Original Paper

Dehydroepiandrosterone-Regulated Testosterone Biosynthesis via Activation of the ERK1/2 Signaling Pathway in Primary Rat Leydig Cells

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Key Words

Dehydroepiandrosterone • Testosterone biosynthesis • ERK signaling pathway • Primary Leydig cell

Abstract

Background: Dehydroepiandrosterone decreases with age and this reduction has been shown to be associated with physical health in human. Some studies have suggested that the effects of DHEA are exerted after it is biotransformed into more biologically-active hormones in peripheral target cells. This study investigated the effects of DHEA on the testosterone biosynthesis and possible signaling pathway mechanism underlying these DHEA effects were also explored in primary rat Leydig cells. **Methods:** Primary Leydig cells were treated with DHEA and then detected testosterone content by RIA and steroidogenic enzymes, ERK1/2 signal pathway factors protein expression level by Western blot. **Results:** Incubation of primary Leydig cells with DHEA significantly increased testosterone content and 3 β -HSD and 17 β -HSD protein expression levels, while aromatase protein expression levels were decreased. Compared with the control group, p-ERK1/2 and p-CREB protein levels were significantly increased in DHEA-treated groups. Testosterone content was significantly decreased in the DHEA-treated group pre-incubated with U0126 (p-ERK1/2 inhibitor). Additionally, the rise in p-ERK1/2, 3 β -HSD and 17 β -HSD protein levels induced by DHEA was reversed when cells were pre-incubated with U0126. Interestingly, no significant difference was found in aromatase protein expression level in cells pretreated with U0126. **Conclusion:** These findings demonstrate that (a) exogenous DHEA might preferentially convert to testosterone rather than estradiol due to the up-regulation of 3 β -HSD and 17 β -HSD protein levels and the down-regulation of aromatase protein level in primary Leydig cells, and (b) the action of DHEA is at least partly associated with the elevation of p-ERK1/2 and p-CREB protein levels.

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Introduction

Dehydroepiandrosterone (DHEA) is the major steroid hormone produced by the adrenal zona reticularis. One of the main characteristics of DHEA is its age-dependent pattern of secretion; its production begins during fetal life, reaches the highest serum levels between the age of 20 and 30 years, and then steadily declines at a rate of 2% to 5% per year, resulting in a decline of up to 80% in old age [1, 2]. Significant clinical interest in the decrease in DHEA with age exists because this reduction has been shown to be associated with physical health [3-5]. Outside of the regular pharmaceutical networks in the United States, DHEA is widely commercially available as a non-prescription dietary supplement [6]. Surprisingly, despite extensive studies linking DHEA conversion to more active androgens and estrogens in either peripheral tissues or target cells, the mechanism of action of DHEA remains elusive. Most of DHEA in humans is synthesized by the adrenal glands, while in rodents active DHEA is locally produced in different tissues [7]. Moreover, the circulating DHEA levels in mice and rats are often unmeasurable, which indicated that in rat endogenous DHEA production is negligible [8]. Although endogenous DHEA secretion could be negligible, there many reported that DHEA could convert to activate steroids hormones in rats [9-11].

The exact mechanisms of DHEA action are unknown, some effects may be due its conversion to androgens and estrogens [12]. By acting as the available precursor that is converted to testosterone and estrogens in various peripheral tissues, DHEA plays a critical physiological role in maintaining steroidogenesis. As a steroid hormone precursor, DHEA can be rapidly converted into androstenedione by 3 β -hydroxysteroid dehydrogenase (3 β -HSD) in peripheral target tissues, and then undergo further conversion to testosterone and estradiol by 17 β -hydroxysteroid dehydrogenase (17 β -HSD) and aromatase, respectively [13-16]. *In vitro* studies using osteoblasts (OBs) demonstrated that DHEA can be metabolized to testosterone by 17 β - and 3 β -HSD and further metabolized to DHT by the 5 α -reductase [17]. Our previous study demonstrated that serum concentrations of testosterone, estradiol and aldosterone in rats were markedly increased following treatment with DHEA [18]. Other studies also showed that the contents of androstenedione and testosterone were significantly increased in aged adults following treatment with DHEA [15, 16]. Although DHEA in primates is secreted primarily by the adrenal cortex, whereas little or no DHEA is produced by the adrenal cortex in mice and rats [8], exogenous DHEA can be transformed into active androgens or estrogens in peripheral tissues, which contain various steroidogenic enzymes [15]. In males, ~95% of androgen biosynthesis and secretion occurs in Leydig cells, which are situated in the testicular interstitium. Previous studies have suggested that functional changes in Leydig cells, rather than cell loss, account for the observed reduction in serum testosterone levels [3]. Recent studies from our laboratory also demonstrated that exogenous DHEA inhibited TM-3 (a Leydig cell line) cell growth by decreasing the activity of G6PD and the expression of cyclin mRNA; it also improved TM-3 cell viability by increasing mitochondrial membrane permeability along with the activity of succinate dehydrogenase [19]. The Leydig cells of rats are known to possess 3 β -HSD, 17 β -HSD and aromatase, which catalyze the formation of testosterone and estradiol [20]. However, to our knowledge, little information is available regarding the effects of DHEA on the expression of steroidogenic enzyme protein expression in primary Leydig cells.

In adult steroidogenic tissues, the levels of most cell- and tissue-specific steroidogenic enzymes depends mainly on trophic hormonal stimulation, which is mediated by a complex network of signal transduction systems [21]. Extracellular signal-regulated kinases (ERKs) belonging to the family of signaling mitogen-activated protein kinases (MAPKs) were first implicated in the regulation of enzyme proliferation and differentiation [22]. However, several studies suggest that the signaling pathway of ERKs can play a critical role in steroidogenesis [23, 24]. DHEA is known to be a strong activator of the MEK/ERK pathway. Charalampopoulos et al. [25] found that DHEA effects in PC12 cells are membrane-mediated and involve PKA and MAPK pathways but not a nuclear receptor. Chang et al. [9, 26] reported that DHEA-inhibited corticosterone is released from rat adrenal zona fasciculata-

reticularis cells in a manner partly related to the post-cAMP pathway, diminishing the expression of steroidogenic enzymes. Additional studies suggested that DHEA affects the phosphorylation/activation of ERKs by enhancing phosphorylation of the steroidogenic factor-1 (SF-1) and regulating the protein expression of steroidogenic enzymes. Moriguchi et al. [27] reported that DHEA restored reduced ERK phosphorylation concomitant with increased CREB phosphorylation in the hippocampal ca1 region of olfactory bulbectomized mouse. Nevertheless, the molecular mechanisms underlying these DHEA effects in Leydig cells remain unclear.

To better understand the mechanism of DHEA action, a study was carried out to investigate (1) whether DHEA activates the ERK1/2 signaling system in cultured rat primary Leydig cells, which regulate the protein expression of steroidogenic enzymes and, subsequently (2) whether DHEA improves testosterone biosynthesis through activation of the ERK1/2 pathway. This information is necessary to fully understand the cellular and molecular mechanisms by which DHEA exerts its biological effects *in vivo*.

Material and Methods

Animals and materials

Male Sprague-Dawley rats weighing 200±20g were purchased from Shanghai Experimental Animal Center of the Chinese Academy of Sciences (China). Animals were housed one per cage under conditions of constant temperature (25°C), constant humidity (50%) and a 12:12-h light/dark cycle. Animals were maintained on standard rodent chow; food and water were available *ad libitum*. All animal handling procedures were performed in accordance with guidelines established by the People's Republic of China, and all procedures were pre-approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

DHEA, dimethyl sulfoxide (DMSO), penicillin, streptomycin, trypsin and percoll were purchased from Sigma (St Louis, MO, USA); transferrin, L-glutamine and HEPES were obtained from Amresco Inc. (Solon, OH, USA); DMEM/F12 medium and fetal bovine serum (FBS) were purchased from Hyclone (Hyclone, USA); the TRIzol Reagent Kit was purchased from Invitrogen (CA, USA); the M-MLV reverse transcriptase, RNase inhibitor and dNTP mixture were obtained from Promega (Madison, USA); Ex Taq DNA polymerase came from TaKaRa Bio Inc. (Shiga, Japan); rabbit anti-3 β -HSD antibodies and rabbit anti-aromatase were from Abcam (Abcam, USA); rabbit anti-17 β -HSD antibody was purchased from Santa Cruz (Santa Cruz, CA); ERK1/2, p-ERK1/2, CERB, p-CREB, rabbit anti-GAPDH and the p-ERK1/2 inhibitor U1026 were purchased from Cell Signaling Technology (Cell Signaling Technology, USA); goat anti-rabbit IgG, horseradish peroxidase conjugate was purchased from Bioworld Technology Co. (Bioworld Technology Co., USA); protein assay kits were from the Biyuntian Bioengineering Institute (Nanjing, China); the testosterone radioimmunoassay (RIA) kits were purchased from the Beijing Beifang Biotechnology Institution (Beijing, China); and all other reagent details are indicated in the text.

Isolation, purification and identification of Leydig cells

Two rats were killed by decapitation, and their testes were decapsulated under aseptic conditions. The Leydig cells were isolated by enzymatic digestion and purified on a discontinuous Percoll gradient by the method described by Murugesan et al. [28]. In brief, testes were decapsulated using fine forceps without breaking the seminiferous tubules and digested in DMEM-F12 containing collagenase (0.5%) at 37°C for 15min in a thermostated shaking water bath. After this incubation, collagenase-free DMEM-F12 was added to each reaction, and the tubes were allowed stand for 3min at room temperature without mixing. The supernatant was then aspirated using a Pasteur pipette and transferred to sterile centrifuge tubes. This procedure was repeated to remove additional Leydig cells. The supernatants were combined and centrifuged at 2500 x g for 5min at 4°C. After discarding the supernatant, the obtained pellet was resuspended in 2mL of DMEM-F12, which represented a crude testicular interstitial cell suspension. Discontinuous Percoll gradients were used to obtain purified Leydig cells from this crude preparation. Two milliliters of 75% Percoll gradient was added to a graduated centrifuge tube. Above this layer, 70%, 58%, 30% and 5% gradients of Percoll (2mL of each) were gently laid one over the other, taking care to avoid mixing. Two milliliters of

crude Leydig cell suspension was then applied on top of this discontinuous gradient and centrifuged at 3000 x g for 30min at 4°C. After centrifugation, most of the observed purified Leydig cells were in the 30% Percoll gradients. These Leydig cells were transferred to centrifuge tubes containing DMEM-F12. After mixing, the tubes were centrifuged at 2500 x g for 10min at 4°C, and the obtained supernatant was discarded. To remove excess Percoll, the cell pellets were washed three times with excess medium and finally suspended in 1mL of DMEM-F12. The purities of the Leydig cells were assessed by histochemical localization of 3 β -HSD performed according to the method of Aldred and Cooke [29]. The viability of the purified Leydig cells was determined by trypan blue dye exclusion [29].

Primary culture of Leydig cells

Primary Leydig cells were plated in six-well plastic culture plates (Nunc Company, Roskilde, Denmark) at a density of 1 $\times$ 10⁶ cells per well in 2mL of DMEM-F12 medium. Supplements were added using 10% FBS, transferrin (5mg/mL), 2mM L-glutamine and 1.75mM HEPES. The culture medium also contained penicillin (100IU/ml) and streptomycin (100mg/mL). Primary Leydig cells were incubated at 37°C in an atmosphere of 95% air and 5% CO₂. Following 24h of acclimatization to the culture environment, cells were incubated with serum-free medium for 1h before the onset of experimental treatments.

Cell viability assay

Cells grown in 96-well plates (1 $\times$ 10⁴cells/well) were treated with 0, 1, 50 or 100 μ M DHEA and cultured at 37°C for 24h. Then, 20 μ L of MTT (5mg/mL) was added to each well. After 4h of culture, the culture medium was removed and blue formazan crystals that had formed were dissolved in 50 μ L DMSO. The optical density of the formazan generated from MTT was measured at 490nm.

Detection of testosterone content by radioimmunoassay (RIA)

The concentration of testosterone in primary Leydig cells under basal or stimulated conditions was determined using an RIA kit. After culturing for 24 h in DMEM-F12 medium at 37°C, cells were incubated with a dimethyl sulfoxide solution of DHEA (1 μ M, 50 μ M or 100 μ M) for 24 h (n = 6). A solution of 0.1% DMSO was used for the control group. Following incubation, cells were harvested, disrupted ultrasonically in ice, and centrifuged at 2500 x g for 10min at 4°C. The supernatants were then collected and stored for subsequent analysis at -20°C. The testosterone concentrations in the supernatants were determined according to the manufacturer's instructions (intra-variation coefficients <10%, inter-variation coefficients <15%). Experiments were repeated three times and six.

Assay of 3 β -HSD and 17 β -HSD expression by real-time quantitative PCR

Following a 24h equilibration to the culture environment, primary Leydig cells incubated in six-well plates was exposed to DHEA. Thereafter, the old culture medium was removed, and cells were resuspended in DMEM-F12 medium with a DMSO solution of DHEA (1, 50 or 100 μ M). Control cultures received an equal volume of DMSO, which did not exceed 0.25 vol-% in all experiments. Control and treated cells were left for 24 h in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO₂. Experiments were repeated three times, with six samples taken at each time point. Total RNA was immediately extracted from Leydig cells after incubation with DHEA by the TRIZOL reagent kit. The RNA concentration was then quantified by measuring absorbance at 260nm with a photometer (Eppendorf Biophotometer). Ratios of absorption (260/280 nm) for all preparations were between 1.8 and 2.0. Aliquots of RNA samples were subjected to electrophoresis through a 1.4% agarose/formaldehyde gel to verify their integrity. After adjustment of the RNA concentrations to 1 μ g/ μ L by OD value measurement, the aliquots were stored at -80°C.

Two micrograms of total RNA were reverse-transcribed by incubation for 1h at 37°C in a 25 μ L mixture consisting of 100U M-KGV reverse transcriptase, 8U RNase inhibitor, 0.5 μ g of Oligo(dT), 50mM Tris-HCl (pH 8.3), 3mM MgCl₂, 75mM KCl, 10mM DDT and 0.8mM dNTP. The reaction was stopped by heating to 95°C for 5min and quickly cooling the mixture on ice. Real-time RT-PCR was performed with a SYBR Green PCR Master Mix Kit (Toyobo, Osaka, Japan) in an ABI7300 Detection System (Applied Biosystems) according to the manufacturer's instructions. The expression of 3 β -HSD and 17 β -HSD mRNA was determined by real-time quantitative RT-PCR. The relative amount of mRNA for each target gene was determined by calculating the ratio between each mRNA and β -actin mRNA [18]. The thermal cycling routine was 5min at 95°C followed by 40 cycles at 95°C for 30s, 60°C for 30s, and 72°C for 40s in an ABI PRISM 7300 Detection System (Applied

Table 1. Primer sequence of β -actin and targeted gene

Gene	Genbank accession number	Primer sequences (5'-3')	Orientation	Product size (bp)
β -actin	NM_031144	CCCTGTGCTGCTCACCGA	Forward	186
		ACAGTGTGGGTGACCCGTC	Reverse	
3 β -HSD	NM_001007719	ACCCTTTAACTGCCACTTGGTC	Forward	141
		AGTGTCCCGATCCACTCCGA	Reverse	
17 β -HSD	NM_001004209	ATTGGAAGACTGACCGCTACGAA	Forward	93
		CGCCGCTGTTTCCTCGATGCC	Reverse	

Biosystems). According to the comparative threshold cycle (Ct) method, the amount of target mRNA normalized to β -actin relative to an internal control was calculated by $2^{-\Delta\Delta Ct}$; $\Delta\Delta Ct = (Ct_{ij} - Ct_{\beta\text{-actin } j}) - (Ct_{i1} - Ct_{\beta\text{-actin } 1})$, where Ct_{ij} and $Ct_{\beta\text{-actin } j}$ are the Ct for gene i and β -actin in a sample j, respectively, and Ct_{i1} and $Ct_{\beta\text{-actin } 1}$ are the Ct in sample 1 expressed as the standard. The primers (Invitrogen, China) for 3 β -HSD and 17 β -HSD are shown in Table 1.

Western blot analysis

The effects of DHEA on 3 β -HSD, 17 β -HSD, aromatase, CREB and p-CREB were measured by co-incubating the cells with 1, 50 and 100 μ M concentrations of DHEA or vehicle for 24h at 37°C and scraping for the subsequent determination of protein levels. Experiments were repeated three times, with six samples taken at each time point. Protein levels were measured using a bicinchoninic acid (BCA) protein determination kit. Extracted cells (60 μ g) were separated on a 10% SDS-polyacrylamide denaturing gel. Following SDS-PAGE, proteins were electrophoretically transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were then blocked at 25°C for 2h in blocking buffer [2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)-buffered saline (TBS; pH7.6; Tris base 2.42g/l, NaCl 8g/l), 0.1% Tween-20, and 5% non-fat dry milk]. The proteins were incubated overnight at 4°C with rabbit anti-3 β -HSD antibodies (1:1000 dilution; Abcam, USA), rabbit anti-17 β -HSD antibodies (1:1000 dilution; Santa Cruz, CA), rabbit anti-aromatase (1:1000 dilution; Abcam, USA), and rabbit anti-GAPDH (1:1000 dilution; CST, USA). After washing with TBS (pH 8.0) containing 0.1% Tween-20, goat anti-rabbit IgG with horseradish peroxidase conjugate (1:5000, Bioworld Technology Co., USA) in washing solution were added, and the solution was incubated for 1 h at room temperature. The immunoreactive proteins were detected by SuperSignal chemiluminescence. The protein bands were digitally imaged for densitometric quantification with a software program (Eastman Kodak Company, Rochester, NY, USA). Finally, the developed blots were subjected to densitometry using glyceraldehyde-3-phosphatedehydrogenase (GAPDH) as an internal control.

ERK signaling analysis

ERK1/2 and p-ERK1/2 protein levels. After culturing for 24h in DMEM-F12 medium at 37°C, cells were incubated with a dimethyl sulfoxide solution of DHEA (1, 50 and 100 μ M) for 30min. Control cultures received an equal volume of dimethyl sulfoxide, which did not exceed 0.25% (v/v) in any experiment. Primary Leydig cells were pre-incubated with 10 μ M U0126 (inhibitor of p-ERK1/2) or vehicle for 60 min, followed by the addition of 100 μ M DHEA for varying lengths of time in DMEM-F12 medium at 37°C. After incubation, ERK1/2 and p-ERK1/2 protein levels in primary Leydig cells under basal or stimulated conditions were determined by Western blot as described above.

Testosterone content. Primary Leydig cells were pre-incubated with 10 μ M U0126 or vehicle for 60min, followed by the addition of 1, 50 and 100 μ M DHEA for 24h in DMEM-F12 medium at 37°C. After incubation, the testosterone contents of primary Leydig cells were determined according to the manufacturer's instructions (intra-variation coefficients <10%, inter-variation coefficients <15%).

Assay of steroidogenic enzymes. Primary Leydig cells were pre-incubated with 10 μ M U0126 or vehicle for 60min, followed by the addition of 100 μ M DHEA for 24h in DMEM-F12 medium at 37°C. After incubation, the 3 β -HSD, 17 β -HSD and aromatase protein levels in primary Leydig cells under basal or stimulated conditions were determined by Western blot as described above.

Statistical analysis

Data were analyzed with one-way ANOVA and expressed as mean values with standard errors. Treatment differences were subjected to a Duncan's multiple comparison tests. Differences were considered significant at $P < 0.05$. All statistical analyses were performed with SPSS 11.0 for Windows (StatSoft, Inc., Tulsa, OK, USA).

Results

Effect of DHEA on cell viability and testosterone content in primary Leydig cells

In the present study, we first determined the effect of DHEA on the cell viability of primary Leydig cells by MTT method. As shown in Fig. 1A, cells viability increased when primary Leydig cells were treated with 1-100 μ M DHEA for 24h ($P < 0.01$). Testosterone content was not detected in the control group, while a marked increase in testosterone was observed in the DHEA-treated groups ($P < 0.01$) in a dose-dependent manner (Fig. 1B).

Effect of DHEA on 3β -HSD and 17β -HSD mRNA expression

As shown in Fig. 2, the expression of 3β -HSD mRNA was significantly higher in with 50 μ M ($P < 0.05$) and 100 μ M ($P < 0.01$) treatment groups compared to the control group (Fig. 2A). The 100 μ M DHEA-treated cells also had significantly higher levels of 17β -HSD mRNA expression compared to control group ($P < 0.05$) (Fig. 2B). Thus, we used the 100 μ M DHEA treatment to culture primary Leydig cells to study its effect on the protein expression of steroidogenic metabolic enzyme.

Effect of DHEA on steroidogenic metabolic enzyme protein levels

To find out whether increased testosterone affected the protein levels of steroidogenic enzymes, the Leydig cellular steroidogenic enzymes protein expression levels were assayed. As shown in Fig. 3, with the extension of culture time, the protein levels of 3β -HSD, 17β -HSD and aromatase were not changed relative to the control group. The 3β -HSD and 17β -HSD protein levels showed an enhancement trend with treatment with DHEA; the 3β -HSD protein level was significantly increased from 12-48 h ($P < 0.05$; Fig. 3A), and the 17β -HSD protein level significantly increased from 24-48 h ($P < 0.05$; Fig. 3B). However, the aromatase protein level was significantly decreased at 48h ($P < 0.05$; Fig. 3C) with DHEA-treated primary Leydig cells.

Fig. 1. Effect of DHEA on cell viability and testosterone content in primary Leydig cells. (A) Cells were treated with different dosage DHEA for 24h, and then detected the cell viability by MTT methods. (B) Cells were pre-incubated with 10 μ M U0126 or vehicle for 60 min followed by the addition of 1, 50 or 100 μ M DHEA for 24h in DMEM-F12 medium. After incubation, testosterone content was detected using an RIA kit. Values are means $\pm$ SE ($n = 6$). "ND" indicates testosterone concentrations below the detect limit. The significant difference between DHEA treatment group and respective control group was representing with capital letter "B". The difference between DHEA+U0126 and DHEA alone group was representing with "#".

Fig. 2. Effect of DHEA on 3β-HSD and 17β-HSD mRNA expression in primary rat Leydig cells. (A) 3β-HSD and (B) 17β-HSD. Values are means ± SE (n=6). The mean values for DHEA-treated cells were significantly different from those of the control groups; * $P < 0.05$, ** $P < 0.01$.

Fig. 3. Effect of DHEA on the protein expression of steroidogenic metabolic enzymes. Results of statistical analysis for protein expression were normalized to GAPDH. (A) 3β-HSD; (B) 17β-HSD; and (C) aromatase. Values are means ± SE (n = 6). Different letters indicate significant differences in DHEA-treated groups between different treatment times, $P < 0.05$. * indicates the significant difference between DHEA-treated and control groups at the same time point, $P < 0.05$.

Compared to the control group at the same time, 3β-HSD and 17β-HSD protein levels increased ($P < 0.05$; Fig. 3A and B) while the level of aromatase protein decreased ($P < 0.05$; Fig. 3C) when subjected to DHEA incubation for 24-48h in primary Leydig cells.

ERK signaling analysis

ERK1/2 and p-ERK1/2 protein levels. We first determined the effect of DHEA on ERK1/2 and p-ERK1/2 protein levels in primary Leydig cells. Compared to the control group, the results showed that DHEA treatment had no significant effect on total ERK1/2 protein levels, while p-ERK1/2 protein levels were significantly increased in 50 and 100 μM DHEA-treated groups ($P < 0.05$; Fig. 4A). Considering this result along with

Fig. 4. Effect of DHEA on the protein expression of ERK1/2 and p-ERK1/2. (A) Primary Leydig cells were incubated in DMEM-F12 medium with various concentrations of DHEA (1, 50 and 100 μM) or vehicle (0 mM) for 30 min. Values are means $\pm$ SE (n = 6). The mean value was significantly different from that of the control group, * $P < 0.05$, ** $P < 0.01$. (B) Primary Leydig cells were pre-incubated with 10 μM U0126 (inhibitor of p-ERK1/2) or vehicle for 60 min, followed by the addition of 100 μM DHEA for varying lengths of time in DMEM-F12 medium at 37°C. Values are shown as means $\pm$ SE (n = 6). The different capital letters indicate significant differences in the same treated group at different times ($P < 0.01$); the mean value of the treated group was significantly different than the control group at the same time, * $P < 0.05$, ** $P < 0.01$.

effect of DHEA on steroidogenic metabolic enzyme protein levels, we used the 100 μM DHEA treatment to further study its effect on ERK1/2 and p-ERK1/2 protein levels.

Compared to the control, total protein ERK1/2 was unchanged with the extension of culture time, while levels of p-ERK1/2 were significantly higher at an experimental time of 30 and 120 min ($P < 0.01$; Fig. 4B). We also found that the levels of p-ERK1/2 proteins were significantly lower during the experimental period from 10 to 120 min ($P < 0.05$) compared to the control group, which was pre-incubated with U0126 (inhibitor of p-ERK1/2) and then treated by DHEA (Fig. 4B).

Testosterone contents. To determine whether DHEA elevates testosterone content by stimulating p-ERK1/2 protein expression, we examined testosterone content in primary Leydig cells pre-incubated with U0126 or vehicle. The results showed that testosterone contents were significantly increased by treatment with 1-100 μM DHEA compared to controls groups either with or without U0126 incubation ($P < 0.01$; Fig. 1B). However, the testosterone content was significantly decreased ($P < 0.05$) in the 50 or 100 μM DHEA-treated groups with U0126 pre-incubation as compared to DHEA alone (Fig. 1B).

Steroidogenic enzymes protein levels. To investigate whether the change in testosterone content induced by the activation of p-ERK1/2 by DHEA was due to alterations 3β-HSD, 17β-HSD and aromatase protein levels, we performed a Western blot analysis. As shown

Fig. 5. Effect of DHEA on p-ERK1/2-mediated steroidogenic enzymes protein levels. Primary Leydig cells were pre-incubated with 10 μ M U0126 (inhibitor of p-ERK1/2) or vehicle for 60 min, followed by the addition of 100 μ M DHEA for 24 h in DMEM-F12 medium at 37°C. (A) 3 β -HSD; (B) 17 β -HSD; and (C) aromatase. Protein levels were detected by Western blot analysis. Results are the mean of at least three separate Western blots. Values are means (n = 6) with standard errors represented by vertical bars. The different letters indicate significant differences when compared to the respective control group ($P < 0.05$); the mean value was significantly different between groups with and without treatment with U0126, * $P < 0.05$.

in Fig. 5, incubation in 100 μ M DHEA significantly increased ($P < 0.05$) the level of 3 β -HSD protein (Fig. 5A) and 17 β -HSD protein (Fig. 5B), while the level of aromatase protein was decreased ($P < 0.01$) compared to the control group (Fig. 5C).

Compared to the control group, a marked decrease ($P < 0.05$) was observed in 3 β -HSD (Fig. 5A) and 17 β -HSD (Fig. 5B) protein levels in cells pretreated with p-ERK1/2 inhibitors. Interestingly, no significant difference with the control was found in the level of aromatase protein in cells pretreated with p-ERK1/2 inhibitors (Fig. 5C).

Effect of DHEA on the expression of cAMP responsive element binding protein (CREB)

As shown in Fig. 6, no significant change in total CREB protein levels was observed with DHEA treatment, while p-CREB protein levels were significantly increased compared to the control in groups treated by 50 and 100 μ M DHEA ($P < 0.05$).

Fig. 6. Effect of DHEA on the CREB and p-CREB protein expression levels. (A) CREB and (B) p-CREB. Values are means $\pm$ SE ($n = 6$). The mean value was significantly different from that of the control group, * $P < 0.05$, ** $P < 0.01$.

Discussion

The major outcome of this study was demonstrating that DHEA targets the ERK1/2 signaling pathway in rat primary Leydig cells. DHEA directly enhanced the level of p-ERK1/2 and p-CREB proteins, which causes exogenous DHEA to preferentially convert to testosterone rather than estradiol. This preference is due to the up-regulation of 3β -HSD and 17β -HSD protein levels along with the down-regulation of aromatase protein expression level in rat primary Leydig cells. Our findings demonstrate a novel signal pathway for DHEA action in primary Leydig cells, and provide a possible explanation for how DHEA carries out its biological functions.

Present results showed that cells viability was significant increase after 1-100 μ M DHEA treatment in primary Leydig cells, and this indicated that there no any cytotoxic effect of DHEA on Leydig cells. Also, a rapid increase in testosterone content was seen as the concentration increased from 1 to 100 μ M, and maximum testosterone content accumulation was observed in the 100 μ M DHEA-treated group. These data are consistent with our previous study showing that DHEA can lead to high serum levels of testosterone in male rats [18] and higher concentrations of testosterone in TM-3 cells (a Leydig cell line) in a dose- and time-dependent fashion [30]. Leblanc et al. [31] also reported that the administration of DHEA to cynomolgus monkeys significantly increased the serum concentrations of androstenedione and testosterone. DHEA can be converted into androstenedione by 3β -HSD in peripheral target tissues. It can then undergo further conversion to testosterone and estrone by 17β -HSD and aromatase, respectively [10]. The expression of 3β -HSD mRNA was significantly increased by treatment with 50 and 100 μ M DHEA. Cells treated with 100 μ M DHEA also had markedly higher 17β -HSD mRNA expression when compared to the control group.

The adrenal cortex accounts for 75-90% of the body's daily DHEA secretions; the remainder is produced by the testes and ovaries [32]. The sulfated form of DHEA (DHEAS) is a hydrophilic storage form that circulates in the blood, and DHEAS can be inter-converted with DHEA by DHEA sulfotransferases and hydroxysteroid sulfatases [13]. This conversion occurs in a wide range of tissues including the brain, liver, kidney, and gonads. DHEA is then metabolized to androstenedione, testosterone, estrogens, and other biologically active steroids, depending on the tissue. The present study showed that 3β -HSD and 17β -HSD protein levels in primary Leydig cells treated with 100 μ M DHEA were significantly increased at 24-48h, while the aromatase protein expression level were significantly decreased. This result was consistent with our previous study demonstrating the increase in 3β -HSD

and 17 β -HSD protein levels following incubation with DHEA for 24 - 48 h along with the decrease in aromatase protein level in TM-3 cells [30]. These findings may be attributed to the increased delivery of intermediates, resulting in the variation of the protein level of the steroidogenic cascade because the precursor substrates also play an important role in enzyme biosynthesis [33].

Furthermore, the conversion of DHEA to other steroidal hormones depends upon the relative activity of key steroidogenic enzymes. Aromatase is a dual function enzyme that can catalyze the conversion of androstenedione to estrone and convert testosterone to estradiol [34]. It is generally acknowledged that the expression level of 3 β -HSD, 17 β -HSD, aromatase and the hormone changes have potential correlation. Bourguiba et al. [35] demonstrated that androgens up-regulate the expression of aromatase in purified adult rat germ cells, whereas estrogens have the opposite effect. Li et al. [36] confirmed that the sex hormone precursor DHEA, which can be converted into estradiol by the enzyme aromatase, has a protective role against osteoarthritis. Our previous study showed that DHEA treatment significantly increased the content of estradiol in TM-3 cells, while the level of aromatase was decreased following treated with DHEA, and the results indicated that high estradiol concentrations result in down regulation of aromatase expression in TM-3 cells [30]. Given the present results and those from previous studies, although we did not detect estradiol content in the present study, we speculate that the down-regulation of aromatase protein level also may be due to the negative feedback of estradiol in primary Leydig cells. However, further study is needed to more precisely validate this hypothesis. DHEA is reported to be preferentially metabolized to androgens in women and estrogens in men [13, 37]. Labrie [38] reported that DHEA supplementation in middle-aged or older men increases estradiol levels. Albrecht et al. [39] found that estradiol decreases DHEA secretion in pregnant baboons, indicating an estradiol-mediated negative feedback control of DHEA levels. However, *in vitro* studies using OBs demonstrated that DHEA can be metabolized to testosterone by 17 β - and 3 β -HSD, and further metabolized to dihydrotestosterone by the 5 α -reductase types 1 and 2 enzymes [17]. In addition, Takeuchi et al. [11] showed that articular chondrocytes express steroidogenesis-related enzyme genes such as 3 β -HSD, 17 β -HSD, and P450arom; the expression of these compounds did not change after the addition of DHEA, and sex steroid hormones were able to be locally synthesized from DHEA. Thus, we speculate that differences in the metabolism of exogenous DHEA depend on sex, age and target cells, and that the negative feedback effect of steroid concentrations in different tissues on key enzymes in the formation or metabolism of steroids should also be considered.

Our results suggest that exogenous DHEA could preferentially convert to testosterone rather than estradiol due to the un-regulation of 3 β -HSD and 17 β -HSD protein levels and the down-regulation of aromatase protein expression levels in primary Leydig cells. Several studies suggest that the MEK/ERK signaling pathway can play a critical role in steroidogenesis [23, 24]. Nelson-Degrave et al. [24] demonstrated that the MEK/ERK pathway inhibits CYP17 gene expression in human Cal cells. Crosstalk between cAMP/PKA and MEK/ERK pathways has been previously described in the human adrenal NCI-H295R cell line [40]. DHEA may act directly by activating the MAPK signal transduction pathway [41]. Upon extracellular stimulation, the activated ERKs regulate additional signaling kinases that participate in the control steroidogenesis in granulosa-derived cell lines [22]. In the present study, DHEA generated no significant changes in the total ERK1/2 protein levels. Conversely, the p-ERK1/2 protein level was significantly increased by 50 and 100 μ M DHEA treatments. Several studies have shown that DHEA induces both genomic and non-genomic effects involving the ERK1/2 kinase pathway in the regulation of glucose uptake [42] and antiviral activity [42-44]. We also found that the levels of p-ERK1/2 proteins were significantly lower when the primary Leydig cells pre-incubated with U0126 (inhibitor of p-ERK1/2); therefore, we speculate that DHEA, a steroid hormone, may affect the enhancement of p-ERK1/2 protein levels in primary Leydig cells.

As shown in Fig.1, treatment with 50 or 100 μ M DHEA combined with pre-incubation with U0126 resulted in a significant decrease in testosterone content. Western blot results

showed that pre-incubation with U0126 could reverse the increased levels of 3 β -HSD and 17 β -HSD proteins generated by DHEA in primary Leydig cells, while the aromatase protein level was unaffected. DHEA has been identified as a potent agonist for the sigma 1 (σ 1) receptor [45]. Activation of this receptor also enhances the NGF-induced neurite outgrowth by modulating the MEK-ERK and PI3K-Akt-mTOR signaling pathways [46]. However, recent findings indicate that genomic effects of DHEA in PC12 cells are membrane-mediated and involve PKA and MAPK pathways but not a nuclear receptor [25]. These results reinforce the idea that DHEA can activate p-ERK1/2, thus enhancing the expression of 3 β -HSD and 17 β -HSD and improving testosterone biosynthesis in primary Leydig cells.

Previous study have observed that DHEA induce the proliferation of estrogen and androgen receptor positive breast cancer cells, but inhibit the proliferation in estrogen receptor negative cells [47]. Also, Nephew et al. [48] reported that DHEA inhibits estradiol binding to its receptor *in vivo* in yeast. Although DHEA and its downstream conversion product androstenedione are often referred to as adrenal androgens, they actually do not represent androgens since they do not bind with high affinity to classical intracellular the androgen receptor [49]. To date, no nuclear steroid receptor with high affinity for DHEA has been found [49] and the mechanisms by which DHEA operated are not fully understood. Transcription factor network analysis identified CREB as the master regulator in the control of gene expression in steroid-related pathways [50]. Many steroidogenic gene promoters exhibit cAMP response elements (CREs) or CRE-like sequences, highlighting the major role of the cAMP pathway in regulating steroid hormone biosynthesis [51]. In this study, DHEA treatment caused a significant up-regulation of the p-CREB protein level in cultured rat primary Leydig cells. This result was consistent with Moriguchi et al. [27], who reported that DHEA significantly restored the reduced ERK phosphorylation concomitant with increased CREB phosphorylation in hippocampal ca1. Saxena et al. [52] reported that DHEA regulates the MAPK pathway in humans via a novel MAPK phosphatase, which not only controls the activity of MAPKs, but also mediates crosstalk between the cAMP system and the MAPK cascade. However, Ren et al. [53] reported that chronic treatment with DHEA did not affect the cAMP level up-regulated by withdrawal, suggesting the role of a non-cAMP/PKA pathway in the regulation of MAPK activation. Combined with these previous studies, the present results lead us to speculate that DHEA can act via an ERK cascade to up-regulate p-CREB, directly enhancing the 3 β -HSD and 17 β -HSD protein levels. This results in the improvement of testosterone biosynthesis in primary Leydig cells *in vitro*. Further study, however, is needed to more precisely validate this hypothesis.

In summary, using an *in vitro* model to study the effect of DHEA on testosterone biosynthesis in male animals, we found that DHEA could preferentially convert to testosterone rather than estradiol due to the up-regulation of 3 β -HSD and 17 β -HSD proteins and the down-regulation of aromatase protein in primary Leydig cells. This DHEA effect, at least in part, was associated with the elevation of p-ERK1/2 and p-CREB protein levels. On the basis of these results, we speculate that DHEA is a clinical agent for the alleviation of aging-associated pathophysiological conditions in humans; however, this supposition requires further investigation.

Disclosure Statement

All of author has none declared.

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References

- 1 Feldman HA, Johannes CB, Araujo AB, Mohr BA, Longcope C, McKinlay JB: Low dehydroepiandrosterone and ischemic heart disease in middle-aged men: Prospective results from the massachusetts male aging study. *Am J Epidemiol* 2001;153:79-89.
- 2 Genazzani AD, Lanzoni C, Genazzani AR: Might DHEA be considered a beneficial replacement therapy in the elderly? *Drug Aging* 2007;24:173-185.
- 3 Baulieu EE, Thomas G, Legrain S, Lahlou N, Roger M, Debuire B, Faucounau V, Girard L, Hervy MP, Latour F, Leaud MC, Mokrane A, Pitti-Ferrandi H, Triviale C, de Lacharriere O, Nouveau S, Rakoto-Arison B, Souberbielle JC, Raison J, Le Bouc Y, Raynaud A, Girerd X, Forette F: Dehydroepiandrosterone (DHEA), DHEA sulfate, and aging: Contribution of the DHEAge study to a sociobiomedical issue. *P Natl Acad Sci USA* 2000;97:4279-4284.
- 4 Labrie F, Luu-The V, Labrie C, Belanger A, Simard J, Lin SX, Pelletier G: Endocrine and intracrine sources of androgens in women: Inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. *Endocr Rev* 2003;24:152-182.
- 5 Bacsí K, Kosa J, Lazary A, Horvath H, Balla B, Lakatos P, Speer G: Significance of dehydroepiandrosterone and dehydroepiandrosterone sulfate in different diseases. *Orvosi Hetilap* 2007;148:651-657.
- 6 Legrain S, Girard L: Pharmacology and therapeutic effects of dehydroepiandrosterone in older subjects. *Drug Aging* 2003;20:949-967.
- 7 Sorwell KG, Urbanski HF: Dehydroepiandrosterone and age-related cognitive decline. *Age* 2010;32:61-67.
- 8 Wolf OT, Kirschbaum C: Actions of dehydroepiandrosterone and its sulfate in the central nervous system: Effects on cognition and emotion in animals and humans. *Brain Res Rev* 1999;30:264-288.
- 9 Chang L-L, Wun W-SA, Wang PS: Mechanisms of inhibition of dehydroepiandrosterone upon corticosterone release from rat zona fasciculata-reticularis cells. *J Cell Biochem* 2008;104:359-368.
- 10 Labrie F, Luu-The V, Labrie C, Simard J: DHEA and its transformation into androgens and estrogens in peripheral target tissues: Intracrinology. *Front Neuroendocrin* 2001;22:185-212.
- 11 Takeuchi S, Mukai N, Tateishi T, Miyakawa S: Production of sex steroid hormones from DHEA in articular chondrocyte. *Am J Physiol-Endoc M* 2007;293:E410-E415.
- 12 Schwartz AG, Pashko LL: Dehydroepiandrosterone, glucose-6-phosphate dehydrogenase, and longevity. *Ageing Res Rev* 2004;3:171-187.
- 13 Arlt W, Haas J, Callies F, Reincke M, Hubler D, Oettel M, Ernst M, Schulte HM, Allolio B: Biotransformation of oral dehydroepiandrosterone in elderly men: Significant increase in circulating estrogens. *J Clin Endocr Metab* 1999;84:2170-2176.
- 14 Arlt W, Justl HG, Callies F, Reincke M, Hubler D, Oettel M, Ernst M, Schulte HM, Allolio B: Oral dehydroepiandrosterone for adrenal androgen replacement: Pharmacokinetics and peripheral conversion to androgens and estrogens in young healthy females after dexamethasone suppression. *J Clin Endocr Metab* 1998;83:1928-1934.
- 15 Labrie F, Belanger A, Belanger P, Berube R, Martel C, Cusan L, Gomez J, Candas B, Chaussade V, Castiel I, Deloche C, Leclaire J: Metabolism of dheA in postmenopausal women following percutaneous administration. *J Steroid Biochem* 2007;103:178-188.
- 16 Morales AJ, Haubrich RH, Hwang JY, Asakura H, Yen SSC: The effect of six months treatment with a 100 mg daily dose of dehydroepiandrosterone (DHEA) on circulating sex steroids, body composition and muscle strength in age-advanced men and women. *Clin Endocrinol* 1998;49:421-432.
- 17 Wang L, Wang YD, Wang WJ, Li DJ: Differential regulation of dehydroepiandrosterone and estrogen on bone and uterus in ovariectomized mice. *Osteoporosis Int* 2009;20:79-92.
- 18 Song L, Tang X, Kong Y, Ma H, Zou S: The expression of serum steroid sex hormones and steroidogenic enzymes following intraperitoneal administration of dehydroepiandrosterone (DHEA) in male rats. *Steroids* 2010;75:213-218.

- 19 Shen X, Liu L, Yin F, Ma H, Zou S: Effect of dehydroepiandrosterone on cell growth and mitochondrial function in tm-3 cells. *Gen Comp Endocr* 2012;177:177-186.
- 20 Kumar V, Balomajumder C, Roy P: Disruption of LH-induced testosterone biosynthesis in testicular leydig cells by triclosan: Probable mechanism of action. *Toxicology* 2008;250:124-131.
- 21 Hanukoglu I: Steroidogenic enzymes - structure, function, and role in regulation of steroid-hormone biosynthesis. *J Steroid Biochem* 1992;43:779-804.
- 22 Seger R, Hanoch T, Rosenberg R, Dantes A, Merz WE, Strauss JF, Amsterdam A: The erk signaling cascade inhibits gonadotropin-stimulated steroidogenesis. *J Biol Chem* 2001;276:13957-13964.
- 23 Tajima K, Dantes A, Yao Z, Sorokina K, Kotsuji F, Seger R, Amsterdam A: Down-regulation of steroidogenic response to gonadotropins in human and rat preovulatory granulosa cells involves mitogen-activated protein kinase activation and modulation of dax-1 and steroidogenic factor-1. *J Clin Endocr Metab* 2003;88:2288-2299.
- 24 Nelson-Degrave VL, Wickenheisser JK, Hendricks KL, Asano T, Fujishiro M, Legro RS, Kimball SR, Strauss JF, McAllister JM: Alterations in mitogen-activated protein kinase kinase and extracellular regulated kinase signaling in theca cells contribute to excessive androgen production in polycystic ovary syndrome. *Mol Endocrinol* 2005;19:379-390.
- 25 Charalampopoulos I, Margioris AN, Gravanis A: Neurosteroid dehydroepiandrosterone exerts anti-apoptotic effects by membrane-mediated, integrated genomic and non-genomic pro-survival signaling pathways. *J Neurochem* 2008;107:1457-1469.
- 26 Chang LL, Wun WSA, Ho LLT, Wang PS: Effects of dehydroepiandrosterone on corticosterone release in rat zona fasciculata-reticularis cells. *N-S Arch Pharmacol* 2003;368:487-495.
- 27 Moriguchi S, Yamamoto Y, Ikuno T, Fukunaga K: Sigma-1 receptor stimulation by dehydroepiandrosterone ameliorates cognitive impairment through activation of CaM kinase II, protein kinase c and extracellular signal-regulated kinase in olfactory bulbectomized mice. *J Neurochem* 2011;117:879-891.
- 28 Murugesan P, Muthusamy T, Balasubramanian K, Arunakaran J: Polychlorinated biphenyl (aroclor 1254) inhibits testosterone biosynthesis and antioxidant enzymes in cultured rat leydig cells. *Reprod Toxicol* 2008;25:447-454.
- 29 Aldred LF, Cooke BA: The effect of cell damage on the density and steroidogenic capacity of rat testis leydig cells, using an nadh exclusion test for determination of viability. *J Steroid Biochem* 1983;18:411-414.
- 30 Xuehuai Shen, Jian Kang, Jing Han, Guanxing Liu, Haitian Ma, Sixiang Zou: Influence the content of steroid hormones and expression of steroidogenic enzymes with DHEA-treated in tm-3 cells. *J Anim Vet Adv* 2013;12:831-838.
- 31 Leblanc M, Labrie C, Belanger A, Candas B, Labrie F: Pharmacokinetics of oral dehydroepiandrosterone (DHEA) in the ovariectomised cynomolgus monkey. *J Steroid Biochem* 2002;81:159-164.
- 32 Savineau J-P, Marthan R, de la Roque ED: Role of DHEA in cardiovascular diseases. *Biochem Pharmacol* 2013;85:718-726.
- 33 Sanderson T, van den Berg M: Interactions of xenobiotics with the steroid hormone biosynthesis pathway. *Pure Appl Chem* 2003;75:1957-1971.
- 34 Payne AH, Hales DB: Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev* 2004;25:947-970.
- 35 Bourguiba S, Lambard S, Carreau S: Steroids control the aromatase gene expression in purified germ cells from the adult male rat. *J Mol Endocrinol* 2003;31:83-94.
- 36 Li W, Tang L, Xiong Y, Zhou X, Wu L: The chondroprotective effects of dehydroepiandrosterone probably exerted by its conversion to estradiol. *J Steroid Biochem* 2013;134:15-22.
- 37 Frye RF, Kroboth PD, Kroboth FJ, Stone RA, Folan M, Salek FS, Pollock BG, Linares AM, Hakala C: Sex differences in the pharmacokinetics of dehydroepiandrosterone (DHEA) after single- and multiple-dose administration in healthy older adults. *J Clin Pharmacol* 2000;40:596-605.
- 38 Labrie F: DHEA, important source of sex steroids in men and even more in women. *Prog Brain Res* 2010;182:97-148.
- 39 Albrecht ED, Aberdeen GW, Pepe GJ: Estrogen elicits cortical zone-specific effects on development of the primate fetal adrenal gland. *Endocrinology* 2005;146:1737-1744.
- 40 Sewer MB, Waterman MR: cAMP-dependent protein kinase enhances cyp17 transcription via MKP-1 activation in h295r human adrenocortical cells. *J Biol Chem* 2003;278:8106-8111.

- 41 Williams MRI, Ling SH, Dawood T, Hashimura K, Dai AZ, Li H, Liu JP, Funder JW, Sudhir K, Komesaroff PA: Dehydroepiandrosterone inhibits human vascular smooth muscle cell proliferation independent of ARs and ERs. *J Clin Endocr Metab* 2002;87:176-181.
- 42 Liu D, Iruthayanathan M, Homan LL, Wang Y, Yang L, Wang Y, Dillon JS: Dehydroepiandrosterone stimulates endothelial proliferation and angiogenesis through extracellular signal-regulated kinase 1/2-mediated mechanisms. *Endocrinology* 2008;149:889-898.
- 43 Formoso G, Chen H, Kim JA, Montagnani M, Consoli A, Quon MJ: Dehydroepiandrosterone mimics acute actions of insulin to stimulate production of both nitric oxide and endothelin 1 via distinct phosphatidylinositol 3-kinase- and mitogen-activated protein kinase-dependent pathways in vascular endothelium. *Mol Endocrinol* 2006;20:1153-1163.
- 44 Torres NI, Castilla V, Bruttomesso AC, Eiras J, Galagovsky LR, Wachsman MB: In vitro antiviral activity of dehydroepiandrosterone, 17 synthetic analogs and erk modulators against herpes simplex virus type 1. *Antivir Res* 2012;95:37-48.
- 45 Hashimoto K: Sigma-1 receptor chaperone and brain-derived neurotrophic factor: Emerging links between cardiovascular disease and depression. *Prog Neurobiol* 2013;100:15-29.
- 46 Nishimura T, Ishima T, Iyo M, Hashimoto K: Potentiation of nerve growth factor-induced neurite outgrowth by fluvoxamine: Role of sigma-1 receptors, IP3 receptors and cellular signaling pathways. *Plos One* 2008;3: DOI: 10.1371/journal.pone.0002558
- 47 Toth-Fejel SE, Cheek J, Calhoun K, Muller P, Pommier RF: Estrogen and androgen receptors as comediators of breast cancer cell proliferation. *Arch Surg* 2004;139:50-54.
- 48 Nephew KP, Sheeler CQ, Dudley MD, Gordon S, Nayfield SG, Khan SA: Studies of dehydroepiandrosterone (DHEA) with the human estrogen receptor in yeast. *Mol Cell Endocrinol* 1998;143:133-142.
- 49 Widstrom RL, Dillon JS: Is there a receptor for dehydroepiandrosterone or dehydroepiandrosterone sulfate? *Semin in Reprod Med* 2004;22:289-298.
- 50 Vanparys C, Hectors TLM, Blust R, De Coen W: Mechanistic profiling of the cAMP-dependent steroidogenic pathway in the h295r endocrine disrupter screening system: New endpoints for toxicity testing. *Toxicol Lett* 2012;208:174-184.
- 51 Manna PR, Wang XJ, Stocco DM: Involvement of multiple transcription factors in the regulation of steroidogenic acute regulatory protein gene expression. *Steroids* 2003;68:1125-1134.
- 52 Saxena M, Williams S, Tasken K, Mustelin T: Crosstalk between cAMP-dependent kinase and MAP kinase through a protein tyrosine phosphatase. *Nat Cell Biol* 1999;1:305-311.
- 53 Ren X, Noda Y, Mamiya T, Nagai T, Nabeshima T: A neuroactive steroid, dehydroepiandrosterone sulfate, prevents the development of morphine dependence and tolerance via c-fos expression linked to the extracellular signal-regulated protein kinase. *Behav Brain Res* 2004;152:243-250.