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# Hormone and Prostaglandin F2α Regulation of Messenger Ribonucleic Acid Encoding Steroidogenic Acute Regulatory Protein in Human Corpora Lutea

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**Steroidogenic acute regulatory (StAR) protein mediates the rapid increase in steroid hormone biosynthesis in response to tropic hormones by facilitating transport of cholesterol into the inner mitochondrial membrane. Although our laboratory has recently reported on the hormonal regulation of StAR mRNA in the rat ovary, the same regulation in the human corpus luteum requires analysis. To this end, a human StAR complementary DNA (cDNA) probe of 858 bp was generated using reverse transcriptase-PCR and RNA from human corpora lutea. The StAR sequence was confirmed by dideoxy chain-termination sequence analysis. Northern blot analysis using the StAR cDNA probe on human corpora lutea mRNA showed that the probe hybridized to a major 1.6-kb transcript and a minor 4.4-kb transcript. Examination of corpora lutea of different luteal phases revealed that the basal expression of the 1.6-kb transcript was significantly more abundant in the early (days 15–19) luteal phase than in the middle (days 20–23) or late (days 24–28) phases. To examine the hormonal regulation of StAR mRNA, corpora lutea were treated in vitro with increasing concentrations of human chorionic gonadotropin (hCG) or prostaglandin F2α (PGF2α). Following hCG stimulation, both 1.6- and 4.4-kb StAR transcripts were increased. A statistically significant increase of 2.2- and 1.8-fold in the 1.6-kb transcript was seen with hCG concentrations of 50 and 100 mIU/mL, respectively. This increase was coupled with a significant elevation in media progesterone levels. In contrast, PGF2α treatment significantly decreased both StAR messenger ribonucleic acid (mRNA) expression and media progesterone levels at concentrations of 500 and 5000 ng/mL. This investigation demonstrated that StAR mRNA is regulated by tropic hormones and prostaglandins in the human corpus luteum. The parallel change in StAR mRNA in conjunction with a change in progesterone levels further supports StAR's putative role in the regulation of steroidogenesis.**

**Key Words:** Ovary; corpus luteum; cholesterol; StAR.

## Introduction

The first enzymatic step in steroidogenesis is the conversion of the cholesterol substrate to pregnenolone *(1)*. This reaction, catalyzed by the cholesterol side-chain cleavage enzyme system (P450scc), which is located (with its associated electron transport chain) on the matrix side of the inner mitochondrial membrane *(2)*, was thought to be the rate-determining step of steroidogenesis. However, the genuine rate-limiting step in the acute regulation of steroidogenesis appears to be the transfer of the substrate cholesterol from the outer to the inner mitochondrial membrane *(3–7)*. It is hypothesized that the facilitation of this step is mediated by an obligate protein(s) that is synthesized *de novo* in direct response to hormonal stimulation. With this in mind, the search for this acutely synthesized regulatory protein(s) has been ongoing for three decades, and a number of candidate proteins have been proposed. These include sterol carrier protein 2 *(8–10)*, steroidogenesis-activator polypeptide *(11)*, and the mitochondrial peripheral benzodiazepine receptor and its ligand *(12)*. None of these proteins, however, appear to mediate cholesterol transport and steroidogenesis acutely.

Most recently, a family of mitochondrial proteins, first described by Orme-Johnson et al. *(13–15)* in several different steroidogenic cell types, have been implicated as the actual acutely stimulated regulatory proteins. Clark et al. *(16)* successfully cloned, sequenced, and expressed a 30-kDa mitochondrial protein from MA-10 mouse Leydig cells, which they named the steroidogenic acute regulatory protein (StAR). This novel mouse protein appears to be

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required for the acute regulation of hormone-induced steroidogenesis *(17–20)*. Of the potential intracellular cholesterol transport proteins noted above, only StAR has been implicated as the acute hormone-mediated mitochondrial cholesterol transport protein. In response to hormone stimulation, the StAR precursor protein is rapidly synthesized in the cytosol and then it migrates to the mitochondria. "Contact sites" between the outer and inner mitochondrial membranes are thought to be formed as this protein is transferred into the inner mitochondrial compartment. It is during the formation of the "contact sites" and the processing of the StAR protein that cholesterol is thought to be transferred from the outer to the inner mitochondrial membrane, thereby making it available for cleavage by the P450scc.

Sugawara and colleagues *(21,22)* elegantly determined the organization of the human StAR structural gene, and mapped it to chromosome 8p11.2. This gene spans 8 kb and consists of 7 exons and 6 introns. StAR expression is restricted to tissues that carry out mitochondrial sterol oxidations and are subject to acute regulation by cAMP *(22)*. Lin et al. further illustrated the importance of StAR in adrenal and gonadal steroidogenesis by showing that in three individuals with congenital lipoid adrenal hyperplasia, the StAR gene was mutated, which resulted in a truncated, nonfunctional StAR protein *(23)*.

Our laboratory has recently demonstrated that the expression of messenger ribonucleic acid (mRNA) encoding StAR in the rat ovary increased with pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), in parallel with an increase in serum progesterone *(24)*. In a separate study *(25)*, we further noted prostaglandin F2α suppressed the expression of both basal and gonadotropin-stimulated StAR expression in the rat ovary. To investigate the regulation of StAR mRNA in the human corpus luteum, basal levels of luteal StAR expression and StAR expression in the corpus luteum in response to hCG and PGF2α were examined. The concomitant changes in the mRNA levels of P450scc were also examined.

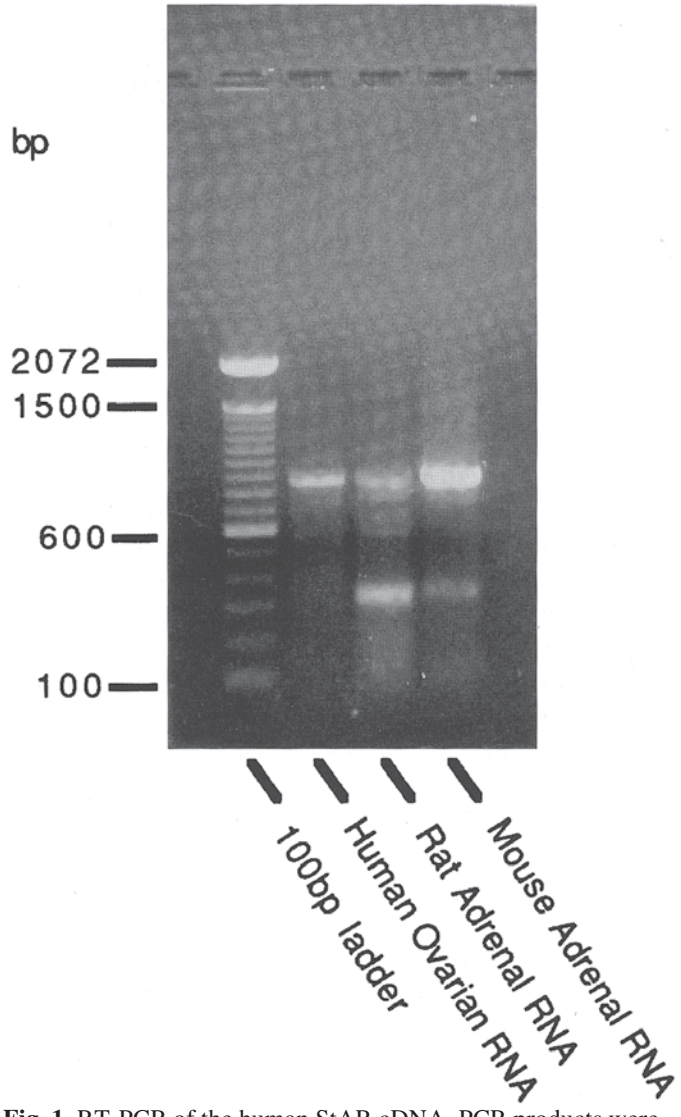


**Fig. 1.** RT-PCR of the human StAR cDNA. PCR products were analyzed by 1% agarose gel electrophoresis. Rat and mouse adrenal RNA were reverse-transcribed to cDNA and amplified by PCR, lanes 3 and 4, respectively.

## Results

A cDNA probe specific for human StAR was generated using reverse transcriptase polymerase chain reaction (RT-PCR) methods. An oligonucleotide primer specific for the 3'-end of human StAR was used to reverse-transcribe human luteal RNA to cDNA. Using oligonucleotides designed from the human StAR cDNA sequence reported by Sugawara et al. *(22)*, a single PCR product was amplified. This PCR product migrated at a similar molecular mass to that of mouse and rat StAR PCR products, which were generated from mouse and rat adrenal RNA using StAR-specific primers *(24)* (Fig. 1). The expected amplicon size of the human StAR cDNA was 858 bp, and was cloned into the TA vector for sequencing and further propagation. Dideoxy chain-termination sequence analysis of the human StAR cDNA indicated that the PCR-amplified sequence was identical to that previously reported *(22)*.

The human StAR cDNA hybridized to rat StAR specific RNA transcripts, suggesting that this probe is valid for the analysis of StAR expression in the human ovary (Fig. 2A). The results of this analysis indicate that the human cDNA probe generated was specific for StAR mRNA and hybridized to a major 1.6-kb transcript and a minor 4.4-kb transcript. The upper transcript in the human appears somewhat larger than the rat upper transcript (3.4 kb). To examine StAR expression throughout the luteal phase, corpora lutea from different stages of development were examined. Basal expression of the 1.6-kb StAR transcript in the human was significantly more abundant in the early luteal phase than

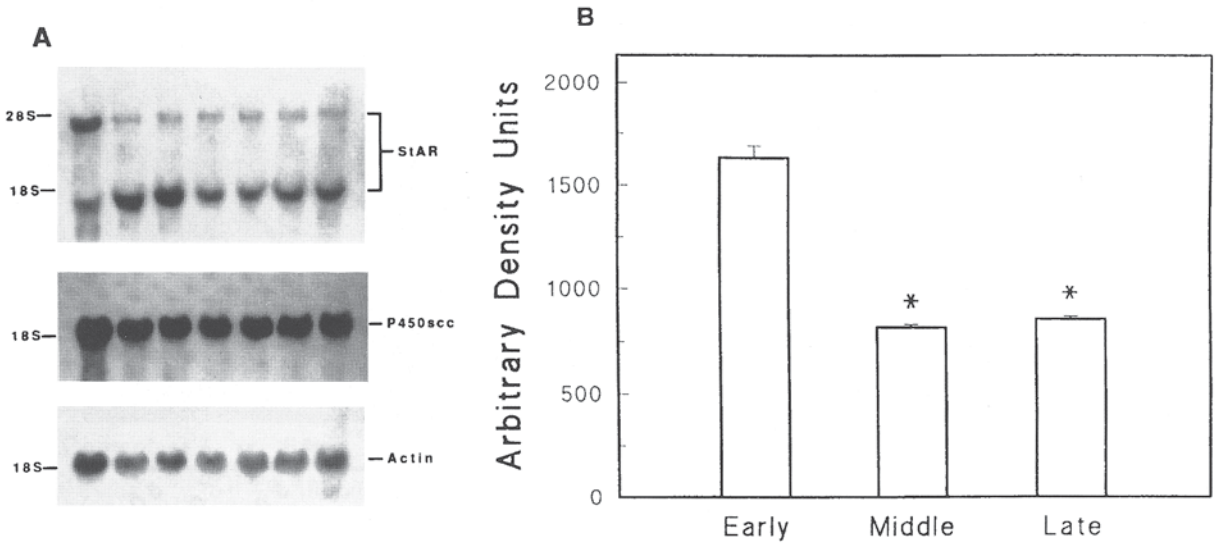


**Fig. 2.** StAR mRNA expression in human early, middle-, and late-luteal phase tissues. **(A)** Rat adrenal RNA is shown for transcript comparison. Northern blot analyses of StAR, P450scc, and β-actin were completed sequentially. **(B)** Densitometric analysis of StAR mRNA levels in human tissues ($n$ = 4/phase). Autoradiographs were scanned with a densitometer and the results presented. Values represent the mean ± SEM of 4 separate corpora lutea/phase. The 1.6-kb StAR transcript was significantly ($p < 0.005$) lower in the middle- and late-luteal phase tissues.

in the middle or late phases (Fig. 2A). Densitometric analysis demonstrated a 50% lower level of the 1.6-kb transcript in the middle or late luteal phase compared to the expression of this transcript in the early luteal phase ($p < 0.005$) (Fig. 2B). In contrast, the level of the 4.4-kb transcript was not different in luteal tissue isolated during the early, middle, or late luteal phases. As expected, the expression of the 2.0-kb P450scc mRNA transcript remained relatively constant throughout the entire luteal phase (Fig. 2A).

Following stimulation with increasing concentrations of hCG, a gradual increase of both 1.6- and 4.4-kb StAR mRNA was demonstrated (Fig. 3). This was accompanied by the emergence of a minor 7.5-kb transcript at the higher hCG concentrations (data not shown). Densitometric analysis of Northern blots probed with the StAR cDNA indicated a statistically significant 2.2- and 1.8-fold increase in the 1.6-kb transcript following hCG treatment at 50 and 100 mIU/mL, respectively ($p < 0.025$) (Fig. 4A). This increase paralleled a concomitant elevation in progesterone levels in the culture media (Fig. 4C). Treatments with low levels of hCG (1 and 10 mIU/mL) produced no effect on StAR or P450scc mRNA expression, whereas concentrations of 50 and 100 mIU/mL resulted in dramatic increases in both StAR and P450scc mRNA levels (Fig. 4A,B).

To determine whether PGF2α could exert its antisteroidogenic effect directly on human luteal StAR mRNA expression, luteal tissue from mid-luteal phase was incubated with increasing doses of PGF2α (0, 5–5000 ng/mL). The results of this experiment indicate that luteal StAR

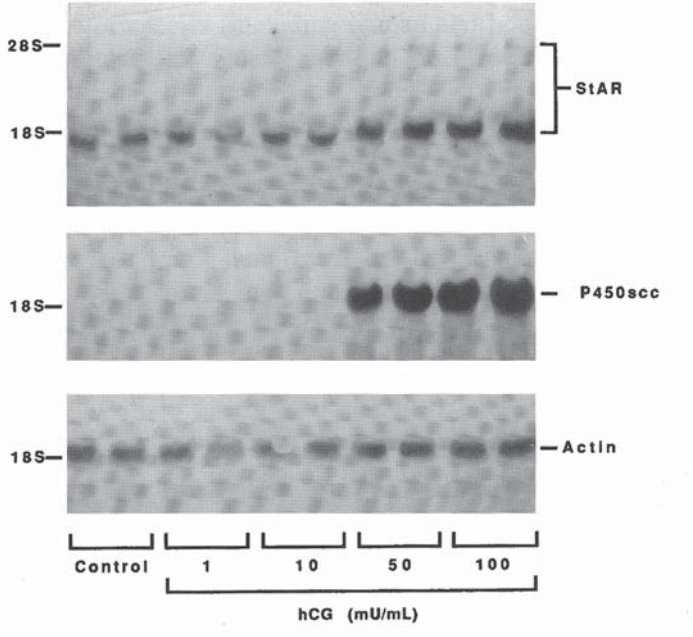


**Fig. 3.** Regulation of StAR mRNA expression in human luteal slices by hCG. Midluteal phase tissue was sliced into <1 mm pieces and incubated with media alone (control) or increasing levels of hCG for 4 h. Northern blot analyses of StAR, P450scc, and β-actin were completed sequentially.

mRNA expression was significantly downregulated (95%; $p < 0.001$; at 500 ng/mL) by PGF2α during a 4-h incubation, whereas actin mRNA levels were unchanged (Fig. 5). The decline in StAR mRNA expression paralleled a significant decrease (50%; $p < 0.001$) in media progesterone lev-

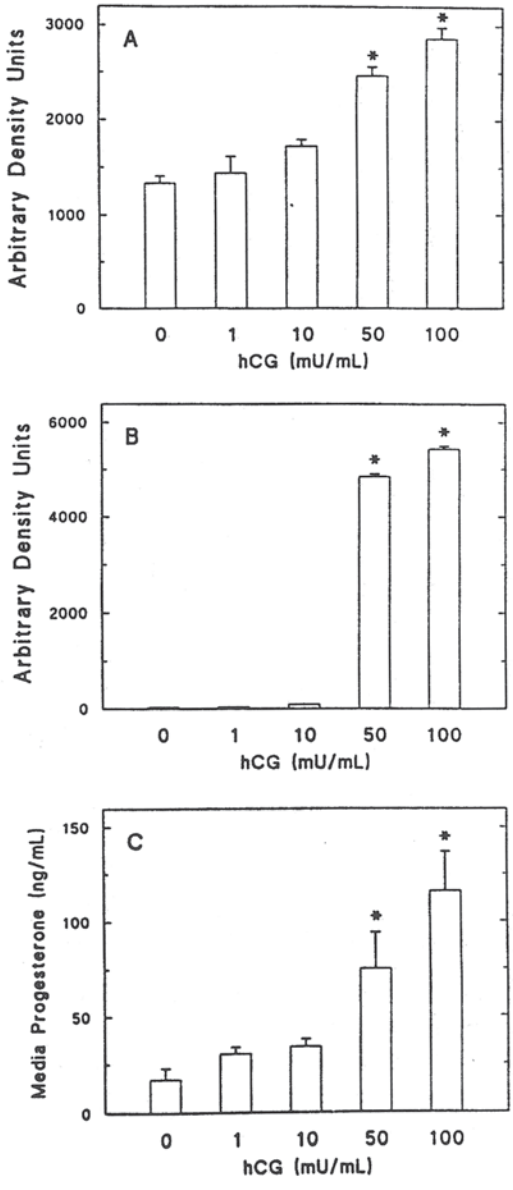


**Fig. 4.** Densitometric analysis of StAR and P450scc mRNA levels in human luteal slices ($n$ = 4/treatment) following hCG incubation. Autoradiographs were scanned with a densitometer and the results presented. **(A)** The 1.6-kb StAR transcript was significantly increased in the luteal slices following hCG treatment at 50 and 100 mIU/mL. **(B)** The P450scc mRNA levels were also increased following hCG treatment. Corresponding media progesterone levels are shown in panel **(C)**. Values represent mean ± SEM of 4 separate cultures. *$p < 0.025$, treatment vs control.

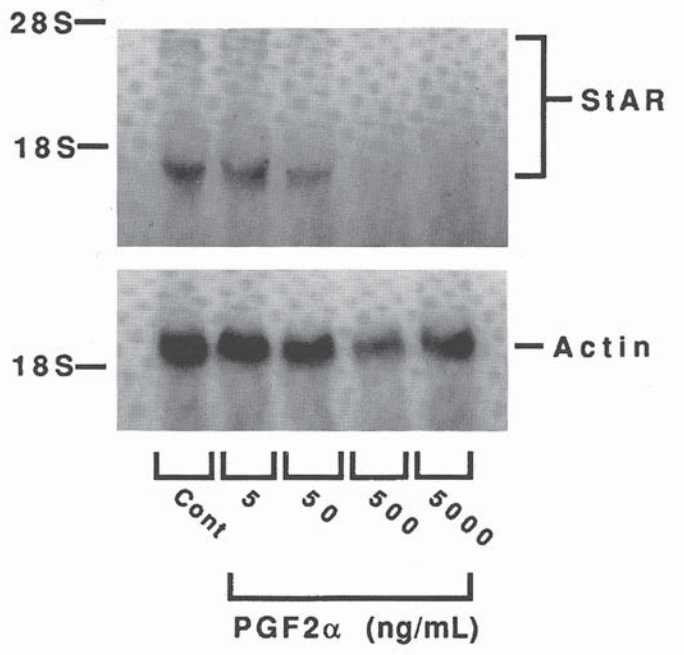


**Fig. 5.** Regulation of StAR mRNA expression in human luteal slices by PGF2α. Midluteal phase tissue was sliced into <1 mm pieces and incubated with media alone (cont) or increasing levels of PGF2α for 4 h. Northern blot analyses of StAR and β-actin were completed sequentially.

els following the 4-h treatment period (Fig. 6). Interestingly, early luteal phase tissue incubated with PGF2α did not show a decline in StAR expression following the 4-h treatment period (data not shown).

## Discussion

A recent commentary by Stocco and Clark *(26)* defines the role of StAR as the long-sought acute regulatory protein in steroidogenesis. Hormonal regulation of StAR mRNA expression in a time- and dose-dependent manner that correlates with steroid production has been characterized in steroidogenic tissues in animals *(13,16,24–29)*. Our experiments examined the regulation of StAR in response to tropic hormone stimulation and PGF2α-mediated repression of StAR in the human corpus luteum.

The human StAR cDNA detected multiple mRNA transcripts in the corpus luteum, the major transcript being at 1.6 kb, consistent with results recently reported by Sugawara et al. *(21)*. A minor 4.4-kb transcript was evident in all stages of the luteal phase, but the 7.5-kb transcript was only seen with hCG stimulation. Basal expression of StAR mRNA was most abundant in the early luteal phase, followed by a decrease seen in the remaining part of the menstrual cycle. This pattern of expression might be correlated with the levels of endogenous luteinizing hormone (LH) during the luteal phase. Immediately following the midcycle surge, which results in ovulation, high levels of LH stimulate the rapid induction of StAR protein and subsequently a cumulative increase in progesterone is seen in the midluteal phase. Lower, but continued tonic secretion of LH after the early luteal phase might explain the falling, but sustained expression in StAR mRNA in the remaining lifespan of the corpus luteum. Other steroidogenic enzymes have also been shown to be regulated by LH *(30)*. However, our data demonstrate a relatively constant expression of P450scc throughout the entire luteal phase. Therefore, the changes in StAR expression appear more likely to be

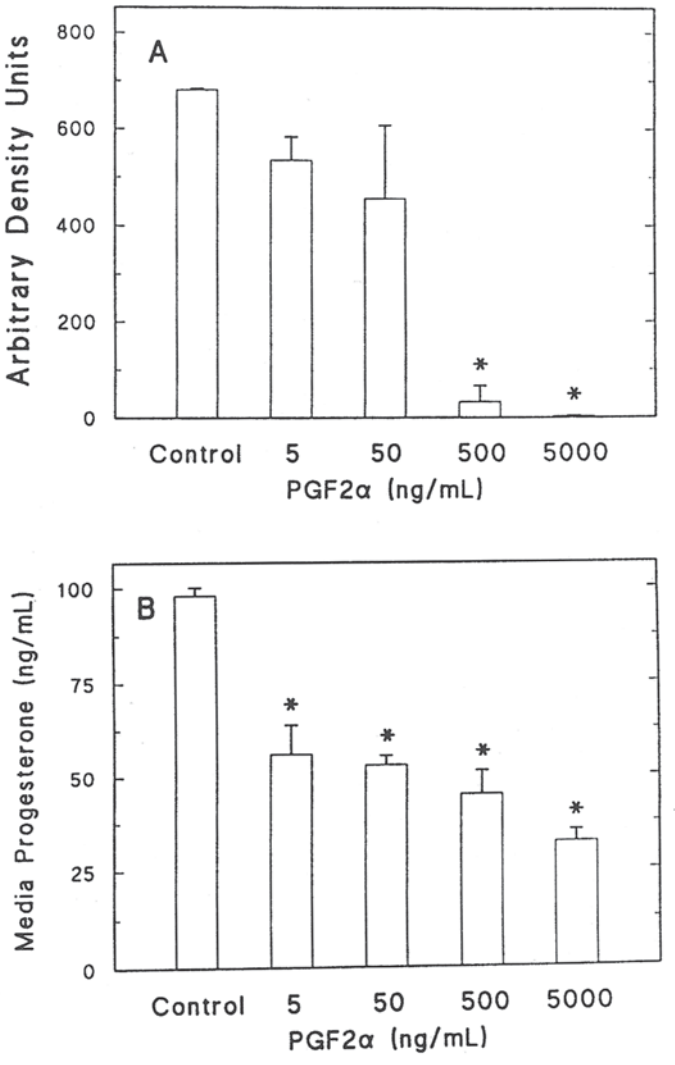


**Fig. 6.** Densitometric analysis of StAR mRNA levels in human luteal slices ($n$ = 4/treatment) following PGF2α incubation. Autoradiographs were scanned with a densitometer and the results presented. **(A)** The 1.6-kb StAR transcript was significantly reduced in the luteal slices following PGF2α treatment at 500 ng/mL or greater ($p < 0.001$). Corresponding media progesterone levels are shown in **(B)**. Values represent mean ± SEM of 4 separate cultures. *$p < 0.001$, treatment vs control.

responsible for the regulation of steroidogenesis rather than a change in P450scc in the corpus luteum.

The rapid increase in steroid hormone biosynthesis in steroidogenic tissues in response to hormone stimulation is well documented *(31–33)*. Our study shows that on hCG stimulation, the expression of the major 1.6-kb StAR mRNA transcript in the human corpus luteum is rapidly enhanced (within 4 h of treatment). The rise in StAR expression paralleled an increase in progesterone levels in the treated media, reaffirming StAR's presumed role in the regulation of steroidogenesis. In contrast to the finding that P450scc is constitutively expressed in luteinized animal ovarian cells independent of hCG stimulation *(24,34,35)*, our results show that P450scc mRNA levels in human corpus luteum slices increased dramatically with hCG treatments at high concentrations of 50 and 100 mIU/mL. Further analysis of the results, however, demonstrated that an increase in StAR expression occurred at a lower concentration of hCG stimulation, compared to the P450scc response. In other words, StAR expression preceded that of P450scc with hormonal stimulation, an observation again consistent with the putative nature of StAR as an acute regulator of steroidogenesis.

If pregnancy does not occur, it is essential for the corpus luteum to regress, allowing initiation of a new reproductive cycle. At the onset of luteal regression, there is a precipitous decline in serum progesterone concentration *(36,37)* followed by a decline in luteal weight *(38,39)*. Regression of the corpus luteum is an example of normal degenerative cellular function, which is a necessary part of the reproductive cycle. In the pig, cow, sheep, and rat, PGF2α is believed to be the physiological agent responsible for causing corpus luteum regression at the end of a nonfertile cycle *(40,41)*. There is growing evidence that PGF2α is also important for corpus luteum involution in humans *(42–44)*. PGF2α has been shown to depress cAMP accumulation and serum progesterone and to antagonize LH-stimulated steroid production in luteal tissue *(45)*. The ability of PGF2α to inhibit luteal cell progesterone production in a variety of species (including human and nonhuman primates) is well documented *(38–45)*. However, its mechanism of action is poorly understood. Our laboratory, in a prior study, demonstrated that PGF2α alters ovarian cholesterol transport capacity as part of its antisteroidogenic action *(46)*. The results of this study show that PGF2α inhibited StAR mRNA expression at high concentrations in the human corpus luteum, in association with a decrease in media progesterone levels.

Following PGF2α-induced luteolysis in sheep and cows, corpora lutea accumulate substantial stores of lipids *(47,48)*, implying that the uptake of cholesterol is not impaired. Recently, Pate and Condon *(49)* concluded that following PGF2α treatment, luteal tissue could bind and internalize lipoproteins, but determined that LDL-derived cholesterol utilization for steroidogenesis was abrogated. In the rat, PGF2α administration also had no effect on lipoprotein binding to luteal membranes, suggesting that PGF2α treatment does not affect the uptake of lipoproteins. Grusenmeyer and Pate *(50)* suggest that PGF2α inhibits progesterone production at a site subsequent to cholesterol transport to the mitochondria, or that it may inhibit cholesterol transfer from the outer to the inner mitochondrial membrane. The current study demonstrates that PGF2α administration leads to a significant reduction in StAR mRNA levels in the human corpus luteum consistent with this prostaglandin altering cholesterol transport to the mitochondria.

In summary, our data provide evidence that tropic hormone stimulation increased StAR mRNA expression, but PGF2α reduced StAR mRNA levels in the human corpus

luteum. An increase in StAR mRNA levels is accompanied by a concomitant increase in progesterone production. These results strengthen the concept that StAR is pivotal in the acute regulation of steroidogenesis. Further characterization of the regulation of StAR at the transcriptional, translational, and posttranslational levels will enhance our understanding of the function of this essential protein.

## Materials and Methods

### *Collection of Corpora Lutea*

Corpora lutea were obtained from women, age 29–41 yr, undergoing total hysterectomy and unilateral or bilateral oophorectomies for benign gynecological conditions at Tampa General Hospital of the University of South Florida (USF). The study was approved by the USF Institutional Review Board. All corpora lutea were obtained from patients without any recent history (3 mo prior to surgery) of hormonal treatment. Once the ovary was removed and examined by the pathologist, the corpus luteum was quickly dissected out, transferred to the laboratory in normal saline solution, and either stored at –80°C until analyzed or immediately processed for hormone/prostaglandin treatment as described in the Hormonal Treatment and Culture of Ovarian Tissues section. Corpora lutea were assigned to early (days 15–19), middle (days 20–24), or late (days 25–28) stage according to endometrial dating of hysterectomy specimens *(51)*.

### *Chemicals*

hCG and prostaglandin F2α were purchased from Sigma Chemical Co. (St. Louis, MO). [$^{35}$S] deoxy-ATP (1348 Ci/mmol) was purchased from DuPont-New England Nuclear (Wilmington, DE). [$\alpha^{32}$P] deoxy-CTP (3000 Ci/mmol) and the Sequenase DNA sequencing kit were obtained from the Amersham Corp. (Arlington Heights, IL). BioMax and XAR-5 films were purchased from Eastman Kodak (Rochester, NY). SeaKem and SeaPlaque agarose were purchased from the FMC Corporation (Rockland, ME), and nylon membrane was obtained from Schleicher and Schuell (Keene, NH). TRI-Reagent, Background Quencher, Formazol, Microcarrier Gel-TR, and High Efficiency Hybridization solution were obtained from Molecular Research Center (Cincinnati, OH). The Random Primed DNA labeling kit and all restriction enzymes were obtained from Boehringer Mannheim (Indianapolis, IN). The Wizard Miniprep and Megaprep DNA purification systems were purchased from Promega (Madison, WI). The kits for RT-PCR were purchased from Perkin Elmer (Norwalk, CT). The TA cloning kit was purchased from Invitrogen (San Diego, CA) and the Sephaglas BandPrep Kit was obtained from Pharmacia Biotech (Piscataway, NJ). An RNA mol-wt marker was purchased from Gibco BRL (Grand Island, NY), and all other chemicals were reagent-grade and were obtained from Fisher Scientific (Norcross, GA) or Sigma Chemical Co.

### *Isolation of the Human StAR Complementary DNA (cDNA) Probe by RT-PCR*

Oligonucleotide primers were engineered based on the recently published human StAR cDNA sequence *(22)*. The 5'-primer (CAATGCTGCTAGCGACATTC) spanned bases 125–144 of the human cDNA and the 3'-primer (CTTCAACACCTGGCTTCAGA) spanned bases 967–986. The 3' (downstream) primer was used to carry out RT-PCR from RNA isolated from human corpora lutea. One microgram of human luteal RNA was reverse transcribed for 1 h at 42°C. This cDNA was then amplified by PCR using both primers. The conditions for PCR were denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 3 min.

### *Characterization of the Human StAR cDNA Probe*

The 858-bp fragment obtained by RT-PCR was cloned into the TA vector (Invitrogen). The StAR cDNA was sequenced using the dideoxy chain-termination method *(52)* with [$^{35}$S]-deoxy-ATP and the Sequenase 2.0 protocol (Amersham). The nucleotide sequence of the RT-PCR product was identical with the StAR cDNA sequence published by Sugawara et al. *(22)*.

### *Human P450scc and β-Actin cDNA Probes*

The human P450scc cDNA was provided by Walter Miller (University of San Francisco, CA) in the PECE vector containing the F2 construct *(53)*. The insert was subcloned into the TA vector (Invitrogen) for use in Northern blot analysis. A 2.0-kb chicken β-actin cDNA probe was used to normalize mRNA levels in all Northern blots *(24)*.

### *Hormonal Treatment and Culture of Ovarian Tissues*

On removal from surgical specimens, corpora lutea were minced to <1-mm pieces. Luteal tissue was transferred to sterile tissue-culture dishes (Falcon, Cockeysville, MD) in equal amounts and incubated in various concentrations of hCG (0, 1, 10, 50, and 100 mIU/mL) or PGF2α (0, 5, 50, 500, 5000 ng/mL) in DMEM medium at 37°C for 4 h on a shaking platform at 100 rpm. A constant 95% $O_2$/5% $CO_2$ environment was maintained during incubation. Tissues were frozen at –80°C at the end of the 4-h experiment until analyzed. The media were frozen at –20°C until assayed for progesterone levels.

### *RNA Isolation*

RNA was prepared from untreated and hormonally treated luteal tissue using a modification of the Chomczynski and Sacchi method *(54)* (TRI-Reagent Method, Molecular Research Center). Tissue (<200 mg) was homogenized in 1 mL of TRI-Reagent with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY), and centrifuged at 11,000*g* for 15 min at 4°C. RNA was precipitated from the aqueous phase with isopropanol, and the

RNA pellet was washed in 75% ethanol and resuspended in Formazol (Molecular Research Center). RNA was quantified by absorbance at 260 nm in a Beckman DU-70 spectrophotometer (Palo Alto, CA).

For Northern blot analysis, equal amounts of total RNA (20 μg) were denatured at 65°C (15 min) and loaded onto 1% agarose gels containing 3% formaldehyde. Ethidium bromide staining of the gel confirmed that the ribosomal RNAs (18S and 28S subunits) were intact and that equal amounts of RNA were loaded in each lane. Following size fractionation, RNA was blotted onto a nylon membrane (0.45-μm pore size) by capillary transfer for 16 h, and RNA was fixed to the membrane by UV crosslinking (0.3 J/cm$^2$).

### *Northern Blot Hybridization*

Northern blot hybridizations were performed using cDNAs labeled with [$\alpha^{32}$P] deoxy-CTP using the Random Primed DNA labeling method. Northern blots were prehybridized at 62°C for 3 h in a 1 *M* NaCl, 1% SDS solution with Background Quencher (Molecular Research Center). Hybridization was completed in High Efficiency Hybridization Solution (Molecular Research Center) containing the radiolabeled probe ($1 \times 10^6$ cpm/mL; SA = $2 \times 10^8$ dpm/μg DNA) at 62°C for at least 16 h. Blots were washed one to two times at room temperature (5 min) in 1X SSC/1% SDS. RNA:cDNA hybrids were visualized on BioMax film using two intensifying screens and a 24-h exposure period. The RNA transcript size was determined by comparison to an RNA mol-wt marker run adjacent to the sample RNA lanes. Blots were stripped and reprobed sequentially with actin and P450scc cDNAs. Densitometric analysis was performed on the 2.0-kb β-actin transcript for the standardization of RNA loading.

### *Progesterone Assay*

Progesterone was measured by ELISA using the progesterone assay kit (Boehringer Mannheim, Indianapolis, IN) with the ES300 Automated ELISA Reader (Boehringer Mannheim) as previously described *(25)*.

### *Data Analysis*

The Northern blot results were quantitatively analyzed using a Hoefer Scanning Densitometer (Hoefer Instruments, San Francisco, CA). Minor variations in RNA loading were corrected for using the β-actin cDNA. Data from these individual parameters were compared by analysis of variance (AVONA) followed by the Student-Newman-Keuls multiple-comparison test when applicable *(55)*. All analyses were completed using the Statview program with graphics (Abacus Concepts, Berkeley, CA) on a MacIntosh IIci computer. A $p < 0.05$ was considered significant for all tests.

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