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RESEARCH ARTICLE

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Human chorionic gonadotropin (hCG) regulates the expression of Steroidogenic acute regulatory protein (StAR) via the ERK1/2 pathway

Abstract It has previously been shown that Human Chorionic Gonadotropin (hCG) can stimulate steroidogenesis in Leydig cells. In the present study, the mechanisms of hCG–stimulated steroidogenesis in Leydig cells of immaturated pigs were investigated. It was found that both hCG and 8-Br-cAMP could enhance the expression level of both the Steroidogenic acute regulatory protein (StAR) and mRNA, and increase the activity of extracellular signal-regulated kinase1/2 (ERK1/2) significantly depending on stimulating time. However, the effect of 8-Br-cAMP was more significant than that of hCG. While appending the inhibitor of Protein Kinase A (PKA) to Leydig cells in culture, the expression level of StAR protein, mRNA and the activity of ERK1/2 began to drop significantly, but the level of StAR mRNA could still be detectable. While appending the inhibitor of MAPK (PD98059), the expression level of StAR protein and mRNA declined significantly. These results infer that at the beginning of hCG stimulation, hCG increases the level of StAR protein by cAMP-PKA. With prolonged stimulating time, hCG increases the level of StAR protein through cAMP-PKA-ERK1/2.

Keywords human chorionic gonadotropin (hCG), steroidogenic acute regulatory protein cAMP, ERK

1 Introduction

In the process of steroid synthesis, human chorionic gonadotropin (hCG) could induce Leydig cells to produce testosterone. According to Clark and Stocco (1997), the action of hCG in regulating testosterone synthesis was performed in two ways: its acute effect could be shown in a few minutes, or a chronic-effect could be observed at least in a few hours. In the chronic period, hCG increased transcriptional level of steroid synthetase to enhance testosterone synthesis. However, in the acute period, hCG would increase testosterone synthesis mainly by influencing the transfer of cholesterol from the outer membrane to the mitochondrial intimal membrane. Recent research suggests that Steroidogenic acute regulatory protein (StAR) was the key regulatory factor in the process of transferring cholesterol from the mitochondrial outer membrane to the inner membrane (Stocco, 1997). Our results also showed that hCG could regulate the testosterone synthesis in vitro in Leydig cells by affecting the level of StAR, but it was still unclear how hCG regulated the expression of StAR (Wang et al., 2005).

The effect of hCG in testosterone synthesis was mediated through receptors. Although there was no specific receptor of hCG in Leydig cells, the affinity of hCG binding Lutropin Hormone (LH) receptor was high and could activate G protein through the cAMP signal pathway when combined with the receptor. Cooke (1999) found that besides the cAMP signal pathway, gonadotropin also transmitted signals through the inflow of Ca^{2+}, Cl^{-} and phosphoinositol signaling, although these processes could not participate in steroid synthesis. Dewi et al. (2001) thought that hCG increased the level of cAMP in human luteining cells and activated ERK1/2(extracellular signal-regulated kinase) in a time- and dose-dependent way. The effect of hCG could be repressed by a protein kinase A (PKA) inhibitor, which indicated that hCG activated ERK1/2 through the cAMP-PKA signal pathway. It was not clear whether the role of hCG was important in activating cAMP-PKA-ERK1/2 in Leydig cells and whether activation of the signaling way had an influence on the expression of StAR. In this experiment, Leydig cells from piglets of 2–3 weeks old were used to study whether hCG participated in the regulation of StAR gene expression and the relation between the two signal pathways.

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2 Materials and methods

2.1 Materials

Experimental materials were DMEM/F-12Hams (Hyclone), DL-α-Tocopherol (Merck), Insulin-Transferring-Sodium (Roche), Sodium Bicarbonate (Sigma), collagenase type I (Roche), ^{125}I-testosterone determination kit, ^{3}H-cAMP determination kit (Chinese Institute of Atomic Energy), hCG (Penglai Huatai CO., LTD), biotinylation rabbit IgG produced in goat and Alkaline phosphatase strepatvielin (Zhongshan Biotechnology CO., LTD, China), Trizol (Dingguo Biotechnology CO., LTD, China), AMV reverse transcriptase, *Taq* enzyme and DNA marker (Promega), StAR antibody kindly provided by professor Stocco DM (Texas Tech University Science Center, Texas, U.S.A.), Antibody of *p*-ERK(E-4) (SantaCruz), and PD98059 (Calbiochem), Protein Kinase A Heat Stable inhibitor (Calbiochem) and Isoform α (Calbiochem). Testes of Changbai piglets of 2–3 weeks old were collected at Chongqing.

2.2 Cell cultures

Leydig cells were prepared from immature porcine testes (2–3 weeks old) by collagenase treatment according to Wang et al. (2005). Briefly, decapsulated testes were minced and washed in DME/Ham's F-12 medium collagenase dissociation (0.5 mg/mL, 90 min at 32°C), and cells were washed by centrifugation (1 500 r/min for 10 min). The pellet was then resuspended and subjected to two successive sedimentations for 5 and 15 min. The crude interstitial cells were recovered from the supernatants and placed in cell filters (0.5 mm). The purity of Leydig cells was more than 90% as determined by histochemical 3β-hydroxysteroid dehydrogenase staining. Leydig cells were plated in Falcon and cultured at 32°C under a humidified condition of 5% CO_2, 95% air in DME/Ham's F-12 medium (1:1) containing sodium bicarbonate (1.2 mg/mL), 15 mm 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), and gentamicin (20 mg/mL). This medium was supplemented with insulin (2 mg/mL), transferrin (5 mg/mL), and α-tocopherol (10 mg/mL).

2.3 Immunoblot

According to the process introduced by Wang et al. (2005), the protein and the RNA of the cells collected were extracted, with protein concentration determined by the method of Bradford. The gel was electrophoresed under the condition of concentration gel 15 mA and separation gel 15 mA in about 1.5 and 3–4 h respectively. The gel was later transferred for 2 h under a voltage of 45 V, referring to the way of immunoblot stated by Mauduit et al. (1998), and compared with the OD value of 0 min as the standard to determine the results of the photodensitometry. The concentration of the antibody of anti-StAR protein, P-ERK and secondary antibody was 1:500, 1:350, 1:5 000, respectively.

2.4 RT-PCR

The hexamer primers (5 μmol/L) were selected randomly. The first chain was obtained by the effect of dNTP (0.2 μmol/L), M-MLV (10 U/μL), RNasein inhibitor (1 μL), and RNA (3 μg) at 37°C for 1 h. PCR reaction was performed with cDNA (1 μL), *Taq* (0.01 U/μL), dNTP (50 μmol/L), and the selected primer (2 μmol/L), and the total volume of the reaction system was 25 μL. The product was electrophoresed on 1.5% AG. The primer of the StAR gene was designed according to the records of Mauduit (1998). With β-actin as the internal standard, a relative abundance of the StAR mRNA was determined by calculating the ratio of StAR/β-actin. The PCR reaction condition was the same as that stated by Wang et al. (2005).

2.5 Statistic analysis of data

Each treatment was designed with three replications, and each trial was repeated three times. The results were represented by $\bar{x} \pm \mathrm{SD}$ and the data were compared with *F*-*Q* or *t* test.

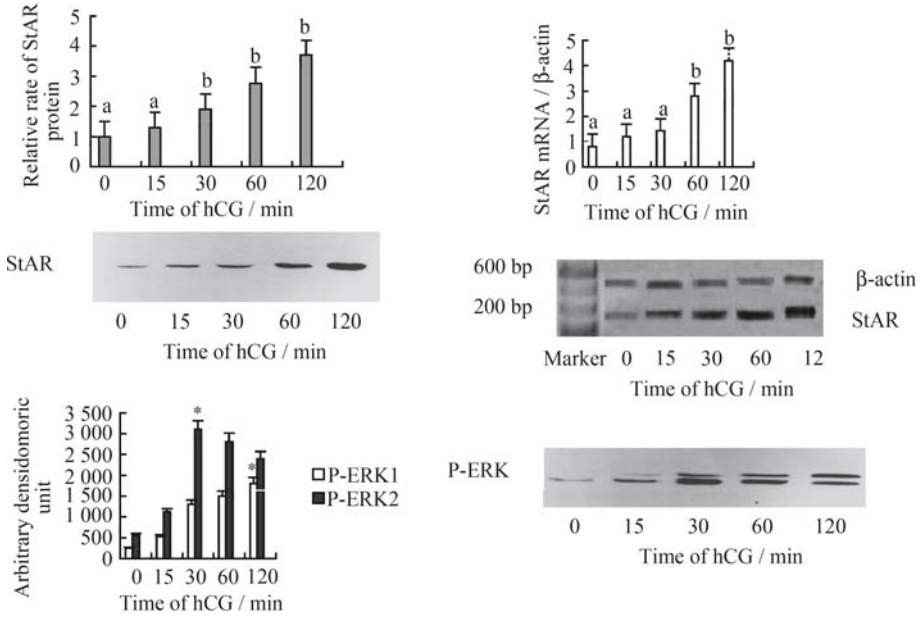
3 Results

3.1 Influence of hCG on the expression of StAR, StAR mRNA and the activity of ERK1/2

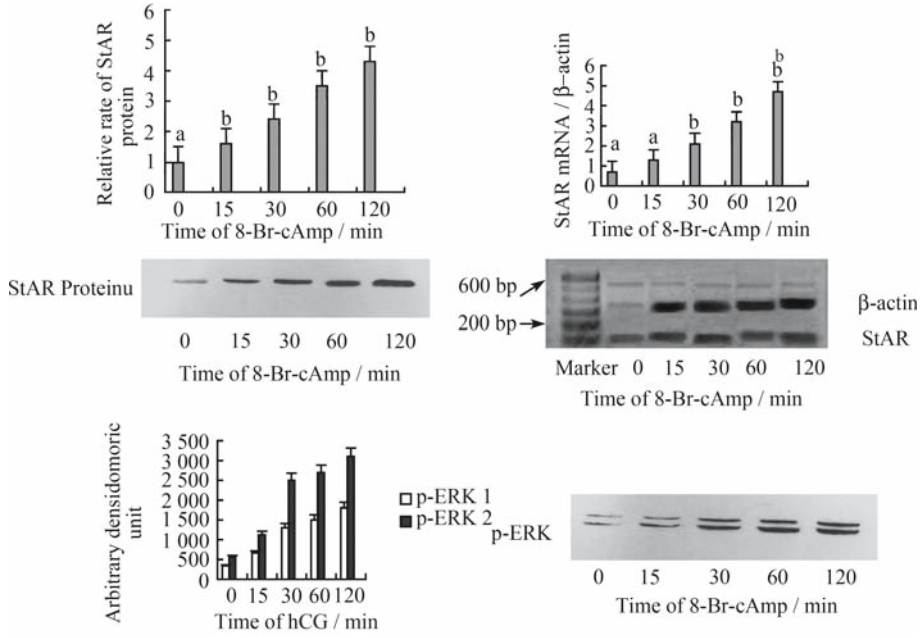
With the increase of stimulus duration, the concentration of the StAR protein was increased gradually .The difference between 0 and 30 min was striking ($P < 0.05$), and StAR mRNA concentration was getting higher with the lengthening of time. However, the difference between 0 and 30 min was not striking, but significant between 0 and 60 min (Fig. 1). There were two isomer ERKs: ERK1 and ERK2. The activity of P-ERK1 (the band of the larger molecular weight) slowly increased, while the concentration of P-ERK2 (the band of the smaller molecular weight) increased more quickly. At 60 min, the concentration of P-ERK2 began to decrease, but the tendency of the total activity of ERK was still increasing.

3.2 Effect of 8-Br-cAMP to StAR protein, mRNA and ERK1/2 activity

With an increase of 8-Br-cAMP stimulus duration, the concentration of the StAR protein increased gradually. The effect of 8-Br-cAMP was faster than that of hCG. The difference between 0 min and 15 min was striking ($P < 0.05$). As for StAR mRNA, its concentration was getting higher with the elongation of time. However, the difference between 0 and 15 min was striking, and more significant between 0 and 60 min (Fig. 2). The activity of P-ERK1 (the band of the largest molecular weight) increased slowly, while the concentration of P-ERK2 (the band of the smallest molecular weight) increased quickly. However, the tendency of the total activity of ERK was still increasing.



Notes: a, b and * denote statistical difference at $P < 0.05$ level of significance.
Fig. 1 Effect of hCG on StAR mRNA, protein and ERK1/2 activity



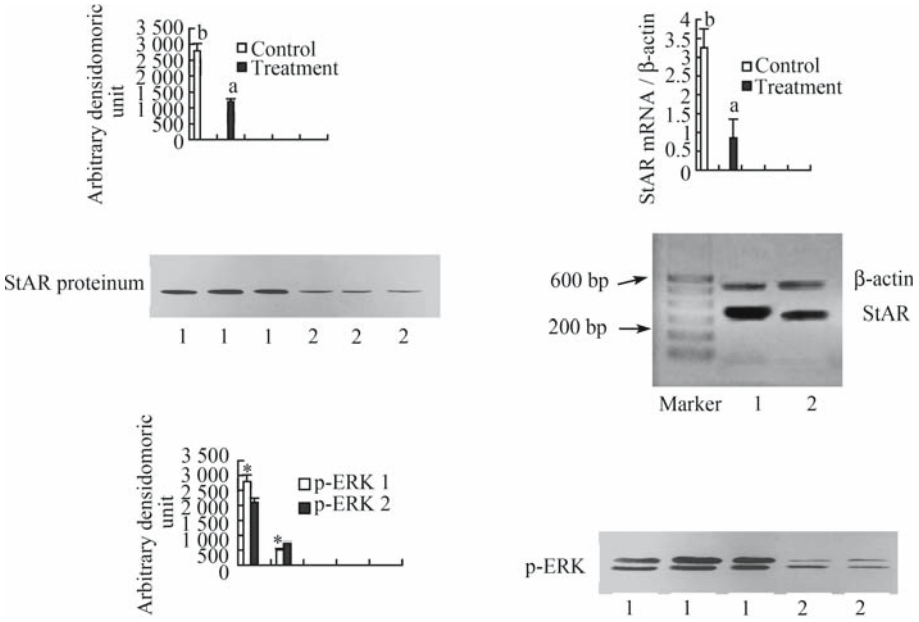
Notes: a and b denote statistical difference at $P < 0.05$ level of significance.
Fig. 2 Effect of 8-Br-cAMP on StAR mRNA protein and ERK1/2 activity

3.3 Influence of PKI on StAR mRNA , StAR protein and the activation of ERK1/2

When adding a PKA inhibitor to Leydig cells, the quantity of StAR protein decreased significantly. The concentration of StAR protein was only 40% of the control group ($P < 0.01$) (Fig. 3). On the other hand, the level of StAR mRNA also decreased. The relative abundance of StAR mRNA in the control group was (3.3 ± 0.51), but fell (0.85 ± 0.05) after adding PKI, which decreased by 74.2% ($P < 0.01$). After two hours, the activity of P-ERK1 and P-ERK2 all decreased significantly.

3.4 Influence of PD 98059 on StAR protein and StAR mRNA

PD98059 could permeate the cell envelope and inhibit MEK1/2, which was the upstream kinase of ERK. When adding PD98059 to Leydig cells, the activation of ERK was blocked. In this experiment, PD 98059 could decrease the

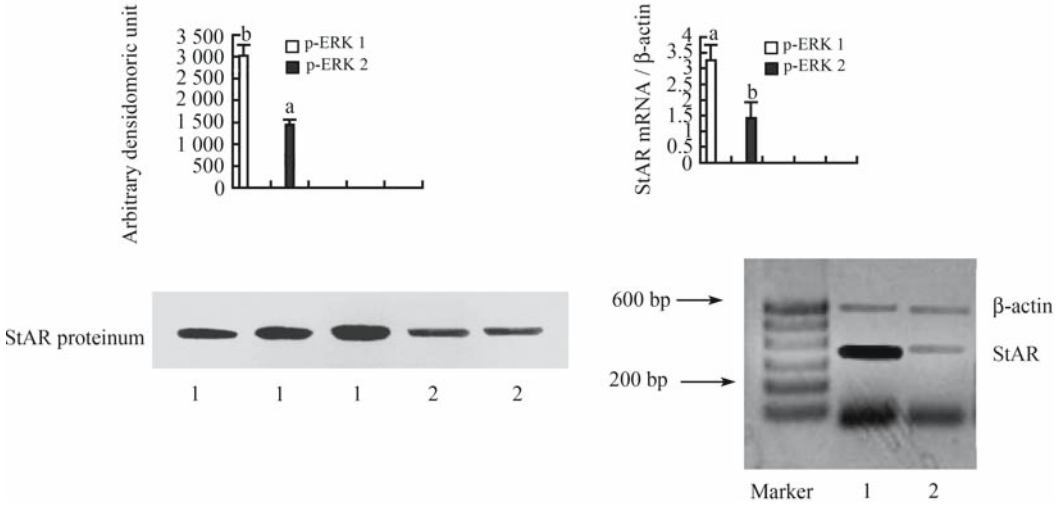


Notes: 1 and 2 represent control and treatment respectively; a, b and * denote statistical difference at $P < 0.05$ level of significance.
Fig. 3 Effect of PKI on StAR mRNA, protein and ERK1/2 activity

quantity of StAR protein significantly by 52.3% of the control ($P < 0.01$). Moreover, the level of StAR mRNA also decreased by 56.7% ($P < 0.01$) compared with that in the control group.

4 Discussion

In the steroid synthesizing cell, the mRNA of StAR was initially translated to the precursor of 37 ku (p37). p37 had three sites of phosphorylation, two of which were regulated by PKA/calmodulin kinase II, and the rest was regulated by PKA under the function of which phosphorylation was done, forming pp37 and pp30. pp30 was the active form of StAR. Thus, the capability of transferring cholesterol in Leydig cells could be reflected directly by detecting the change in 30 ku. Irina et al. (2001) found the main function of Br-cAMP was promoting the StAR phosphorylation in the acute period, but Br-cAMP had no significant effect on translation and processing of StAR in 15 min. The increase in cholesterol transfer might result from the formation of pp30. In this experiment, we discovered that with an increase of stimulus time, hCG and 8-Br-cAMP could increase the quantity of maturation protein 30 ku without any obvious change in StARm RNA. The results indicated that at the initial stage, the site of hCG and 8-Br-cAMP regulated the content of pp30 mainly after being translated. This supported the result of Irina et al. (2001). Therefore, we considered that the role of hCG in



Notes: 1 and 2 represent control and treatment, respectively; a and b denote statistical difference at $P < 0.05$ level of significance.
Fig. 4 Effect of PD 98059 on StAR mRNA protein

the initial period was to phosphorylate the site of Ser/Thr via cAMP-PKA, produce pp37, and convert to pp30, which promoted the transfer of cholesterol and the synthesis of testosterone through the lysis of mitochondrion protein.

The cis-acting element of SREBP, SF-1, Sp1, C/EBP, GATA-4, DAX-1 and other transcription factors resided in the 5'noncoding region of StAR. Adjusting the activity of those transcription factors could affect the transcription of the StAR gene (Tremblay and Viger, 2001). In our study, we found that the quantity of StAR mRNA increased gradually while extending the effective time of hCG (50 IU/mL) and 8-Br-cAMP (3×10^{-3} mol), which indicated that increasing hCG and 8-Br-cAMP stimulating duration could accommodate the content of StAR by affecting the transcription. Dewi et al. (2001) discovered that in human luteining granular cells, hLH, hCG, choleratoxin (CT), forskolin and (Bu) 2cAMP could activate ERK1/2. The activation of ERK1/2 would be repressed with pretreating cells with PKA inhibitor. ERK1/2 inhibitor, PD 98059 and U 0126 could inhibit the activation of ERK1/2 in the inducting period of hLH or hCG, but could not affect the secretion of progesterone under the stimulation of hLH, hCG or 22R-oxycholesterol. This indicated that the activation of ERK1/2 could impact the expression of StAR. Moore et al. (2001) found that in the process of FSH stimulating, U0126 could increase the expression of StAR and the secretion of progesterone, but inhibit the secretion of estradiol in the primary cultured rat granulosa cell. We also found that cAMP-PKA could adjust the level of StAR mRNA related to the activation of ERK1/2. When inhibiting the activity of ERK1/2, the level of StAR mRNA could be regulated by inhibiting PKA activity, which proved that cAMP-PKA could accommodate the activity of the transcription factor through ERK1/2 and participate in the transcription regulation of StAR.

The effect of ERK1/2 in the genetic expression of StAR was not the same as in different experiments. Seger et al. (2001) found that hCG could activate the ERK in rLHR-4 through cAMP-PKA, and the inhibitor of ERK could promote the expression of StAR. Tajima et al. (2003) found that in highly luteinized granulosa cells, GTH would activate the ERK cascade. PD98059 and UO126 could increase the level of progesterone, and StAR mRNA could activate SF-1 and DAX-1. Our study also indicated that the activation of ERK promoted expression of the StAR gene. Our results confirmed that hCG could activate ERK through cAMP-PKA, but the effect on the expression of the StAR gene was quite different, reflecting activation of the passive and positive transcription factor. In our experiment and Tajima's experiments, the stimulating effect of tropic hormones had not reached the maximum, and the positive regulatory factor had an important role in the process. The activation of ERK promoted expression of the StAR gene, the production of steroids through increased activity of SP-1, and other positive regulatory factors. In Seger's (2001) experiment, the high content of steroid under the stimulation of hCG damaged the cells, activating in passive factors in cells. The activated ERK would activate DAX-1 and other unknown inhibitive factors. DAX-1 combined with the hairpin structure in 5'flanking sequence of the StAR gene, interfering with the transcription. The activated DAX-1 also affected SF-1 directly, which inhibited transcription of the StAR gene (Reinhart et al., 1999).

In our tests, we also discovered that the inhibitor of ERK1/2 could not suppress the effect of PKA, which indicated that the effect of cAMP-PKA was carried out partly through ERK1/2 cascade. However, it was not clear whether there was another protein kinase in the process. On the other hand, the inhibitor of PKA did not inhibit StAR transcription, although it was not clear whether it depended on PKA completely. Possible reasons were the pathway independence on PKA, or basic transcript activity in Leydig cells. The answer to these questions would be helpful in understanding the expression and regulatory mechanism of StAR genes.

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