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·Original Article·

Cox7a2 mediates steroidogenesis in TM3 mouse Leydig cells

Liang Chen[1], Zhong-Cheng Xin[1], Xin Li[2], Long Tian[1], Yi-Ming Yuan[1], Gang Liu[1], Xue-Jun Jiang[3], Ying-Lu Guo[1]

[1]*Andrology Center of Peking University First Hospital, Beijing 100009, China*
[2]*Department of Surgery, People's Hospital of Zhangqiu, Zhangqiu 250200, China*
[3]*Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China*

Abstract

Aim: To investigate the regulatory function of Cox7a2 on steroidogenesis and the mechanism involved in TM3 mouse Leydig cells. **Methods:** The cDNA of Cox7a2 was cloned from TM3 mouse Leydig cells. It was subcloned to pDsRed-Express-N1 and transfected back into TM3 mouse Leydig cells for Cox7a2 overexpression by transient gene transfection. Steroidogenesis affected by overexpressed Cox7a2 was studied by ELISA. To elicit the mechanism of this effect, expression of steroidogenic acute regulatory (StAR) protein and reactive oxygen species (ROS) were examined by Western blot and fluorometer, respectively. **Results:** The cDNA of Cox7a2 (249 bp) was cloned from Leydig cells and confirmed by DNA sequencing. After constructed pDsRed-Express-N1-Cox7a2 was transfected back into TM3 mouse Leydig cells, Cox7a2 inhibited not only luteinizing hormone (LH)-induced secretion of testosterone but also the expression of StAR protein. At the same time, Cox7a2 increased the activity of ROS in TM3 mouse Leydig cells. **Conclusion:** Cox7a2 inhibited LH-induced StAR protein expression, and consequent testosterone production, at least in part, by increasing ROS activity in TM3 mouse Leydig cells. *(Asian J Androl 2006 Sep; 8: 589–594)*

Keywords: Cox7a2; fusion protein; steroidogenesis; steroidogenic acute regulatory protein; reactive oxygen species; Leydig cell

1 Introduction

Progressive decrease of testosterone production is a common phenomenon in aging men, and is accompanied by a series of clinical manifestations, such as decreased libido, erectile dysfunction (ED), osteoporosis, fatigue, depression and alterations in mood and cognition [1, 2]. The syndrome of these clinical symptoms is referred to as partial androgen deficiency in aging male (PADAM) [2, 3]. Although it has been suggested that the decline of serum testosterone plays a major role in PADAM, other factors might also be involved [3–6]. Leydig cells are the main producer of testosterone in mammalian testes; however, the mechanism underlying the age-related functional deficiency of Leydig cells in steroidogenesis remains unclear [2–4].

It is well established that mitochondria contain proteins and enzymes essential for testosterone biosynthesis. For example, steroidogenic acute regulatory (StAR) protein, which mediates the rate-limiting step in steroidogenesis, is located in the inner membrane of mitochondria [7–9].

During mitochondrial electron transport reactions, the by-products, reactive oxygen species (ROS), can be produced and cumulated continuously [4–6]. Prolonged exposure to ROS can cause cumulative oxidative damage, which is thought to be one of the major causes of cellu-

Correspondence to: Dr Zhong-Cheng Xin, Andrology Center of Peking University First Hospital, Beijing 100009, China.
Tel/Fax: +86-10-6618-2822
E-mail: xinzc@bjmu.edu.cn
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lar aging and reductive ability to produce testosterone in Leydig cells [5, 6].

Cox7a2 is the seventh subunit of the nuclear-coded polypeptide chains of cytochrome c oxidase in the mitochondrial respiratory chain [10, 11]. Published studies demonstrate that *Cox7a2* gene is significantly upregulated in testes of aged men [10], but the connection between the increase of Cox7a2 and the decrease of testosterone production in testes of aged men is unknown.

The aim of the present study is to investigate the effect of Cox7a2 on steroidogenesis and the involved mechanism. TM3 mouse Leydig cells (TM3 cells) are used for the study of steroidogenesis because these cells express luteinizing hormone (LH) receptors and StAR protein and are responsive to LH [12, 13]. Cox7a2 overexpressed in TM3 cells by transient transfection of recombinant Cox7a2 cDNA plasmid. LH-induced testosterone production is observed in these cells overexpressing Cox7a2. To elicit the influence of Cox7a2 on steroidogenesis, ROS level and the expression of StAR protein are also measured.

2 Materials and methods

2.1 Materials and apparatus

TM3 mouse Leydig cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Oligo-dT and Pfu DNA polymerase were obtained from Promega (Madison, WI, USA). Restriction endonucleases were from New England Biolabs (Beverly, MA, USA). Recombinant plasmid DNA purification kit and effectene transfection reagent were from Qiagen (Valencia, CA, USA). CM-H2DCFDA was from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Fluorescence imaging microscopy system (ECLIPSE TE2000) was the product of Nikon (Tokyo, Japan). Rabbit anti-StAR antibody was from Santa Cruz (Santa Cruz, CA, USA). Testosterone ELISA kit was from DRG (Marburg, Germany). LH was bought from Sigma (St. Louis, MO, USA).

2.2 Methods

2.2.1 Plasmid construction

TM3 cells were cultured in F12-DME medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 5% horse serum and 2.5% fetal calf serum. 5.0×10^6 cultured TM3 cells were washed with PBS solution and were collected by centrifugation (1 000 × *g* for 3 min). Total RNA of TM3 cells was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Three micrograms of total RNA were reversely transcribed to first strand cDNA using oligo (dT) as the primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA) [14]. The coding region of mouse Cox7a2 cDNA was obtained by polymerase chain reaction (PCR) using the first strand cDNA as the template. The forward primer was 5'-gaagatctatgttgcggaatctgctggcc-3' and the reverse primer was 5'-cggaattcgcgttctgcttcttgggga-3'. After being digested with EcoRI and BglII, the PCR product was cloned into the plasmid pDsRed-Express-N1 (BD Biosciences Clontech, Franklin Lakes, NJ, USA). The recombinant plasmid was further confirmed by dideoxynucleotide sequencing.

2.2.2 Cell transfection

TM3 cells were cultured in the above medium and split into 60 mm dishes. When the cells reached 65% confluence, the transfection was performed using effectene transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, with vector as a control. Cox7a2-pDsRed-Express-N1 (1.5 μg) and pDsRed-Express-N1 (1.5 μg) were diluted separately in Buffer EC (the enhancer and the DNA-condensation buffer) to a total volume of 150 μL and 8 μL enhancer was added and mixed by vortexing for 1 s. After incubating at 20ºC for 5 min, the 25 μL effectene transfection reagent was added to the DNA-enhancer mixture and the mixture was vortexed for 10 s. During the waiting time, the growth medium of the plates was gently aspirated and 3 mL of fresh growth medium was added to the cells. While being incubated at 20ºC for 10 min, 1 mL growth medium was added to the transfection complexes, which were then added to the cells in the 60 mm dishes. Finally, the cells with the transfection complexes were incubated under an atmosphere containing 95% air and 5% CO_2 at 37ºC. After transfected for 12 h, the cells were treated with LH 12 h (100 ng/mL) for another 12 h. Then the red fluorescence was acquired through Cy3 channel (excitation: 557 nm; emission: 579 nm). The transfection efficiency was estimated by cell counting under fluorescence microscope, and Cox7a2 expression was determined by reverse transcriptase (RT)-PCR (forward primer: 5'-cgtcagattgggcagag-3'; reverse primer: 5'-ggaaatgaagccacagc-3'). The same procedure as described above were performed for total RNA extraction and RT-PCR. The cell transfection was repeated for three times.

2.2.3 Evaluation of StAR protein and testosterone production

Samples of culture medium in process 2.2.2 were as-

sayed for testosterone release using a testosterone ELISA kit (DRG, Marburg, Germany), according to the manufacturer's instructions. Testosterone level was normalized to protein concentration for each sample and was expressed as nanogram/mL media. Each sample was quadruplicated for testosterone measurement and the experiment was repeated for three times. Part of the cells was lysed in cold TGH lysis buffer (1% Triton X-100, 10% glycerol, 20 mmol/L Hepes [pH 7.4], 100 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L Na_3VO_4 and 50 mmol/L NaF). The lysate was separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was incubated in a blocking buffer (Tris Buffered Saline [TBS] containing 5% nonfat dry milk) for 1 h, followed by wash with TBS containing 0.1% Triton X-100 (TBST) (3 × 10 min). Subsequently, the membranes were probed with rabbit anti-StAR antibody (incubation overnight at 4ºC, 1:500 TBST, 1% milk), rabbit anti-β-actin antibody (incubation overnight at 4ºC, 1:5 000 TBST, 1% milk). The secondary antibody was the horseradish peroxidase conjugated goat anti-rabbit IgG antibody. Blotted antibodies were then visualized by enhanced chemoluminescence (Amersham, Piscataway, NJ, USA). The experiment was repeated for three times. To determine the linear range of the chemiluminescence signals, several X-ray radiographs were analyzed, and densitometry was performed for quantification by using AlphaImager 2200 and Alpha Ease image software (Alpha Technology, San Francisco, CA, USA). StAR protein expression level was normalized to that of β-actin and was expressed as the optical density ratio of the StAR band to the β-actin band for further analysis.

2.2.4 Measurement of reactive oxygen species (ROS) production

CM-H2DCFDA (Invitrogen, Carlsbad, CA, USA), an acceptable cell-permeant indicator of ROS, was used to assess the ROS level within the TM3 cells [5]. Cells in process 2.2.2 were harvested by centrifugation (1 000 × *g* for 3 min) and washed in serum free medium (pH 7.2, buffered in Hepes) three times. The cells were re-suspended in serum free culture medium to 10^5/mL. The cells were added 2 μL of 0.5 mmol/L CM-H2DCFDA (final concentration) and incubated at 37ºC for exact 10 min. Finally, the cells were plated into the 96-well plate. The cells incubated with 1 mmol/L H_2O_2 for 30 min at 37ºC were used as positive controls [15, 16]. The relative level of ROS within the cells was analyzed by microplate fluorometer (excitation: 488 nm, emission: 535 nm) (BMG labtechnology, Offenburg, Germany), and the data were expressed as the relative numerical values by detecting the increase of fluorescence. Each sample was quadruplicate for ROS measurement and the experiment was replicated three times.

2.2.5 Statistical analysis

Data were presented as means ± SME of testosterone production and ROS relative level in three independent experiments carried out in quadruplicate under each sample or of the ratio of StAR to β-actin integrated density of three separate experiments. To compare the significance of the two means obtained from LH-treated and LH-untreated samples, statistical analysis was performed using the unpaired *t*-test. The significant differences between various means (more than two means) were determined by analysis of variance using the statistical package SPSS 11.0 for Windows (SPSS, Chicago, IL, USA). $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Expression of fusion fluorescence protein in TM3 cells

An image of red fluorescence Cox7a2 was observed 24 h post transfection, as shown in Figure 1. Among 200 cells counted, 85% of them were stained with red fluorescence. The transfection efficiency of the three separate experiments was similar to each other. The expression of Cox7a2 was also determined by RT-PCR. An approximate four-fold increase of expressing Cox7a2 was demonstrated (data not shown).

3.2 Cox7a2 inhibited LH-induced increase of testosterone synthesis

Upon overexpressed Cox7a2, we examined the testosterone synthesis in TM3 cells in presence or absence of LH. Testosterone synthesis induced by LH stimulation was inhibited in TM3 cells overexpressing Cox7a2 ($P < 0.01$, compared with non-transfected and vector-transfected TM3 cells with LH stimulation). Testosterone concentration in the media did increase dramatically in non-transfected or vector-transfected TM3 cells with LH stimulation ($P < 0.01$, compared with their respective counterparts without LH stimulation) (Figure 2). Each experiment was performed independently for three

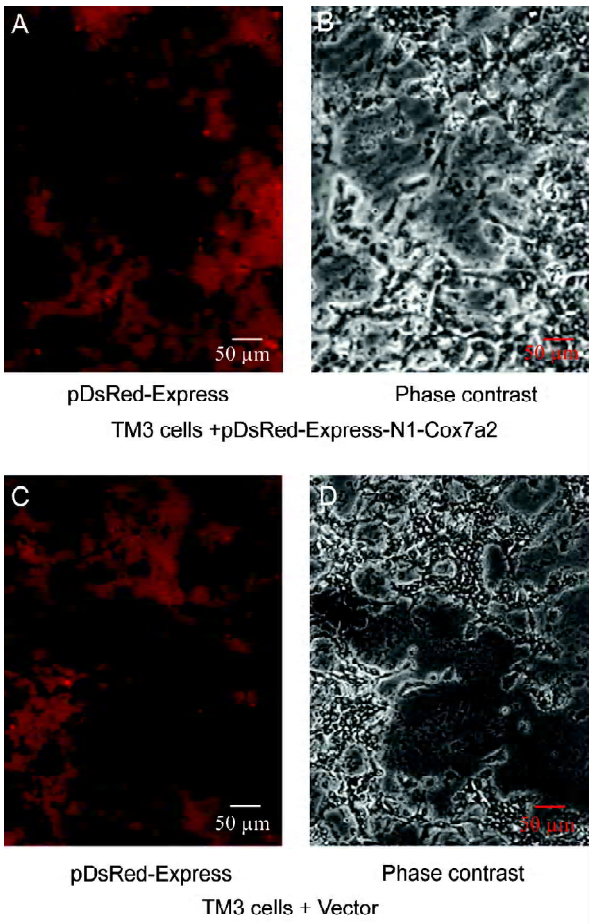

Figure 1. Expression of Cox7a2 fusion fluorescence protein was investigated by living cell fluorescence imaging. TM3 mouse Leydig cells (TM3) were transfected with the recombinant plasmid. Cells were split onto coverslip and living cells images were acquired by fluorescence microscopy via Cy3 channel 24 h post transfection, showing a microscopic field of fluorescence (A) and phase contrast (B) images. (C) and (D) showed the expression of the vector fluorescence protein and phase contrast images. By cell counting, approximately 85% cells expressed the fusion protein. Scale bar = 50 μm.

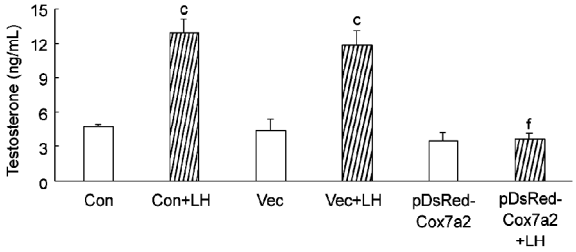

Figure 2. Cox7a2 inhibited testosterone production in TM3 mouse Leydig cells (TM3 cells). Data are represented as mean ± SME of three independent experiments. $^{c}P < 0.01$ (in non-transfected and vector-transfected cells), compared with their respective counterparts; $^{f}P < 0.01$ (in Cox7a2-overexpressed cells), compared with the vector-transfected and non-transfected cells with luteinizing hormone (LH) stimulation. Con, control, non-transfected cells; Vec, vector-transfected cells.

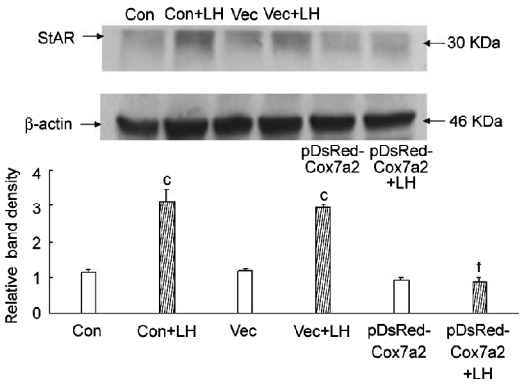

Figure 3. Cox7a2 suppressed the expression of steroidogenic acute regulatory (StAR) protein in TM3 cells. TM3 cells with transfection of either Cox7a2 or vector were lysed and immunblotted with antibodies indicated. Each StAR and β-actin band in three separate Western blot results were quantitated by densitometric scanning. The optical density ratio of StAR bands to β-actin bands is presented. Data are represented as mean ± SME of three independent experiments. $^{c}P < 0.01$ (in non-transfected and vector-transfected cells), compared with their respective counterparts; $^{f}P < 0.01$ (in Cox7a2-overexpressed cells): compared with the vector-transfected and non-transfected cells with luteinizing hormone (LH) stimulation. Con: control, non-transfected cells; Vec, vector-transfected cells.

times and the results were similar.

3.3 Cox7a2 inhibited LH-induced increase of StAR protein

StAR protein mediates the transfer of cholesterol from outer mitochondrial membrane to inner mitochondrial membrane where the cholesterol is cleaved to pregnenolone, a rate-limiting step in testosterone synthesis [7–9]. Therefore, we investigated the amount of StAR protein in TM3 cells overexpressing Cox7a2 in presence or absence of LH. Expression of StAR protein did increase considerably in non-transfected or vector-transfeced TM3 cells with LH stimulation ($P < 0.01$, compared with their respective counterparts without LH stimulation). The induced increase of StAR protein by LH stimulation did not occur any more in TM3 cells overexpressing Cox7a2 ($P < 0.01$, compared with non-transfected and vector-transfected TM3 cells

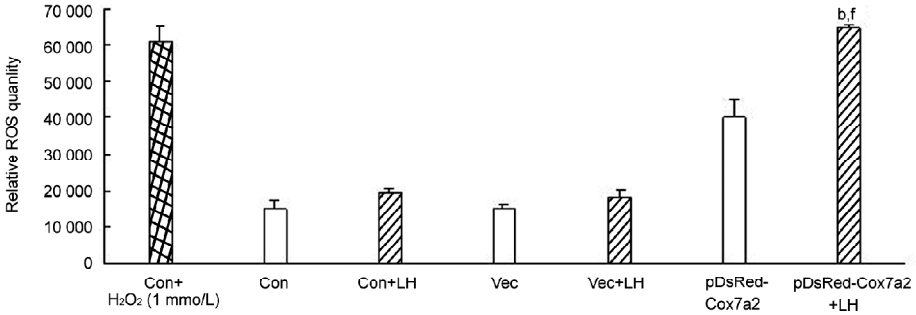

Figure 4. Cox7a2 enhanced reactive oxygen species (ROS) production in TM3 mouse Leydig cells (TM3 cells). The cells treated with H_2O_2 (1 mmol/L, 30 min) were used as positive controls. Data are represented as mean ± SME of three independent experiments. $^bP < 0.05$, compared with TM3 cells overexpressing Cox7a2 without luteinizing hormone (LH) stimulation; $^fP < 0.01$, compared with non-transfected and vector-transfected cells with LH stimulation. Con: control, non-transfected cells; Vec, vector-transfected cells.

with LH stimulation) (Figure 3). Each experiment was performed independently for three times and the results were similar.

3.4 Cox7a2 enhanced ROS production

ROS, mainly produced in the respiratory chain of mitochondria, are involved in negatively regulating the expression of StAR protein [5, 6, 15, 16]. We examined the possible connection between ROS level and Cox7a2 overexpression. As shown in Figure 4, stimulated by LH, the level of ROS increased significantly in TM3 cells overexpressing Cox7a2 ($P < 0.05$, compared with TM3 cells that overexpressed Cox7a2 fusion protein without LH stimulation; $P < 0.01$, compared with non-transfected and vector-transfected cells with LH stimulation). In non-transfected and vector-transfected cells, the production of ROS changed little with or without LH stimulation. Each experiment was performed independently for three times and the results were similar.

4 Discussion

Testosterone production in Leydig cells depends on LH stimulation. Upon binding to its receptor, LH initiates the translocation of cholesterol into the mitochondria and triggers the process of androgen biosynthesis with the involvement of upregulated StAR protein [7–9, 17, 18]. Our findings (Figures 2, 3) on testosterone production in TM3 cells in response to LH treatment supports the previous observations. Most importantly, we find that Cox7a2 plays a role in mediating testosterone synthesis. Cox7a2 is able to inhibit androgen production induced by LH (Figure 2).

At the same time, our findings also demonstrate that Cox7a2 inhibits the expression of StAR protein induced by LH in TM3 cells (Figure 3). Considerable evidences indicate that the expression of StAR protein is the key regulatory event delivering cholesterol to the inner mitochondrial membrane, which constitutes the rate-limiting step in testosterone synthesis [6–9]. Therefore, our results suggest that the inhibiting effect of Cox7a2 on testosterone production might be, at least, a result of the disruption of the expression of StAR protein (Figure 3), that is, the disruption of the cholesterol-transferring process. StAR protein is primarily translated as a larger molecule (37 kDa) containing mitochondria targeting precursor in the amino terminus [8, 9]. During the cholesterol transferring process, the larger StAR molecule is transferred into mitochondria and transformed into the mature form (30 kDa) [8, 9]. In the present study, we used rabbit anti-StAR antibody, which recognized the 30 kDa StAR protein. Therefore, the translocation of StAR protein onto mitochondria might also be influenced by Cox7a2, which needsto be further validated.

The data suggest that the inhibiting effect of Cox7a2 on StAR protein expression might be partially involved in the enhancement of ROS level. Former studies indicate that ROS mediates mitochondrial perturbation of Leydig cell, decreases the expression of StAR protein and results in the inhibition of testosterone production [15, 16]. Our findings on TM3 cells overexpressing Cox7a2 are consistent with these observations. By analysis of ROS level, we reveal that the decrease of testosterone production and expression of StAR protein upon overexpression of Cox7a2

are possibly because of the increased production of ROS in TM3 cells (Figure 4).

However, the mechanism of steroidogenesis in Leydig cells is complicated and remains obscure so far [6, 7]. Some recent reports indicate that the peripheral-type benzodiazepine receptor is also an indispensable element mediating the delivery of cholesterol to the inner mitochondrial membrane [19, 20]. Further research should be performed to fully understand the steroidogenic process.

In summary, data presented here reveal an unknown role of Cox7a2 in the regulation of the expression of StAR protein, and in its consequent mediating androgen biosynthesis. In TM3 cells, the negative regulatory effect of Cox7a2 on steroidogenesis is, at least, a result of the increase of ROS production.

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