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Expression of steroidogenic acute regulatory protein and its regulation by interferon-gamma in rat corpus luteum

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Abstract The steroidogenic acute regulatory protein (StAR) is the key regulatory protein of steroidogenesis. *De novo* synthesis of StAR protein is required for intramitochondrial translocation of cholesterol to the cytochrome P450 side chain cleavage enzyme which is located on the matrix side of the inner mitochondrial membrane. This is the rate-limiting step of steroid biosynthesis. Using *in situ* hybridization and immunohistochemistry we studied StAR expression in various stages of the corpora luteal and its regulation by interferon-gamma (IFNγ) in the adult pseudopregnant rat. The results indicated that expression of StAR in the corpora luteal was correlated with progesteron production and IFNγ was capable of inhibiting its expression.

Keywords: corpus luteum, StAR, *in situ* hybridization, immunohistochemistry, IFNγ.

Biosynthesis of all steroids begins with the cleavage of cholesterol to form pregnenolone. This reaction is catalyzed by the cytochrome P450 side chain cleavage enzyme ($P450_{scc}$)[1,2]. An increase in the activity of the $P450_{scc}$ enzyme has been considered as a rate-limiting step in gonadotropin-induced steroidogenesis. Several subsequent observations indicated, however, that the rate-limiting step was the delivery of cholesterol to the inner mitochondrial membrane and the $P450_{scc}$ [3]. StAR is believed to be the key regulator of steroid biosynthesis[4,5]. The appearance of StAR has been found to be precisely correlated with steroid production spatially and temporally[6,7]. The expression of StAR protein in MA-10 mouse Leydig tumor cells and COS-1 cells in the absence of hormone stimulation results in a significant increase in steroid production[8,9].

IFNγ is capable of inhibiting granulosa cell differentiation and steroidogenesis in rats[10], and of affecting luteal function by enhancing prostaglindin $F_{2\alpha}$ ($PGF_{2\alpha}$) synthesis[11]. Because acute regulation of steroid production in the CL is mediated by StAR, and the expression of StAR is regulated by both cAMP[5] and $PGF_{2\alpha}$[12], IFNγ may directly influence StAR expression.

1 Materials and methods

(i) Animals. Sprague-Dawley rats were obtained from the Institute of Zoology, Chinese Academy of Sciences. To obtain corpora luteal of the pseudopregnant, adult females were mated with vasectomized males. Three females were kept together with one male and vaginal smears were carried out daily. Corpora luteal were designated day 1 (D1) of pseudopregnancy on the day when a vaginal plug was found. The animals were decapitated on D1, D2, D4, D6, D8, D10 and D13 and the ovaries were removed and fixed in formalin fluid for *in situ* hybridization and immunohistochemistry. The

groups of animals were injected s.c. with IFNγ 500, 1 000, 5 000, 10 000 IU respectively on D4, the animals were decapitated 24 h later and the ovaries were removed and fixed in formalin fluid. The ovaries were embedded in paraffin prior to sectioning (6 μm) according to the standard procedures.

(ii) *In situ* hybridization. The digoxigenin (DIG) RNA labeling kit and the reagents used for DIG detection, except noted, were purchased from Boehringer Mannheim GmbH Biochemica (Mannheim, Germany). Mouse StAR cDNA was kindly provided by Dr. Douglas M. Stocco (Texas Tech University Health Science Center, Texas, USA). The plasmids were linearized with the appropriate endonucleases and labeled by *in vitro* transcription. Both antisense and sense StAR and LHR RNA probes were labeled. The ovaries left for *in situ* hybridization were fixed in Bouin's fluid and embedded in paraffin prior to sectioning (6 μm), according to standard procedures. The deparaffinized sections were treated with 8 μg/mL proteinase K (E. Merck, Darmstadt, Germany) for 10 min and washed in PBS for 5 min. Sections were then fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS for 5 min and washed in PBS for 10 min. Before hybridization, the sections were dehydrated through a graded ethanol series and allowed to air-dry. The sections were prehybridized with 50% formamide and 2×SSC for 2 h at room temperature, then hybridized overnight with DIG labeled StAR RNA probe in hybridization buffer (10 mmol/L Tris-HCl, pH 7.5, 2 × SSC, 50% deionized formamide, 1×Denhardt's, 2.5 mmol/L DTT, 5% dextran sulfate, 250 μg/mL yeast tRNA, and 0.5% SDS) at 48℃. After hybridization, the sections were thoroughly washed in 2 ×, 1×, and 0.1 × SSC, each for 2 ×15 min at 40℃. The sections were then rinsed in DIG buffer I (0.1 mol/L maleic acid, 150 mmol/L NaCl, pH 7.5) for 5 min, and blocked with 1% blocking reagent in DIG buffer I for 1 h, incubated with alkaline phosphatase conjugated anti-DIG Ig G diluted 1 : 500 in DIG buffer I containing 1% blocking reagent for 1 h, washed in DIG buffer I for 3 × 5 min. The bound antibody was detected by a standard immuno-alkaline phosphatase reaction, using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) as substrate, for 2—6 h. The sections were dehydrated through a graded series of ethanol, cleared in xylene, and then mounted. For control hybridization, the sections were hybridized with StAR sense RNA probe.

(iii) Immunohistochemistry (ABC). Immunohistochemistry was carried out with a Vectastain ABC (avidin-biotin peroxidase) kit (Vector Laboratories, Burlingame, CA) as recommended by the manufacturer. Deparaffinized sections were incubated with 3% H_2O_2 for 10 min and then incubated with 10% normal goat serum (NGS) in PBS for 30 min. The primary antibody of StAR was diluted in PBS containing 10% NGS and incubated with the section for 1 h (control groups were incubated with 10% NGS in PBS instead of primary antibodies). Then the sections were washed in PBS three times (5 min each), incubated with biotinylated second antibody for 1 h, and washed in PBS three times (5 min each). After incubation with avidin-biotin-peroxidase complex in PBS for 1 h and washed in PBS three times for 5 min each, sections were incubated in diaminobenzidine tetrahydrochloride in 0.05 mol/L Tris-HCl (pH 7.2) with 0.01% H_2O_2 for 2—7 min. The sections were dehydrated through a graded series of ethanol, cleared in xylene, and then mounted.

2 Results and discussions

The expression of StAR antigen in the CL of pseudopregnant rat was low on D1 and D2, and increased significantly on D4, and maintained a high level on D6. The antigen decreased slightly on D8, and decreased further on D10 and thereafter (fig. 1). The expression of StAR mRNA maintained a high level from D1 to D6 and decreased from D8 to D13 (fig. 2). The changes of StAR mRNA were correlated with the changes of its protein. Treatment of the animals with IFNγ 500 IU did not affect the StAR expression as compared with that in the control group. Treatment with IFNγ 1000 IU decreased the antigen production, only high doses of the cytokine (5 000—10 000 IU) were capable of significantly inhibiting the StAR expression in the CL (fig. 3).

Our previous reports demonstrated that both StAR mRNA and protein increased steadily from D1—D4 in the CL of gonadotropin-treated rat, reached the highest level on D4 and then dropped sharply on D8 when luteolysis took place. StAR mRNA and protein levels in the CL of pregnant rats were also high during the early luteal development (D2, D4), increased even further on D9, and decreased on D13

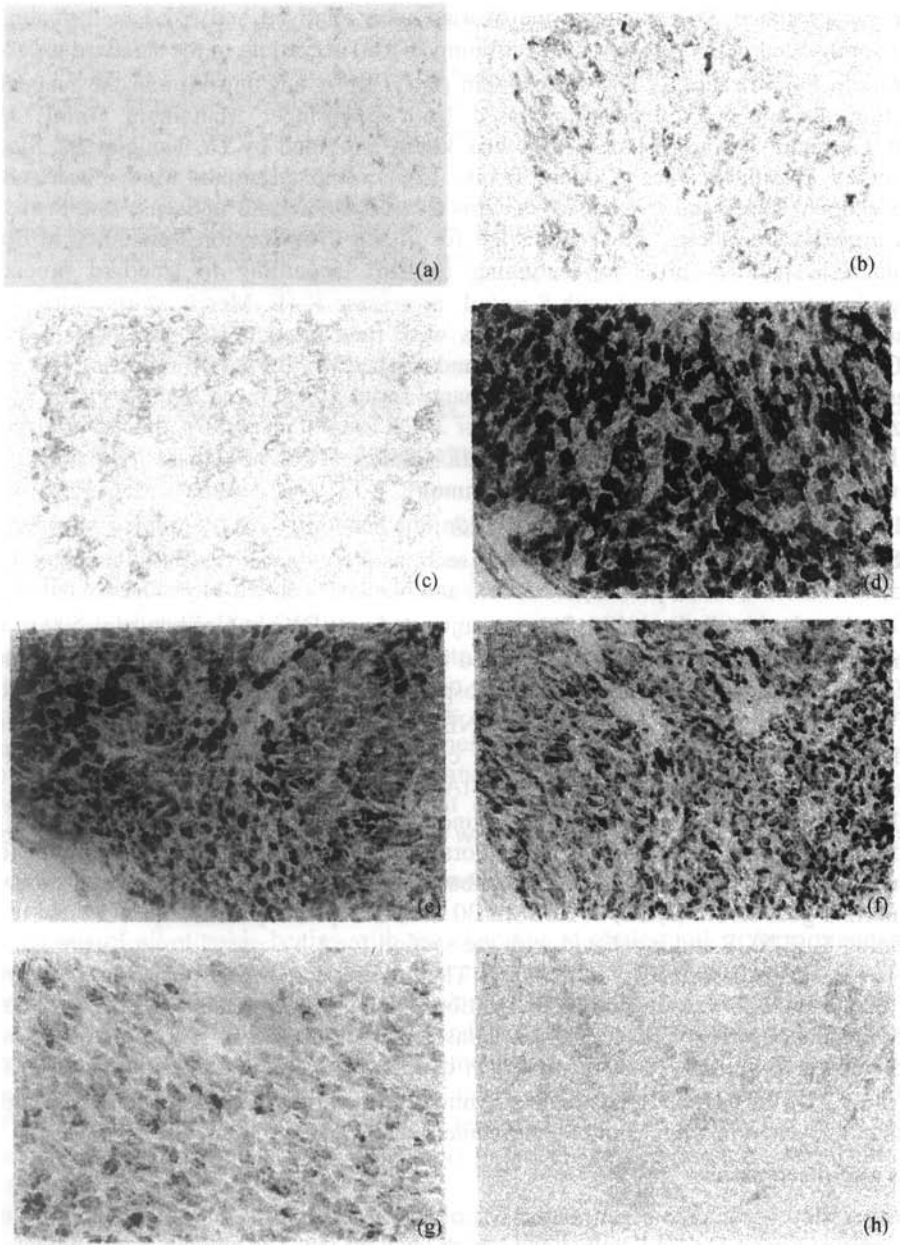

Fig. 1. Expression of StAR antigen in the CL of adult pseudopregnant rat. (a) Negative control; (b) D1; (c) D2; (d) D4; (e) D6; (f) D8; (g) D10; (h) D13, ×200.

when luteolysis took place[13]. The present results were consistent with our previous reports. The expression of StAR coincided well with the progesteron production in the CL[14]. Therefore, StAR can be regarded as a functional marker of CL development[15—17].

StAR precursor is synthesized in the cytosol with signal sequence that targets it to mitochondria and mediates its import[18]. The signal sequence is then cleaved and StAR processed to its mature form. C-terminal is thought to play an important role in transferring cholesterol[19] and it is possible that StAR plays its transmembrane role together with other factors.

The CL on D4 was most sensitive to the cytokine stimulation, that is why we chose the animals on

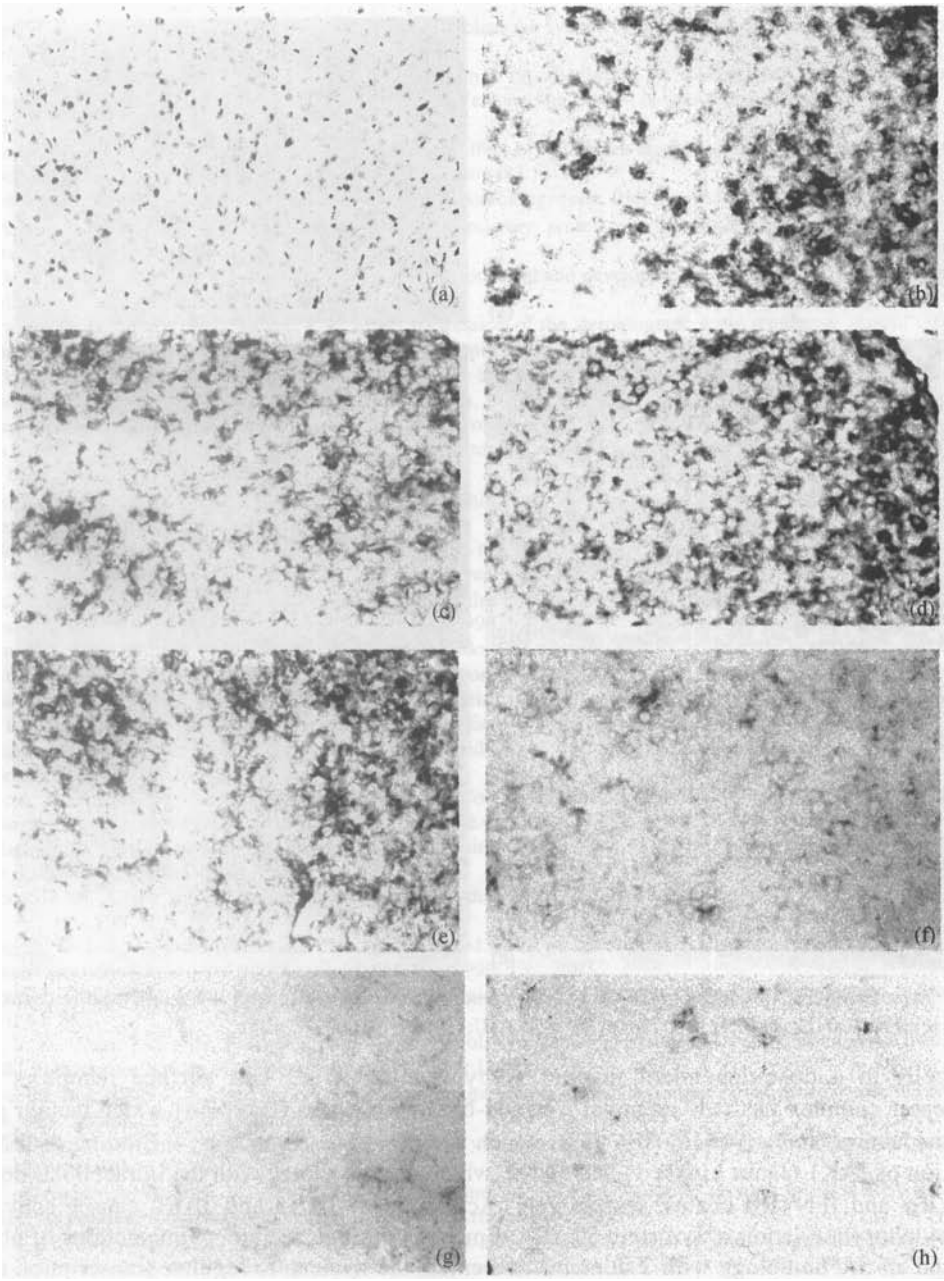

Fig. 2. Expression of StAR mRNA in the CL of adult pseudopregnant rat. (a) Negative control; (b) D1; (c) D2; (d) D4; (e) D6; (f) D8; (g) D10; (h) D13, ×200.

D4 with the IFNγ injection. The results indicated that 500 IU of IFNγ did not affect the StAR antigen expression. Increase in dose of the cytokine inhibited StAR expression. Treatment of the animals with IFNγ 5 000—10 000 IU significantly decreased StAR expression. It has been reported that IFNγ inhibited the synthesis of progesteron production, in bovine[20] and rat[21]. Effect of IFNγ on CL function may be through two ways: to induce the expression of Class II MHC antigens[22] or to increase the prostaglandin synthesis[23]. PGF2α inhibits StAR expression[24] and affects the synthesis of progesterone. Recent report has shown that IFNγ is capable of inhibiting the StAR expression in rat

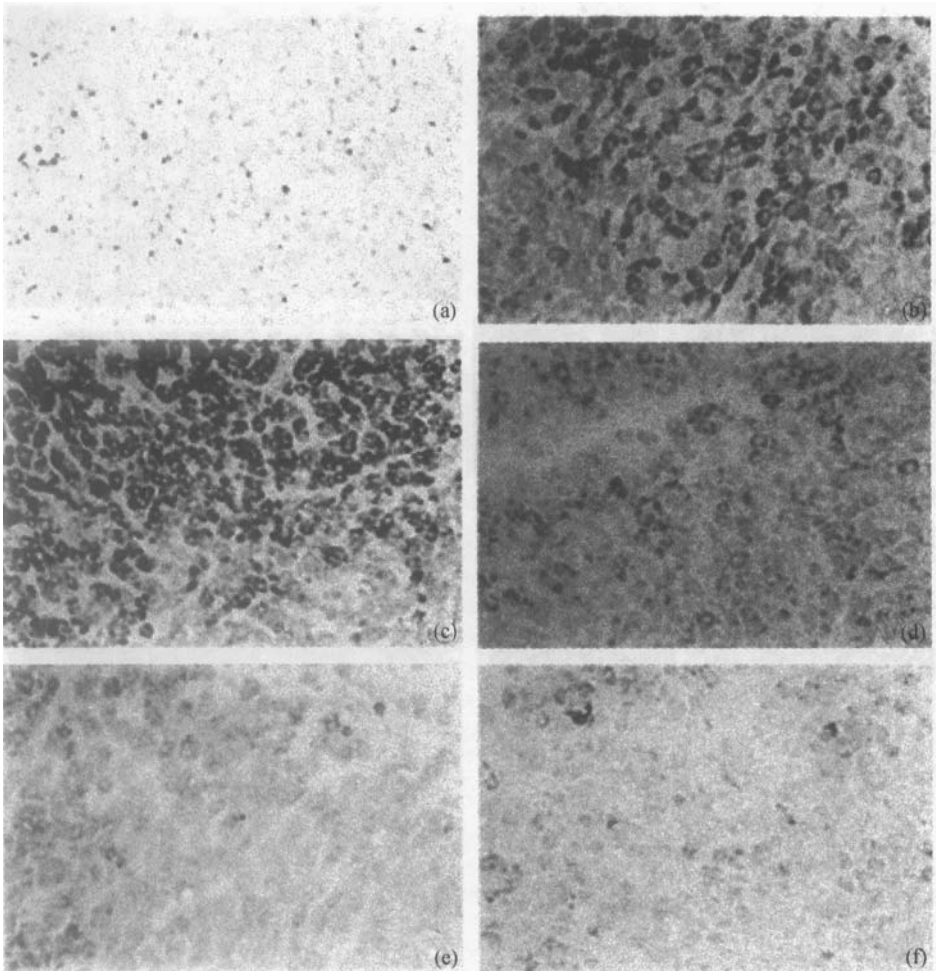

Fig. 3. Expression of StAR antigen in the CL of adult pseudopregnant rat treated with IFNγ. (a) Negative control; (b) control; (c) IFNγ 500 IU; (d) IFNγ 1000 IU; (e) IFNγ 5000 IU; (f) IFNγ 10 000 IU, ×200.

Leydig cells in a dose-dependent manner[25] by binding to its cell surface receptors[26]. The IFNγ-receptor complex has two subunits: a ligand-binding α-chain (IFNγ Rα) which cannot generate signal transduction; and a β-chain (IFNγ Rβ) which is required for signaling[26]. Binding of IFNγ leads to activation of JAK1 (Janus kinase 1) and JAK2, which are associated with the intracellular domain of the IFNγ Rα and IFNγ Rβ chains, respectively. Activation of JAK1 and JAK2 causes activation of STAT-1 by phosphorylation of tyrosine. STAT-1 dimerizes through reciprocal interactions of phosphotyrosine and an Src homology with 2 domains and enters the nucleus to regulate transcription of many different genes containing γ-activated sequence elements[26,27]. As both IFNγ Rα and IFNγ Rβ mRNAs have been identified in the rat testis[28], and IFNγ has been shown to regulate immediately the early gene expression in Sertoli cells by phosphorylation of STAT-1 protein[29,30], it is possible that the inhibitory effects of IFNγ on StAR gene expression may be mediated by similar JAK/STAT pathways. Until now, however, there are no reports available to show the presence of IFNγ Rα and IFNγ Rβ in rat CL, further study is, therefore, needed to clarify the mechanism of the IFNγ effect on StAR expression in the CL.

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