# Interleukin-1 inhibits Leydig cell steroidogenesis without affecting steroidogenic acute regulatory protein messenger ribonucleic acid or protein levels

**T Lin, D Wang** and **D M Stocco**[1]

Medical and Research Services, WJB Dorn Veterans' Hospital and the Department of Medicine, University of South Carolina School of Medicine, Columbia, South Carolina 29208, USA and [1]Texas Tech University Health Sciences Center, School of Medicine, Department of Cell Biology and Biochemistry, Lubbock, Texas 79430, USA

(Requests for offprints should be addressed to T Lin, Department of Medicine, University of South Carolina School of Medicine, Medical Library Bldg. Suite 316, Columbia, South Carolina 29208, USA)

## Abstract

The rate-limiting step of steroidogenesis is the transport of the substrate cholesterol from the outer to the inner mitochondrial membrane which involves a cycloheximide-sensitive newly synthesized protein. A protein believed to carry out this function was recently cloned from MA-10 mouse Leydig tumor cells and named the steroidogenic acute regulatory protein (StAR). In the present study, we evaluated the expression and regulation of StAR in primary cultures of rat Leydig cells. StAR mRNA was expressed in Leydig cells as two major transcripts of 3·8 and 1·7 kb and one minor transcript of 1·2 kb. Induction of StAR mRNA transcripts could be detected as early as 30 min after the addition of human choriogonadotropin (hCG) with peak levels attained between 2 and 4 h. hCG in concentrations of 0·1–10 ng/ml caused a dose-dependent increase in StAR mRNA expression. hCG administered at a dose of 10 ng/ml increased the 3·8 kb StAR mRNA level about 14-fold and the 1·7 kb StAR mRNA level about 13·6-fold. hCG-stimulated StAR mRNA was associated with increased StAR protein levels as determined by immunoblot analysis (a 4·5-fold increase). Murine interleukin-1α (mIL-1α) at a concentration of 100 ng/ml inhibited hCG-induced cytochrome P450 side-chain cleavage (P450 scc) mRNA expression and testosterone formation almost completely. Interestingly, mIL-1α had no effect on hCG-induced StAR mRNA or protein levels. Furthermore, mIL-1α (10 ng/ml) decreased conversion of (*22R*)-hydroxycholesterol to testosterone while the conversion of pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone and androstenedione to testosterone were not affected. These results indicate that the major inhibitory effect of IL-1 on Leydig cell function occurs at the level of P450 scc.

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## Introduction

The rate-limiting step in steroidogenesis is the transport of the substrate cholesterol from the outer to the inner mitochondrial membrane which involves a cycloheximide-sensitive newly synthesized protein (for a review, see Stocco & Clark 1996). Several proteins have been proposed as the acute regulator. One of these candidate proteins was recently cloned from MA-10 murine Leydig tumor cells and was named the steroidogenic acute regulatory protein (StAR) (Clark *et al.* 1994). Transient transfection of both steroidogenic and non-steroidogenic cells with StAR cDNA directly stimulated steroid production in the absence of trophic hormone stimulation (Clark *et al.* 1994, 1995, Sugawara *et al.* 1995). In patients with lipoid congenital adrenal hyperplasia, adrenal and gonadal steroidogenesis is impaired because of inefficient transport of cholesterol into the mitochondria. In a number of patients with this condition, mutations of several types in the StAR gene were found, and to date, mutations in the StAR gene are its only known cause (Lin *et al.* 1995, Tee *et al.* 1995, Bose *et al.* 1996). These findings provide compelling evidence that the StAR protein plays an important role in steroid hormone synthesis.

Interleukin-1 (IL-1) is one of the major cytokines produced by monocytes and macrophages in response to infection and tissue injury (for a review, see Dinarello 1994). Increased plasma IL-1 concentration and reduced serum testosterone levels are frequently found in patients with critical illness and septicemia (Vogel *et al.* 1985, Woolf *et al*, 1985, Cannon *et al.* 1990). We and others have reported previously that IL-1 is a potent inhibitor of luteinizing hormone/human choriogonadotropin (hCG)-induced androgen formation by Leydig cells (Calkins *et al.* 1988, 1990, Verhoeven *et al.* 1988, Hales 1992, Mauduit

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*et al.* 1992). IL-1 decreases hCG-stimulated cAMP and testosterone formation (Calkins *et al.* 1988). IL-1 also reduces 8-bromo-cAMP-induced testosterone formation and hCG-induced cytochrome P450 side-chain cleavage (P450 scc) mRNA expression in rat Leydig cells (Calkins *et al.* 1988, Lin *et al.* 1991). The inhibitory effect of IL-1 can be reversed by IL-1 receptor antagonist (Lin *et al.* 1991). In the present study, we evaluated the expression and regulation of both StAR mRNA and protein synthesis in primary cultures of rat Leydig cells by IL-1.

## Materials and Methods

### *Materials*

Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture (F12) were obtained from Gibco–BRL (Grand Island, NY, USA). BSA, penicillin, streptomycin and collagenase (type I) were from Sigma Chemical Co. (St Louis, MO, USA). Percoll was from Pharmacia (Piscataway, NJ, USA). The StAR cDNA probes were prepared as described previously (Clark *et al.* 1994). The P450 scc cDNA probe was kindly provided by Dr JoAnne Richards (Houston, TX, USA). [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol) was obtained from ICN Biochemical (Irvine, CA, USA). Materials for molecular biology were obtained from Promega (Madison, WI, USA) and Gibco–BRL. Murine IL-1$\alpha$ (mIL-1$\alpha$) was purchased from Genzyme Corp. (Cambridge, MA, USA). Highly purified hCG (13 000 units/mg) was kindly provided by Dr Patricia Morris (Population Council, Rockefeller University, New York, NY, USA).

### *Isolation and culture of Leydig cells*

For isolation of purified Leydig cells, male Sprague–Dawley rats were obtained from Charles River (Raleigh, NC, USA). Rats were kept in an air-conditioned room with a controlled light cycle (12 h light/12 h darkness). They were fed dry rat chow and water *ad libitum*. The protocol was approved by the local animal study committee. The testes were removed after rats were rendered unconscious in a $CO_2$ precharged chamber. Highly purified Leydig cells were isolated from rat testes using the combination of arterial perfusion, collagenase digestion, centrifugal elutration and Percoll gradient centrifugation as described by Klinefelter *et al.* (1987) with minor modification (Lin *et al.* 1990). As assessed by 3$\beta$-hydroxysteroid dehydrogenase staining, over 97% of the cells were stained positive for Leydig cells (Payne *et al.* 1980). Purified Leydig cells were resuspended in DMEM/F12 with 0·5% BSA, 15 mM Hepes, 100 U/ml penicillin and 100 μg/ml streptomycin. Leydig cells ((8–10) × $10^6$ cells) were plated in 50 mm culture wells (Costar, Cambridge, MA, USA) and incubated at 37 °C in a humidified atmosphere of 95% air/5% $CO_2$. mIL-1$\alpha$ (1–100 ng/ml) was then added. After 24 h in culture, the medium was removed and replaced with fresh medium. mIL-1$\alpha$ and/or hCG (10 ng/ml) were added and cultures were continued for an additional 30 min to 6 h. To evaluate the effects of mIL-1$\alpha$ on the conversion of steroid precursors to testosterone, purified Leydig cells ($10^5$/ml) were cultured with or without mIL-1$\alpha$ (10 ng/ml) for 24 h. After medium change, cells were cultured with or without testosterone precursors ((22*R*)-hydroxycholesterol+hCG, pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone or androstenedione; $10^{-6}$ M) and mIL-1 (10 ng/ml) 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. Testosterone levels were assayed as previously described (Lin 1985). The highly specific testosterone antibody cross-reacted only 7% with 5$\alpha$-dihydrotestosterone and less than 1% with other steroids (pregnenolone, 17$\alpha$-hydroxypregnenolone, progesterone, 17$\alpha$-hydroxyprogesterone, androstenedione and dehydroepiandrosterone).

### *RNA extraction and Northern blot analysis*

Total cellular RNA was extracted using the acid guanidinium thiocyanate–phenol–chloroform method as described previously (Chomczynski & Sacchi 1987). For Northern blot analyses, 20 μg total cellular RNA was denatured with 6% formaldehyde and 50% formamide, run on a 1% agarose gel containing 2·2 M formaldehyde and then transferred on to a Nytran membrane (0·45 μm; Schleicher and Schuell, Keene, NH, USA) (Lin *et al.* 1992). Hybridization was carried out with (1–5) × $10^7$ c.p.m. of the StAR, P450 scc or $\beta$-actin cDNA probes labeled with [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol) by using a Random Primers DNA Labeling System (Gibco–BRL). The membranes were then exposed to Kodak XAR5 film with an intensifying screen at −70 °C. The autoradiograms were quantified by densitometric scanning using a Bio-Rad video densitometer, model 620. Expression of $\beta$-actin mRNA, which was not affected by any of these treatments, was used as the internal control for each specimen (Wang *et al.* 1994). Results are expressed as arbitrary units of StAR or P450 scc/actin mRNA ratios.

### *Isolation of mitochondria and Western blot analysis*

Leydig cells were homogenized in TSE buffer (0·25 M sucrose, 10 mM Tris–HCl, pH 7·4, 0·1 mM 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 quantification 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 mouse polyclonal antiserum to a 10-amino acid segment (amino acids 88–98) of the 30 kDa StAR protein (Clark *et al.* 1994). The specific signal was detected by chemiluminescence using the Renaissance kit from Dupont–NEN (Boston, MA, USA). The integrated optical density of the bands was quantified using the BioImage Visage 2000 computer-assisted image analysis system (BioImage, Ann Arbor, MI, USA). Blots were also stained for the mitochondrial enzyme cytochrome oxidase to correct for possible differences in protein loading of the gels.

All experiments were repeated at least three times. One-way ANOVA followed by Neuman-Keuls multiple comparison tests were used for statistical analyses (GraphPad Prism, version 2·01). A *P* value equal to or less than 0·05 was considered to be significant.

## Results

### *Expression of StAR mRNA in Leydig cells*

Expression of StAR mRNA has not been characterized previously in primary cultures of rat Leydig cells, therefore we first evaluated the expression of StAR mRNA. StAR mRNA was expressed in Leydig cells as two major transcripts, of 3·8 and 1·7 kb, and one minor transcript of 1·2 kb (Fig. 1). StAR mRNA levels were induced by hCG in a time-dependent manner. They increased as early as 30 min after the addition of hCG (Fig. 1), peaked at between 2 and 4 h and remained elevated at 6 h (Fig. 1). Consequently, a 4 h stimulation with hCG was used in all subsequent studies. StAR mRNA levels were higher in freshly isolated Leydig cells than in cells that had been in culture for 24 h (Fig. 2). After 48 h in culture, they had decreased even further. Levels of the 3·8 kb transcript had decreased to 26% of the control by 24 h and to 10% by 48 h in culture (Fig. 2). hCG markedly induced StAR mRNA expression even in Leydig cells that had been in culture for 48 h (Fig. 2). Figure 3 shows the dose–response relationship of hCG-induced StAR mRNA expression. hCG at concentrations of 0·1–10 ng/ml caused a dose-dependent increase in expression of StAR mRNA. At a concentration of 10 ng/ml, hCG increased the 3·8 kb StAR mRNA level about 14-fold and 1·7 kb mRNA about 13·6-fold.

### *Effects of mIL-1α on hCG-induced StAR mRNA expression*

Since IL-1 inhibits hCG-induced testosterone formation, we evaluated the effects of IL-1 on hCG-induced StAR mRNA expression. When Leydig cells were treated with mIL-1α (1, 10 or 100 ng/ml), hCG-induced testosterone formation was markedly reduced in a dose-dependent manner, decreasing from 161 ± 13 ng/ml (mean ± S.E.) to 9 ± 0·7 ng/ml ($P<0·01$) on the addition of 100 ng/ml mIL-1α. Even though P450 scc mRNA expression was almost completely inhibited by mIL-1α, StAR mRNA levels were not affected (Fig. 4). Table 1 shows the effects of mIL-1α on the conversion of steroid precursors to testosterone. mIL-1 (10 ng/ml) inhibited hCG-induced

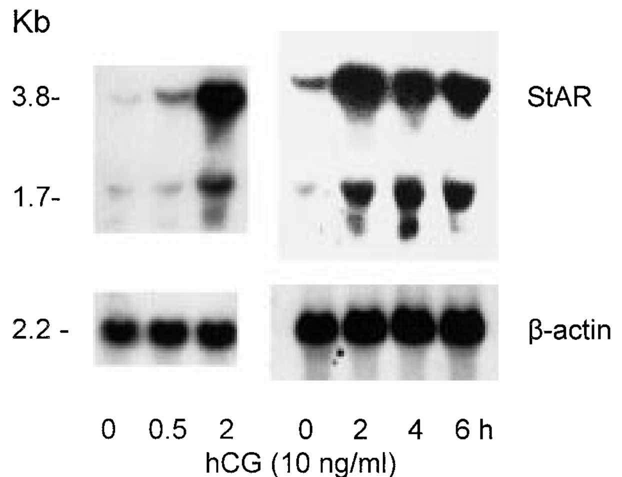


**Figure 1** Time course of hCG-induced StAR mRNA expression. Purified Leydig cells ((8–10) × $10^6$ cells/50 mm dish) were cultured for 24 h. After medium change, cells were treated with hCG (10 ng/ml) for 0·5, 2, 4 or 6 h. Total RNAs were extracted for Northern blot analyses. Each lane contains 20 µg total RNA. The blot was first hybridized with [α-$^{32}$P]dCTP-labeled StAR cDNA probes. After exposure and development of the autoradiogram, the blot was stripped of StAR cDNA probes and rehybridized with β-actin probes.

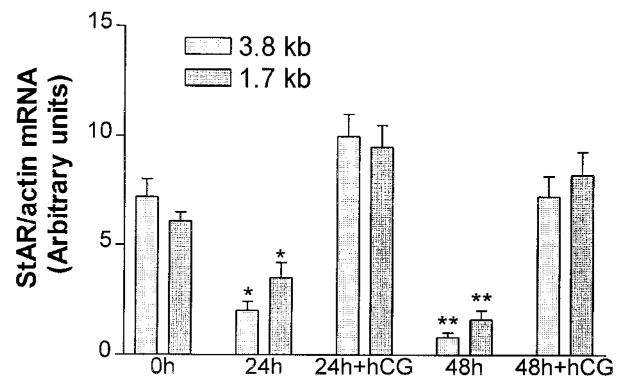


**Figure 2** Effects of *in vitro* culture on StAR mRNA levels. Total RNAs were extracted from freshly isolated Leydig cells and cells that had been in culture for 24 or 48 h. After medium change, hCG (10 ng/ml) was added and cells were cultured for an additional 4 h. Total RNA was then extracted for Northern blot analyses. The blot was first hybridized with an [α-$^{32}$P]dCTP-labeled StAR cDNA probe. The blot was then stripped and reprobed with an actin probe. Results are the mean ± S.E. of StAR/actin mRNA ratios of three separate experiments. *$P<0·05$, **$P<0·01$ as compared with freshly isolated Leydig cells (time 0).

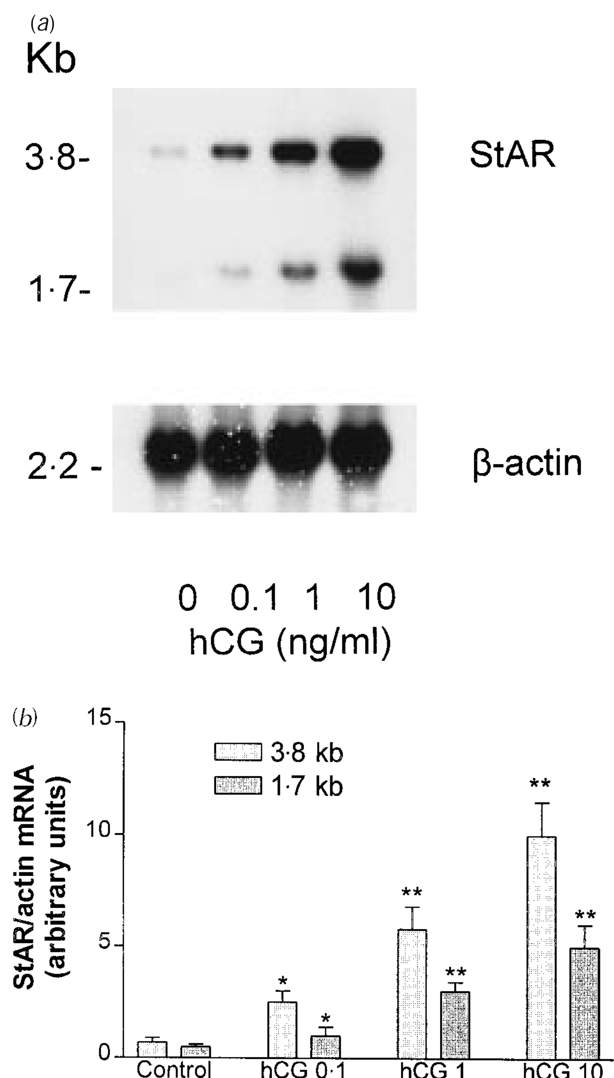


**Figure 3** Dose–response of hCG-induced StAR mRNA levels. Purified Leydig cells were cultured for 24 h. After medium change, hCG (0·1, 1 or 10 ng/ml) was added and total RNAs were extracted after 4 h. (*a*) A representative Northern blot. (*b*) StAR/actin mRNA ratios. Results are the mean ± S.E. of three separate experiments. *$P<0.05$ and **$P<0.01$ as compared with controls.

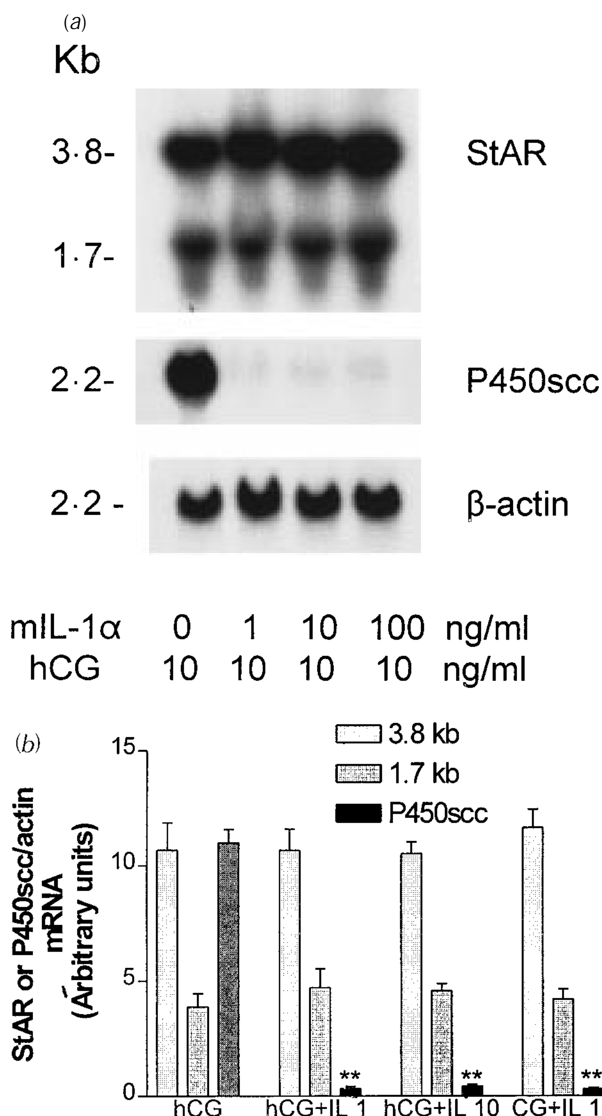


**Figure 4** Effects of mIL-1α on hCG-induced StAR and P450scc mRNA expression. Purified Leydig cells were cultured with or without mIL-1α (1, 10 or 100 ng/ml) for 24 h. After medium change, cells were treated with or without mIL-1α and/or hCG (10 ng/ml) for an additional 4 h. Total RNAs were extracted for Northern blot analyses. (*a*) A representative Northern blot. (*b*) StAR/actin or P450scc/actin mRNA ratios. Results are the mean ± S.E. of three separate experiments. **$P<0.01$ as compared with cells treated with hCG only.

testosterone formation and the conversion of (22*R*)-hydroxycholesterol to testosterone, while the conversion of pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone and androstenedione to testosterone were not affected. This indicates that the major inhibitory effect of mIL-1α on Leydig cell steroidogenesis is at the level of P450 scc.

*Effects of hCG and mIL-1α on StAR protein levels*

The immunoblot analysis of StAR protein expressed in Leydig cells is depicted in Fig. 5. Basal StAR protein levels were low after 24 h in culture, and hCG (10 ng/ml) markedly increased these levels (4·5 ± 0·8-fold increase; $n=4$; $P<0.01$ as compared with basal controls). The increase was seen as early as 2 h after the addition of hCG (data not shown) and peaked between 4 and 6 h, which correlated with the increased StAR mRNA levels. Concentrations of mIL-1α up to 100 ng/ml had no effect

**Table 1** Effects of mIL-1α on the conversion of steroid precursors to testosterone. Results are the mean ± S.E. of triplicate incubations

| | **Testosterone** (ng/ml) | |
|---|---|---|
| | − mIL-1α | +mIL-1α |
| **Steroid precursor** | | |
| Control | 11·2 ± 1·1 | — |
| hCG (10 ng/ml) | 135 ± 6·8 | 13·2 ± 1·6* |
| (22*R*)-Hydroxycholesterol | 45·6 ± 3·4 | 8·4 ± 0·7* |
| (22*R*)-Hydroxycholesterol+hCG | 138 ± 7·4 | 12·8 ± 1·3* |
| Pregnenolone | 141 ± 5·8 | 128 ± 4·6 |
| 17-Hydroxypregnenolone | 126 ± 6·1 | 118 ± 4·1 |
| Dehydroepiandrosterone | 110 ± 4·2 | 120 ± 3·6 |
| Androstenedione | 134 ± 5·6 | 140 ± 6·4 |

*$P<0·01$ compared with Leydig cells cultured without mIL-1α.

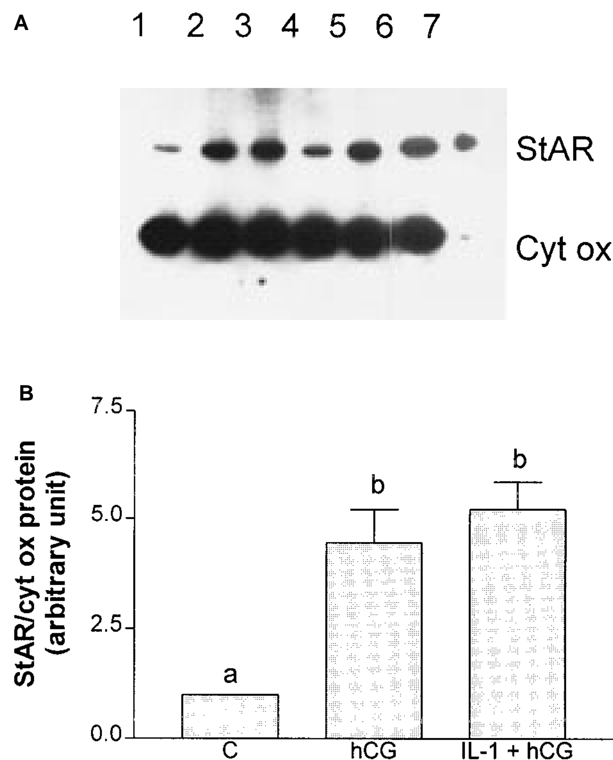


**Figure 5** Immunoblot of StAR protein. Mitochondrial protein (90 µg) was analyzed in each sample. Mitochondria were isolated from Leydig cells as described in Materials and Methods. (A) A representative blot. Lane 1, control; lane 2, hCG (10 ng/ml) for 4 h; lane 3, hCG (10 ng/ml) for 6 h; lane 4, control; lane 5, hCG (10 ng/ml) for 4 h; lane 6, hCG+mIL-1α (10 ng/ml) for 4 h; lane 7, MA-10 cell mitochondria as a positive control. (B) StAR/cytochrome oxidase (cyt ox) protein ratio. Results are the mean ± S.E. of four separate experiments. Bars not labeled with the same alphabetical letters are significantly different at $P<0·01$.

on hCG-induced StAR protein levels (5·2 ± 0·6-fold increase; $n=4$; $P>0·05$ compared with cells treated with hCG only).

## Discussion

In the present study, we demonstrate that StAR mRNA is expressed in rat Leydig cells as two major transcripts of 3·8 and 1·7 kb and a minor transcript of 1·2 kb. After hCG stimulation, both 3·8 and 1·7 kb StAR mRNA levels begin to increase within 30 min and peak between 2 and 4 h. Increased StAR mRNA levels are associated with increased StAR protein as determined by immunoblot. Even though mIL-1α markedly decreases P450 scc mRNA levels and inhibits testosterone formation, it has no significant effect on either StAR mRNA expression or protein levels.

Induction of steroidogenesis by pituitary trophic hormones is mainly mediated by cAMP. The acute effect of cAMP results in an increased mobilization and transport of cholesterol from the outer to the inner mitochondrial membrane, where it is converted to pregnenolone by the P450 scc (for a review, see Stocco & Clark 1996). Chronic effects of trophic hormones involve increased transcription of the genes that encode the steroidogenic enzymes and serve to maintain optimal capacity for steroid production (for reviews, see Miller 1988 and Simpson & Waterman 1988). Recent studies suggest that StAR, a 30 kDa mitochondrial protein, is an essential component of the acute response of steroidogenic cells to trophic hormone (Stocco & Clark 1996). StAR expression is rapidly increased by trophic hormone and correlates with the acute stimulation of steroidogenesis (Clark *et al.* 1994). In the present study, we found that StAR mRNA is expressed as two major transcripts of 3·8 and 1·7 kb and one minor transcript of 1·2 kb in rat Leydig cells. The difference in the transcript lengths may be attributable to a difference in the length of the 3′-untranslated regions (Hartung *et al.* 1995). In rat Leydig cells, hCG increases both the 3·8 kb and 1·7 kb StAR mRNAs. StAR protein levels increase as early as 2 h after the addition of hCG and peak between 4 and 6 h.

Most studies suggest that IL-1 inhibits hCG-stimulated steroidogenesis (for a review, see Saez 1994). However, the mechanisms of action may differ depending on the species or culture systems. In mouse Leydig cells, IL-1 causes a dose-dependent inhibition of cAMP-stimulated P450c17, P450 scc and 3β-hydroxysteroid dehydrogenase expression (Hales 1992). However, IL-1-mediated inhibition of testosterone biosynthesis is primarily caused by the inhibition of P450c17 expression (Hales 1992). In immature porcine Leydig cells, IL-1α is a potent inhibitor of hCG-stimulated testosterone formation (Mauduit *et al.* 1992). Furthermore, inhibitory effects of IL-1 could be reversed by the addition of (22*R*)-hydroxycholesterol, suggesting that IL-1 might affect the transport of cholesterol into the mitochondria (Mauduit *et al* 1992). However, the activity of steroidogenic enzymes was not determined directly. In our present study, we found that mIL-1α inhibited hCG-induced testosterone

formation and the conversion of (22*R*)-hydroxycholesterol to testosterone, while the conversion of pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone and androstenedione to testosterone were not affected. This further confirms that the major inhibitory effect of mIL-1α on rat Leydig cell steroidogenesis is at the level of P450 scc.

The effects of endotoxin on StAR protein have been reported by Bosmann *et al.* (1996). Adult male mice were treated with 200 μg lipopolysaccharide (LPS) intraperitoneally. Blood and testes were collected after 2 or 24 h. Within 2 h of injection of LPS, there was a greater than 90% decrease in serum testosterone and StAR protein levels. However, there was no inhibition of StAR mRNA expression at 2 or 24 h after LPS injection. P450 scc and P450c17 mRNA levels decreased at 24 h but not at 2 h. Therefore acute decreases in serum testosterone levels were associated with decreased StAR protein and this inhibition appeared to occur at the level of translation. In contrast, we found that, even though mIL-1α almost completely inhibits hCG-stimulated P450 scc expression and testosterone formation, neither StAR mRNA nor protein levels are affected. It is possible that *in vivo* administration of LPS increases cytokine production and alters the hypothalamic–pituitary–adrenal axis in addition to its direct effects on Leydig cells. Increased glucocorticoid in response to *in vivo* administration of LPS also has a direct inhibitory effect on Leydig cell steroidogenesis.

In conclusion, StAR mRNA is expressed in rat Leydig cells. hCG induces StAR mRNA expression and protein synthesis in a time- and dose-dependent manner. Our study provides further evidence that StAR is involved in the acute response of steroidogenic cells to trophic hormone stimulation. IL-1 inhibits P450 scc mRNA expression and testosterone formation but has no effect on either StAR mRNA or protein levels.

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