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Upregulation of Human Chorionic Gonadotrophin-Induced Steroidogenic Acute Regulatory Protein by Insulin-Like Growth Factor-I in Rat Leydig Cells

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Insulin-like growth factor-I (IGF-I) plays an essential role in reproductive function. Leydig cells express specific IGF-I receptors, and IGF-I enhances human chorionic gonadorphin (hCG)-induced testosterone formation. In the present study, we evaluate the effect of IGF-I on the gene expression and protein levels of steroidogenic acute regulatory protein (StAR), the rate-limiting step in steroidogenesis. StAR mRNA is expressed in rat Leydig cells as two major transcripts of 3.8 and 1.7 kb. StAR mRNA levels (both 3.8 and 1.7 kb) were markedly induced about 20-fold by hCG (10 ng/mL). Concomitant addition of IGF-I (50 or 100 ng/mL) and hCG (10 ng/mL) resulted in significant increases in StAR and cytochrome P450 side-chain cleavage (P450scc) mRNA levels, whereas lower doses of IGF-I (1 or 10 ng/mL) had small effects. Synergistic effects of IGF-I and hCG on StAR mRNA levels were confirmed by ribonuclease protection assay (RPA). IGF-I (100 ng/mL) enhanced hCG- and 20 OH-cholesterol + hCG-induced testosterone formation, whereas the conversions of pregnenolone, 17-OH pregnenolone, dehydroepiandrosterone, and androstenedione to testosterone were not affected. This suggests that the major effect of IGF-I is at the steps of StAR and P450scc, whereas other steroidogenic enzymes are not affected. To evaluate whether increased StAR mRNA levels induced by IGF-I and hCG are associated with increased StAR protein levels, we carried out Western blot analyses. Basal StAR protein levels were low after 24 h in culture. hCG (10 ng/mL) increased StAR protein by 4.5-fold. In the presence of IGF-I (100 ng/mL), hCG-induced StAR protein levels were further increased. In conclusion, our present study demonstrated that IGF-I enhances Leydig cell steroidogenesis by upregulating hCG-induced StAR gene expression and protein production.

Key Words: Leydig cell; insulin-like growth factor-I; steroidogenic acute regulatory protein (StAR).

Introduction

Although luteinizing hormone (LH) is the major regulatory factor of Leydig cell function, many other growth factors and cytokines also modulate Leydig cell steroidogenesis (for review, *see* ref. *1*). Indeed, insulin-like growth factor-I (IGF-I) is known to play an essential role in reproduction (for review, *see* ref. *2*). The mice in which the IGF-I and IGF-I receptor genes have been inactivated exhibit a marked reduction of the size of reproductive organs and are infertile *(3)*. Serum testosterone levels are markedly decreased to about 18% of normal in mice homozygous for a target mutation of *igf1* gene *(4)*. Furthermore, the basal and LH-stimulated production of testosterone by testicular parenchyma is reduced in comparison with wild-type controls *(4)*. We and others have reported previously that Leydig cells express specific IGF-I receptors and that IGF-I enhances hCG-stimulated testosterone formation *(2)*. Furthermore, high levels of IGF-I and IGF binding protein 2, 3, 4, 5, and 6 mRNA are expressed in Leydig cells *(2)*, suggesting that IGF-I and its binding proteins may have autocrine and paracrine effects.

The intracellular targets of IGF-I action, however, remain undetermined. Recently, it was found that the steroidogenic acute regulatory protein (StAR) mediates the rate-limiting step in steroidogenesis, the transport of substrate cholesterol from the outer to the inner mitochondrial membrane (for review, *see* ref. *5*). Transient transfection of both steroidogenic and nonsteroidogenic cells with StAR cDNA directly stimulated steroid production in the absence of trophic hormone stimulation *(6–8)*. In the present study, we evaluated whether IGF-I has any effect on StAR gene expression in rat Leydig cells.

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Results

Leydig cells were cultured with or without IGF-I (100 ng/mL) for 24 h. After medium change, IGF-I (100 ng/mL) and/or hCG (10 ng/ml) was added and cultures were continued for an additional 4 h. Basal testosterone levels were 5.2 ± 0.5 ng/ml (mean ± SE, $n = 3$), in the presence of human chorionic gonadotrophin (hCG) (10 ng/mL), testosterone levels were markedly increased (110.7 ± 12.7 ng/mL) (Fig. 1A). IGF-I enhanced hCG-induced testosterone formation (170 ± 18.2 ng/mL; $P < 0.01$ as compared with cells treated with hCG only). StAR mRNA was expressed in Leydig cells as two major transcripts, 3.8 and 1.7 kb (Fig. 1B). Both 3.8 and 1.7 kb StAR mRNA levels were markedly induced about 20-fold by hCG (10 ng/mL) (Fig. 1B,C). IGF-I (1, 10, 50, or 100 ng/mlL) alone had a very small effect on StAR or cytochrome P450 side-chain cleavage (P450scc) mRNA expression (data not shown). However, concomitant addition of IGF-I (50 or 100 ng/mL) and hCG resulted in significant increases in StAR mRNA levels (32 ± 6 and 42 ± 8%, respectively; $P < 0.05$ as compared with cells treated with hCG only). This indicates that IGF-I enhances hCG-induced StAR and P450scc mRNA expression.

The effect of IGF-I on hCG-induced StAR mRNA level was confirmed by the ribonuclease protection assay (RPA). Leydig cells were cultured with or without IGF-I (100 ng/mL) for 12 or 24 h. hCG (10 ng/mL) was then added, and cultures were continued for an additional 4 h before RNA extraction. As shown in Fig. 2, hybridization with [^{32}P]-labeled StAR cRNA, followed by RNase digestion and PAGE, resulted in protected StAR and actin RNA bands corresponding to sizes of approx 180 and 125 bp, respectively. hCG in a concentration of 10 ng/mL increased StAR mRNA levels by 2.5-fold. Treatment of Leydig cells with IGF-I (100 ng/mL) for 12 and 24 h further enhanced hCG-induced StAR mRNA levels.

To evaluate the effects of IGF-I on other steroidogenic enzymes, the conversions of steroid precursors to testosterone were investigated. IGF-I (100 ng/mL) increased hCG-stimulated testosterone formation and the conversion of 20-OH cholesterol to testosterone, whereas the conversions of pregnenolone, 17-OH pregnenolone, dehydroepiandrosterone, and androstenedione to testosterone were not affected (Table 1). This indicates that the major effect of IGF-I on the steroidogenic pathway is at the steps of StAR and P450scc, whereas other steroidogenic enzymes were not affected.

To evaluate further whether increased StAR mRNA levels induced by IGF-I and hCG are associated with increased StAR protein levels, we carried out Western blot analyses using specific StAR protein antibody. The immunoblot analysis of StAR protein expressed in Leydig cells is depicted in Fig. 3. Basal StAR protein levels were low after 24 h in culture. hCG (10 ng/mL) markedly increased StAR protein levels by 4.5-fold. StAR protein level was further

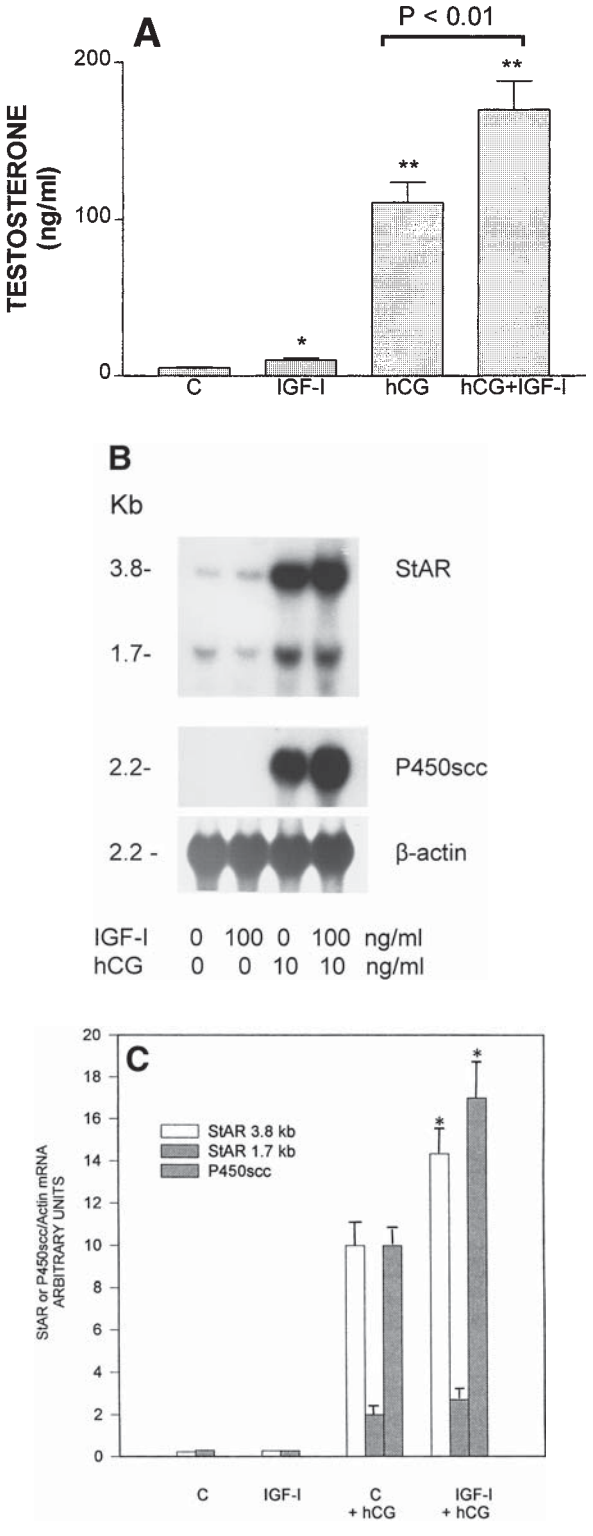

Fig. 1. Effects of IGF-I on hCG-induced testosterone formation, StAR, and P450scc mRNA expression. Purified Leydig cells were cultured with or without IGF-I (100 ng/mL) for 24 h. After medium change, cells were cultured with or without IGF-I (100 ng/mL) and hCG (10 ng/mL) for an additional 4 h. **(A)** Results are the mean ± SE of three separate experiments. *$P <$ 0.05, **$P < 0.01$ as compared with controls. **(B)** A representative Northern blot. **(C)** StAR or P450scc/β-actin mRNA ratios in arbitrary units. Results are the mean ± SE of three separate experiments. *$P < 0.05$ as compared with cells treated with hCG only.

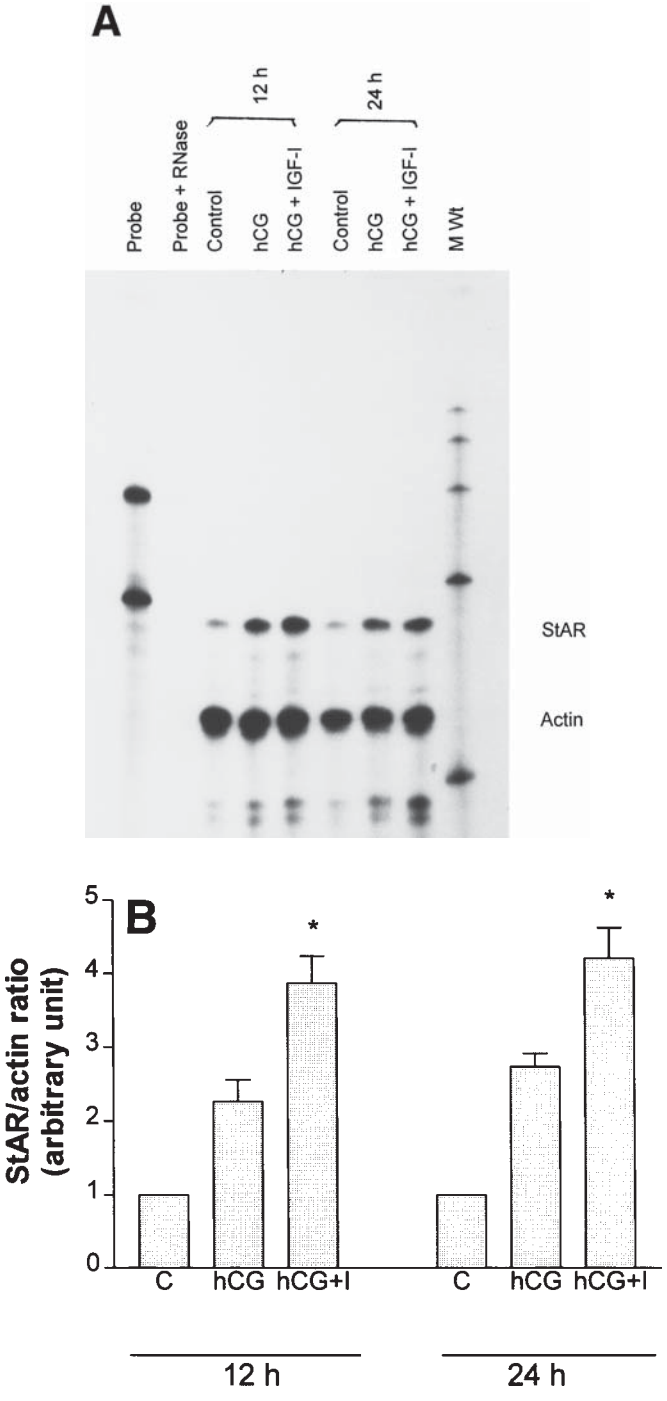

Fig. 2. Ribonuclease protection assay. **(A)** A representative autoradiogram showing the effect of IGF-I on StAR mRNA expression. Rat Leydig cells were treated with or without IGF-I (100 ng/mL) for 12 or 24 h. hCG (10 ng/mL) was then added, and cultures were continued for an additional 4 h before RNA extraction. Ten-microgram aliquots of total RNA were subjected to RNase protection assay as described in Materials and Methods. Lane 1 and 2 show mouse StAR riboprobe and rat β-actin riboprobe, which were carried through the assay in the presence of only nontarget yeast RNA and were subsequently incubated without and with RNase, respectively. Probes in lane 1 were diluted before loading. **(B)** StAR/actin mRNA ratios in arbitrary units. Results are the mean ± SE of three separate experiments. *$P < 0.05$ as compared with cells treated with hCG only. I: IGF-I.

increased by the concomitant addition of hCG (10 ng/mL) and IGF-I (100 ng/mL).

Discussion

In the present study, we demonstrate that StAR mRNA is expressed in rat Leydig cells. StAR gene expression is markedly increased in the presence of hCG. Furthermore, IGF-I enhances hCG-stimulated Leydig cell steroidogenesis by upregulating StAR and P450scc gene expression. Increased StAR gene expression was also found to be associated with increased production of StAR protein.

Biosynthesis of steroid hormones begins with the enzymatic cleavage of the side chain of cholesterol to form pregnenolone. This reaction is catalyzed by the P450scc, which is part of the cholesterol side-chain cleavage enzyme system and is located on the matrix side of the inner mitochondrial membrane. The true rate-limiting step in steroidogenesis is the delivery of the substrate, cholesterol, from the outer to the inner mitochondrial membrane and the P450scc enzyme *(5)*. Transfection experiments of MA-10 cells revealed that the expression of the cDNA-derived StAR protein resulted in increased steroid production in MA-10 cells in the absence of hormone stimulation. StAR is a mitochondrial phosphoprotein that is first synthesized as a 37 kDa precursor in the cytosol and is quickly targeted to and imported into the mitochondria where the signal sequence is removed yielding the 30-kDa mature form of the protein. In addition, when COS-1 cells were cotransfected with P450scc, adrenodoxin, and the cDNA for the 37-kDa StAR precursor protein, there was a sixfold increase in the conversion of cholesterol to pregnenolone. These results confirm a direct role for the StAR protein in hormone-regulated steroid production. Northern and Western analyses demonstrated that StAR mRNA and protein were induced concomitantly via a cAMP-mediated mechanism that paralleled the acute production of steroid hormone in MA-10 mouse Leydig tumor cells *(7)*. In the present study, we demonstrated that hCG-induced testosterone formation is tightly coupled with the induction of StAR mRNA levels in rat Leydig cells.

Regulation of StAR gene expression has been extensively studied in the adrenals and ovaries *(5)*. Sugawara et al. reported that a cAMP analog stimulated StAR mRNA in luteinized human granulosa cells *(8)*. Juengel et al. reported that serum progesterone levels and corpus luteum StAR mRNA levels decrease after hypophysectomy, which could be restored by the administration with LH *(9)*. Prostaglandin F2α and PMA, agents that cause luteal regression, decrease StAR transcripts *(9)*. In the rat ovary, Sandhoff and McLean demonstrated that both StAR expression and serum progesterone levels increased in parallel in response to trophic hormone stimulation, whereas P450scc mRNA levels were unchanged *(10)*. In porcine granulosa cells, treatment with FSH or IGF-I alone had a small stimulatory

Table 1
Effects of IGF-I on the Conversions of Steroid Precursors to Testosterone[a]

	Testosterone, ng/mL	
		IGF-I, 100 ng/mL
Control	10.8 ± 1.4	
hCG (10 ng/mL)	127.0 ± 5.6	189.0 ± 15.0[b]
20-OH cholesterol + hCG	83.0 ± 10.2	231.0 ± 7.3[b]
Pregnenolone	56.3 ± 1.3	60.3 ± 2.2
17-OH pregnenolone	58.2 ± 1.5	59.3 ± 1.8
Dehydroepiandrosterone	62.0 ± 1.8	64.2 ± 2.9
Androstenedione	60.0 ± 2.5	62.8 ± 3.3

[a]Purified Leydig cells (1.5×10^5/mL) were cultured with or without IGF-I (100 ng/mL) for 24 h. After medium change, cells were cultured with or without IGF-I (100 ng/mL), hCG (10 ng/mL), and 20-OH-cholesterol, pregnenolone, 17-OH pregnenolone, dehydroepiandrosterone, or androstenedione in concentrations of 1 μ*M*. Cultures were continued for an additional 24 h. Testosterone levels were measured in the supernatants. Results are the mean ± SE of triplicate incubations.

[b]$P < 0.01$ as compared with Leydig cells cultured without IGF-I.

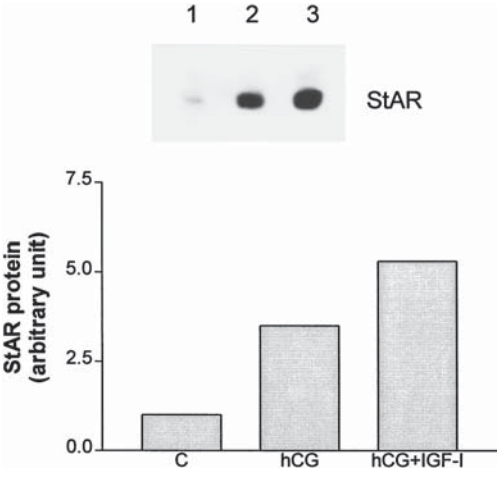

Fig. 3. Western immunoblot of StAR protein. Mitochondrial protein (90 μg) was analyzed in each sample. Mitochondria were isolated from Leydig cells as described in Materials and Methods. Lane 1, control; lane 2, hCG (10 ng/mL); lane 3, hCG (10 ng/mL) + IGF-I (100 ng/mL). Similar results were observed in two other separate experiments. This is a representative blot.

effect on StAR mRNA accumulation at 12, 24, or 48 h. Concomitant addition of follicle-stimulating hormone (FSH) and IGF-I synergistically increased StAR mRNA levels by 24 h, with a maximal effect at 48 h *(11)*. Our present study demonstrated that StAR mRNA levels in Leydig cells were rapidly induced by hCG. hCG-stimulated StAR mRNA levels were further increased as early as 12 h after the addition of IGF-I. Synergistic effects of IGF-I and hCG on StAR mRNA expression are associated with increased StAR protein production. IGF-I has no effect on the conversions of pregnenolone, 17 OH-pregnenolone, dehydroepiandrosterone, and androstenedione to testosterone. This suggests that the major effect of IGF-I is at StAR and P450scc, whereas other steroidogenic enzymes are not affected.

The role of IGF-I in male sexual maturation and fertility has been unclear. Serum IGF-I levels of male little (*lit*) mice carrying a missense mutation of GH-releasing hormone receptor gene are only 10% of normal, but these mice are fertile *(12–14)*. Furthermore, steroidogenesis and spermatogenesis are normal *(15)*. Additionally, fertility is not impaired in homozygous *dr/dr* rats, even though serum IGF-I concentration is only 13% of normal *(16,17)*. However, compelling evidence that IGF-I is essential in male reproduction is provided by *igf1* knockout mice *(3,4)*. The Leydig cells of the *igf1* (–/–) mutants appear to be condensed and clustered into small groups. The volume of these cells was reduced to about 50% of normal, whereas their number was reduced to only 33% of normal. Most significantly, serum testosterone levels of mutants were decreased to about 18% of normal. Furthermore, basal and LH-stimulated testosterone production by testicular cells in organ culture were reduced by 38 and 72%, respectively, over a 3-h period *(3,4)*.

Our present study demonstrated that StAR mRNA is expressed in primary cultures of rat Leydig cells and its expression is markedly induced by the addition of hCG. IGF-I and hCG were found to have synergistic effects on StAR gene expression. In conclusion, IGF-I enhances hCG-induced testosterone formation by upregulating StAR gene expression and protein production.

Materials and Methods

Materials

The StAR cDNA probes were prepared as described previously *(6)*. The P450scc cDNA probe was kindly provided by JoAnne Richards (Houston, TX). [α-^{32}P]-Deoxycytidine triphosphate (dCTP, 3000 Ci/mmol) and [α-^{32}P]-CTP (3000 Ci/mmol) were obtained from ICN Biochemical (Irvine, CA). Materials for molecular biology were obtained from Promega (Madison, WI) and Gibco-BRL (Grand Island, NY). IGF-I was obtained from Amgen (Thousand Oaks, CA). Highly purified hCG (13,000 U/mg) was kindly provided by Patricia Morris (the Population Council, Rockefeller University, New York, NY).

Isolation and Culture of Leydig Cells

Male Sprague-Dawley rats were obtained from Charles River (Raleigh, NC). Highly purified Leydig cells were isolated from rat testes using the combination of arterial perfusion, collagenase digestion, centrifugal elutriation, and Percoll gradient centrifugation as described by Klinefelter et al. *(18)* with minor modification *(19)*. The protocol was approved by the local animal study committee. By 3β-HSD staining, over 97% of the cells stained

positive for Leydig cells *(20)*. Purified Leydig cells were resuspended in DMEM/F12 with 0.5% BSA, 15 m*M* HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin. Leydig cells ($4–5 \times 10^6$ cells) were plated in 50-mm culture wells (Costar, Cambridge, MA) and incubated at 37°C in a humidified atmosphere of 95% air–5% CO_2. IGF-I (1–100 ng/mL) was then added. After 12–24 h in culture, the medium was removed and replaced with fresh medium. IGF-I and/or hCG (10 ng/mL) were added, and cultures were continued for an additional 4 h. To evaluate the effects of IGF-I on the conversions of steroid precursors to testosterone, purified Leydig cells (1.5×10^5 cells/mL) were cultured with or without IGF-I (100 ng/mL) for 24 h. After medium change, cells were cultured with or without IGF-I (100 ng/mL), hCG (10 ng/mL) and 20-OH-cholesterol, pregnenolone, 17-OH pregnenolone, dehydroepiandrosterone, or androstenedione in concentrations of 1 μ*M*. Cultures were continued for an additional 24 h. Culture media were then centrifuged, and the supernatants were saved at –20°C for testosterone assay. More than 95% of the cells remained viable as determined by trypan blue exclusion.

RNA Extraction and Northern Blot Analysis

Total cellular RNA was extracted using the acid guanidinium thiocyanate phenol-chloroform method as described previously *(21)*. For Northern blot analyses, 20 μg of total cellular RNA were denatured with 6% formaldehyde and 50% formamide, run on a 1% agarose gel containing 2.2*M* formaldehyde, and then transferred onto a Nytran membrane (0.45 μm, Schleicher and Schuell, Keene, NH) *(22)*. Hybridization was carried out with $1–5 \times 10^7$ cpm of the StAR, P450scc, or β-actin cDNA probes labeled with [α-^{32}P]-dCTP (3000 Ci/mmol) by using a Random Primers DNA Labeling System (Gibco-BRL). The membranes were then exposed to Fuji RX x-ray film with an intensifying screen at –70°C. The autoradiograms were quantified by densitometric scanning using a Bio-Rad (Hercules, CA) video densitometer, Model 620. The expression of β-actin mRNA level, which was not affected by any of these treatment was used as the internal control for each specimen *(23)*. Results are expressed as arbitrary units of StAR or P450scc/actin messenger RNA (mRNA) ratios.

Ribonuclease Protection Assay

A 180-bp mouse StAR cDNA fragment was PCR-amplified using primers corresponding to the mouse cDNA sequence bases 542–562 and 702–722 (sense primer, 5'-AGAGGATTGGAAAAGACACGG-3'; antisense primer, 5'-GCTCTGATGACACCACTCTGC-3'), and cloned into a pCRII vector using the TA cloning kit (Invitrogen Corp., San Diego, CA). The insert was sequenced manually by the dideoxynucleotide chain-termination method (Sequenase version 2.0 kit, US Biochemical Corp., Cleveland, OH). The StAR antisense riboprobe (cRNA), approximately 288 bases in length, was transcribed using the Maxiscript in vitro transcription kit (Ambion, Austin, TX). Before transcription, the DNA template was linearized with *Bam*H1. Transcription was carried out for 1 h at 37°C using 0.5 μg DNA template with [^{32}P]CTP (ICN, Costa Mesa, CA) and T7 polymerase. Rat β-actin RNA antisense riboprobe was synthesized using Ambion's pTRI-β-actin-125-rat template, T7 polymerase, and the Maxiscript in vitro transcription kit.

RNase protection assays were performed using the HybSpeed RPA kit (Ambion). In brief, preliminary experiments were performed to determine saturating quantities of each riboprobe used in subsequent experiments. Total RNA (10 μg) from rat Leydig cells was resuspended in hybridization buffer containing saturating concentrations of StAR riboprobes and rat β-actin riboprobes. The mixture was heated to 95°C for 5 min, and samples were hybridized for 1 h at 68°C. Samples were then treated with RNase A/T1 mixture for 1 h at 37°C, precipitated, resuspended in formamide-containing gel-loading buffer, and run on 5% polyacrylamide-8*M* urea gels. In each gel, five [^{32}P]CTP-labeled RNA transcripts synthesized from Century Marker Templates (Ambion) with lengths of 100, 200, 300, 400, and 500 bases were run simultaneously with samples in a separate lane as size standards. Gel were exposed overnight and up to 3 d to Fuji RX film at –70°C with intensifying screens.

Isolation of Mitochondria and Western Blot Analysis

Leydig cells were homogenized in TSE buffer (0.25*M* sucrose, 10 m*M* Tris, pH 7.4, 0.1 m*M* EDTA) on ice using 20 strokes in a Dounce homogenizer. Lysates were centrifuged at 600*g* for 15 min at 4°C. The supernatant was centrifuged at 10,000*g* for 15 min at 4°C. The resulting pellets were then resuspended in 1 mL ice-cold buffer. A 100 μL aliquot of this was used for protein quantitation by the Bio-Rad protein dye assay. The remaining mitochondrial suspension was pelleted by centrifugation at 10,000*g* for 15 min at 4°C and then lyophilized. Western blot analyses were performed as previously described using a mouse polyclonal antisera to a 10 amino acid segment (amino acids 88–98) of the 30 kDa StAR protein *(6)*. The specific signal was detected by chemiluminescence using the Renaissance kit from Dupont NEN. The integrated optical densities of the bands were quantitated using the BioImage Visage 2000 computer-assisted image analysis system (BioImage, Ann Arbor, MI).

All experiments were repeated at least three times. One-way analysis of variance followed by Neuman-Keuls multiple-comparison tests were used for statistical analyses (GraphPad Prism, version 2.01). A P value of ≤ 0.05 was considered to be significant. Statistical analyses on Western blots were not performed. Similar results were obtained in three separate experiments, and only one representative blot was presented.

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