAMP-stimulated P4 production. Since neither P450scc nor 3β-HSD are affected by VPA treatment, a putative target of VPA action could be the expression of StAR protein. To test this hypothesis, StAR protein levels were evaluated in Y1 adrenocortical cells incubated in the presence or absence of VPA. The exposure to the drug (0.5 and 1.0 mM) reduced the action of 8Br-cAMP on the levels of StAR protein (Fig. 4). This effect is due, at least in part, to an action of VPA on StAR gene expression, since VPA reduced StAR mRNA levels (Fig. 5, panel A) and also StAR promoter activity (Fig. 5, panel B). Thus, this study strongly demonstrates that VPA impairs StAR expression, leading to the decrease of stimulated-P4 production.

4. Discussion

In this study we used Y1 adrenocortical cells to determine whether VPA acts directly on this kind of cells since there are reports that indicate that this drug alters gonadal steroid production and the hypothalamus–pituitary–adrenal axis. Indeed, here we highlight the stimulatory action of VPA (0.5 and 1 mM) on basal adrenocortical steroidogenesis. This effect involves a direct action on cholesterol transport across the mitochondrial membrane without the intervention of StAR protein. Besides, this study establishes that VPA reduces ACTH/cAMP-dependent StAR expression.

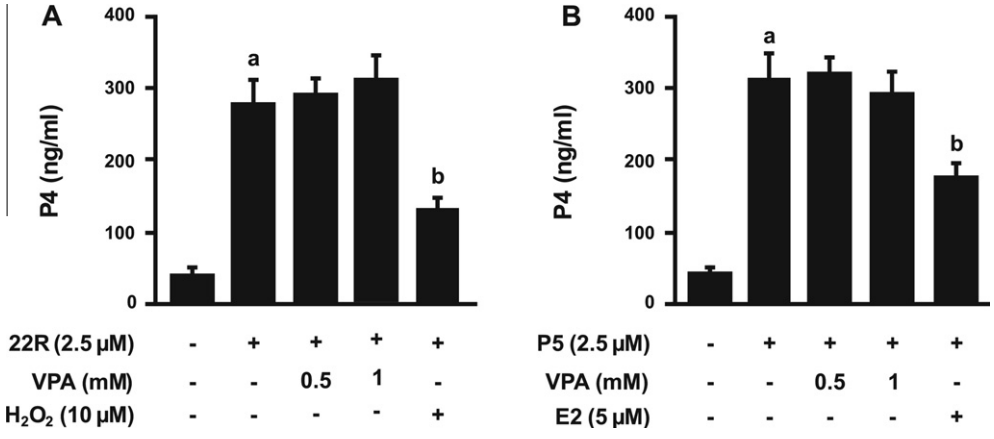

Fig. 3. Effect of VPA on P4 production sustained by 22R(OH)-cholesterol and pregnenolone. Y1 cells were pre-incubated in the absence or in the presence of the indicated concentrations of VPA for 2 h and then incubated with 22R(OH)-cholesterol (22R, 2.5 μM) (panel A) or with pregnenolone (P5, 2.5 μM) (panel B) for 6 h, with or without H_2O_2 (10 μM) or estradiol (E2, 5 μM) as indicated in the panels. Progesterone (P4) concentration was determined in the incubation media by radioimmunoassay. Each data point represents the mean ± SD of a representative experiment, performed in triplicates and repeated three times. Panel A: (a) $P < 0.001$ vs. cells incubated in the absence of 22R(OH)-cholesterol and VPA; (b) $P < 0.01$ vs. cells incubated with 22R(OH)-cholesterol in the absence of VPA. Panel B: (a) $P < 0.001$ vs. cells incubated in the absence of P5 and VPA; (b) $P < 0.05$ vs. cells incubated with P5 in the absence of VPA.

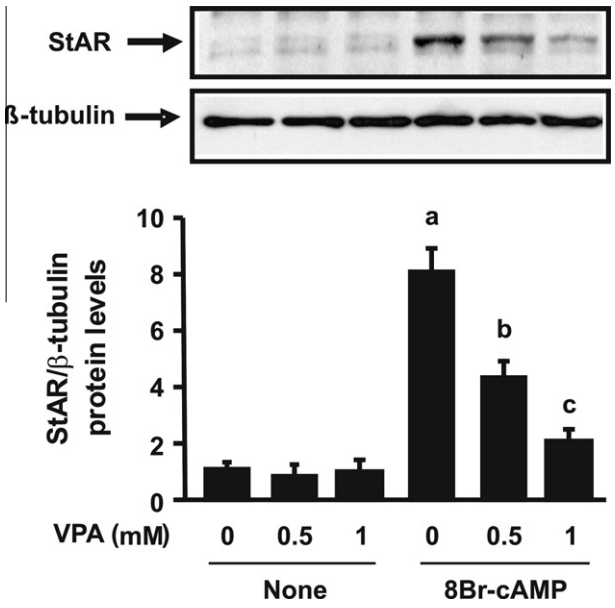

Fig. 4. Effect of VPA on StAR protein levels. Y1 cells were pre-incubated in the absence or in the presence of VPA (0.5 or 1 mM) for 2 h and then incubated with or without 8Br-cAMP (0.3 mM) for 6 h. StAR protein levels were analyzed by Western blot. The intensity of the specific bands was quantified and normalized against the loading controls (β-tubulin) and expressed in arbitrary units. The experiments were repeated three times and the figure shows a representative Western blot (above) and the quantitative representation of the data (means ± SD, $n = 3$) (below). (a) $P < 0.001$ vs. non-stimulated cells incubated in the absence of VPA; (b) $P < 0.01$ and (c) $P < 0.001$ vs. 8Br-cAMP-stimulated cells incubated in the absence of VPA.

VPA, a short-chain fatty acid, is a membrane-disordering agent (Perlman and Goldstein, 1984) able to transversally diffuse across (flip-flop) lipid bilayers (Kessel et al., 2001). Thus, it is possible that these properties of VPA are linked to its ability to modify the diffusion of diverse molecules across the lipid bilayer. Our results showing an increased accessibility of cholesterol to the P450scc complex in isolated mitochondria in the presence of VPA (Fig. 2) clearly show that the drug contributes to cholesterol transport across the inner mitochondrial membrane by a StAR-independent mechanism. In this regard, the action of fatty acids on cholesterol transport has been described elsewhere (Artemenko and Jefcoate, 2004; Castillo et al., 2006; Duarte et al., 2007). Particularly, we described the direct stimulatory action of arachidonic acid on cholesterol transport in steroidogenic cells (Castillo et al., 2006; Duarte et al., 2007).

Regarding the inhibitory action of VPA on stimulated steroidogenesis, this effect is exerted by the reduction of ACTH or cAMP action on StAR protein expression. Even when only 1 mM of VPA reduces the amount of steroids produced by stimulated cells (Fig. 1), VPA at both tested concentrations diminishes the stimulatory capacity of 8Br-cAMP on StAR expression in Y1 adrenocortical cells (Figs. 4 and 5). Our hypothesis is that stimulated steroid production is not inhibited by 0.5 mM of VPA even when StAR protein levels are reduced due to the direct stimulatory action of the fatty acid on mitochondrial cholesterol transport.

It is worth to mention that Nelson-DeGrave and col. did not find modifications of StAR mRNA or protein levels after 24 h of VPA treatment (0.5 mM) of ovarian theca cells stimulated with a maximal concentration of forskolin (Nelson-DeGrave et al., 2004). Additionally, this treatment causes an increase in the levels of P450c17 and P450scc proteins and in their corresponding mRNAs. Thus, VPA does not produce the same effect on the expression of key steroidogenic proteins in Y1 adrenocortical cells and in ovarian cells. Moreover, in non-stimulated adrenocortical H295R cells (human adrenal carcinoma cell line) VPA treatment down-regulates StAR expression and decreases estradiol levels while it does not affect P4 and testosterone production (Gustavsen et al., 2009). Even

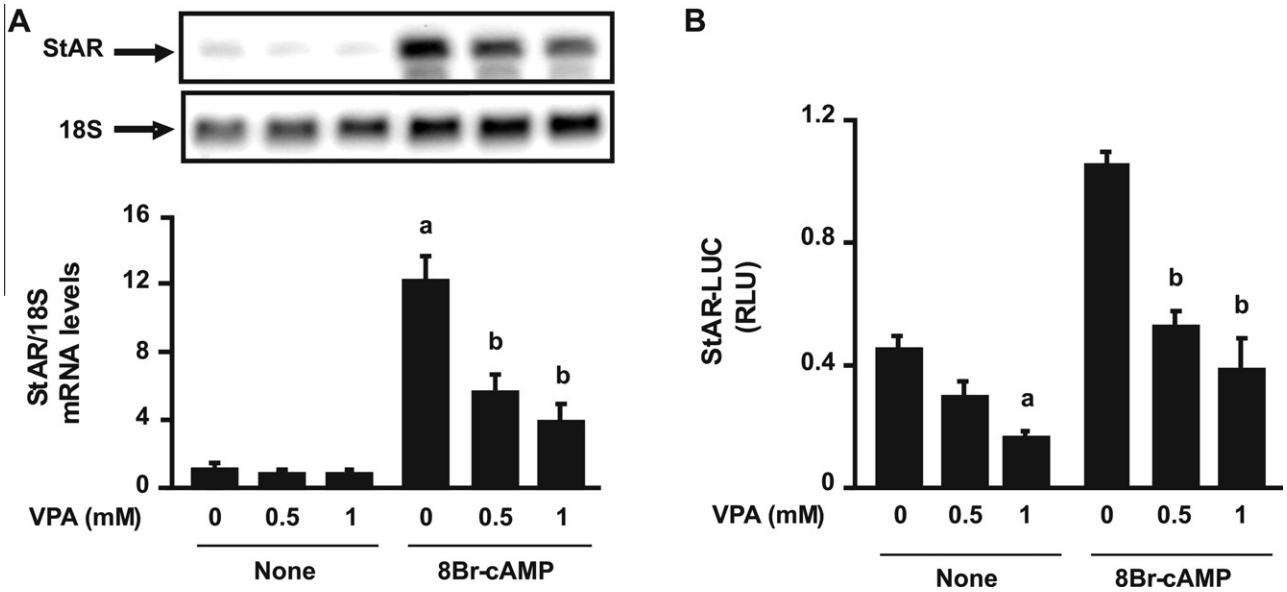

Fig. 5. Effect of VPA on StAR mRNA levels and StAR promoter activity. Panel A: Y1 cells were pre-incubated in the absence or in the presence of VPA (0.5 or 1 mM) for 2 h and then incubated with or without 8Br-cAMP (0.3 mM) for 6 h. StAR mRNA levels were analyzed by Northern blot. The intensity of the specific bands was quantified and normalized against the loading control (18S rRNA) and expressed in arbitrary units. The experiments were repeated three times and the figure shows a representative Northern blot (above) and the quantitative representation of the data (means ± SD, $n = 3$) (below). (a) $P < 0.001$ vs. non-stimulated cells incubated in the absence of VPA; (b) $P < 0.01$ vs. 8Br-cAMP-stimulated cells incubated in the absence of VPA. Panel B: Y1 cells were transiently co-transfected with pGL3 reporter plasmid containing the StAR promoter and pRL *Renilla* luciferase control vector. After 24 h, the cultures were pre-incubated in the absence or in the presence of VPA (0.5 or 1 mM) for 2 h and then incubated with or without 8Br-cAMP (0.3 mM) for 24 h. Firefly luciferase activity was measured in cell extracts and each value was normalized to *Renilla* luciferase activity. Results, expressed in relative light units (RLU), represent the means ± SD of triplicates of one representative experiment. (a) $P < 0.01$ vs. non-stimulated cells incubated in the absence of VPA; (b) $P < 0.001$ vs. 8Br-cAMP-stimulated cells incubated in the absence of VPA.

though these and our results seem to disagree, both studies were performed under different experimental conditions. Moreover, Y1 and H295R cells do not display the same steroid secretion profile (Gazdar et al., 1990; Schimmer, 1979). Thus, the results of both groups are not suitable to be compared.

Acetylation of histones, a process controlled by the concerted action of histone deacetylases (HDACs) and histone acetyltransferases, is a critical regulatory mechanism by which gene expression is regulated (Ellis et al., 2008; Struhl, 1998). In general, increased levels of histone acetylation correlate with transcription activation (Struhl, 1998). Given that VPA inhibits HDACs, VPA treatment could enhance the expression of genes related to steroidogenesis, particularly StAR gene. However, our data demonstrate that the anticonvulsant increases histone acetylation (data not shown) but it does not increase StAR mRNA levels. Moreover, StAR mRNA levels are blunted by this treatment (Fig. 5). Though the elucidation of the mechanism is out of the scope of this study, it is interesting to mention that Chen and col. demonstrated that inhibitors of HDACs, VPA among them, cause an increase in ubiquitination and degradation of the transcription factor SF1 in Y1 adrenocortical cells (Chen et al., 2007). SF1 is necessary for the expression of several proteins related to steroidogenesis such as ACTH receptor, StAR and P450scc. Thus, the mechanism by which VPA disrupts the expression of StAR gene could be related to the described action of this drug on SF1 (Chen et al., 2007).

In summary, this *in vitro* study demonstrates that VPA can alter steroid production in mouse Y1 adrenocortical cells by a mechanism that mainly involves an action on cholesterol access to the inner mitochondrial membrane. It is worth to mention a report that indicates that 6 months of treatment of epileptic children with VPA results in an increase in serum cortisol concentration (Aydin et al., 2005). Whether these clinical findings are related to the direct action of VPA on cholesterol transport described in the present report deserves to be considered. Then, in this context, it could be interesting to include the evaluation of adrenal function in the follow-up of patients under chronic VPA treatment.

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