0021-972X/03/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 88(7):3421–3430

doi: 10.1210/jc.2002-021916

The Steroidogenic Response and Corpus Luteum Expression of the Steroidogenic Acute Regulatory Protein after Human Chorionic Gonadotropin Administration at Different Times in the Human Luteal Phase

PAULINA KOHEN, OLGA CASTRO, ALBERTO PALOMINO, ALEX MUÑOZ, LANE K. CHRISTENSON, WALTER SIERRALTA, PILAR CARVALLO, JEROME F. STRAUSS III, AND LUIGI DEVOTO

Instituto de Investigaciones Materno Infantil y Departamento de Obstetricia y Ginecología, Facultad de Medicina, Universidad de Chile, Hospital Clínico San Borja-Arriarán (P.K., O.C., A.P., A.M., L.D.); and Instituto de Nutrición y Tecnología de Alimentos, Universidad de Chile (W.S.), Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile (P.C.), CP 6519100 Santiago, Chile; and Center for Research on Reproduction and Women's Health, University of Pennsylvania (L.K.C., J.F.S.), Philadelphia, Pennsylvania 19104

This study was designed 1) to assess corpus luteum (CL) steroidogenesis in response to exogenous human chorionic gonadotropin (hCG) at different times during the luteal phase, 2) to examine the effect of hCG on steroidogenic acute regulatory protein (StAR) expression within the CL, 3) to correlate StAR expression and luteal steroidogenic responses to hCG, and 4) to determine whether endogenous LH regulates ovarian steroidogenesis in the early luteal phase. Blood was collected before and after hCG treatment for steroid and hCGβ determinations. CL were obtained at the time of surgery to assess StAR gene and protein expression. During the early luteal phase various women received the GnRH antagonist for 24–48 h; some of them also received hCG 24 h after the GnRH antagonist. A slight steroidogenic response to hCG was observed in early luteal phase; 17α-hydroxyprogesterone, but not progesterone (P4), levels were significantly increased 8 h post-hCG, indicating a differential response by the granulosa and theca-lutein cells. The 1.6- and 4.4-kb StAR transcripts and the 37-kDa preprotein and 30-kDa mature StAR protein did not change post-hCG administration in early luteal phase CL.

In contrast, the StAR 4.4- and 1.6-kb transcripts diminished significantly ($P < 0.05$) after the antagonist treatment. Immunohistochemical staining for StAR protein was weak, particularly in granulosa-lutein cells. Treatment with hCG restored StAR mRNA and protein and plasma P4 levels within 24 h in antagonist-treated women. hCG stimulated the highest plasma concentrations of P4 and estradiol in the midluteal phase, indicating its greatest steroidogenic capacity. Midluteal tissue StAR gene and protein expression increased by 1.6- and 1.4-fold after 24 h of hCG treatment, respectively. Administration of hCG resulted in the greatest increment in plasma P4 (4-fold) and 17α-hydroxyprogesterone (3-fold) levels over baseline in the late luteal phase. This was associated with an increase in StAR mRNA (3.5-fold) and protein (1.8-fold).

Collectively, these data indicate that 1) the hCG-stimulated steroidogenic response is dependent on the age of the CL; 2) the early luteal phase CL is relatively insensitive to exogenous hCG in the presence of normal pituitary gonadotropin support, but becomes responsive when the latter is withdrawn; 3) the hCG-stimulated steroidogenic response in the mid- and late luteal phase is correlated with increased StAR mRNA and protein abundance; and 4) there are differential responses of small and large luteal cells to hCG stimulation that depend upon the age of the CL. (*J Clin Endocrinol Metab* 88: 3421–3430, 2003)

THE CORPUS LUTEUM (CL) is a transient endocrine gland formed from the ruptured ovulatory follicle. In the human, the main function of the CL is to produce progesterone (P4) under the influence of pituitary-derived LH and later trophoblast-derived human chorionic gonadotropin (hCG) during a cycle of conception. LH and hCG bind to and activate a specific glycoprotein membrane receptor on luteal steroidogenic cells to primarily stimulate the adenylyl cyclase/cAMP/protein kinase A pathway, which has been widely recognized as the primary signaling cascade through which gonadotropins affect luteal steroid biosynthesis (1).

It is thought that the rate-limiting step in P4 synthesis is the movement of cholesterol from the outer to the inner mitochondrial membrane where the cytochrome P450 side-chain cleavage system is located (2). Steroidogenic acute regulatory protein (StAR), a cAMP-dependent phosphoprotein expressed in luteal cells, is essential for this sterol translocation process resulting in pregnenolone and P4 synthesis (3).

The steroidogenic response of the CL to exogenous hCG has been tested in several primate species, including the human and monkey (4, 5). Administration of hCG in the midluteal phase achieves the highest P4 levels, whereas treatment during the early luteal phase has relatively little effect, and during the late luteal phase it prevents the decline in luteal steroidogenesis (6). In the clinical setting, hCG administration has been proposed as a treatment for luteal phase defects in unstimulated cycles as well as in cycles stimulated

Abbreviations: AUC, Area under the curve; CL, corpus luteum; E2, estradiol; hCG, human chorionic gonadotropin; 17αOHP, 17α-hydroxyprogesterone; P4, progesterone; SSC, standard saline citrate; StAR, steroidogenic acute regulatory protein; T, testosterone.

with clomiphene citrate or human menopause gonadotropin (7). In women, hCG administration in a regimen that mimics hCG concentrations after spontaneous embryo implantation sustains the steroidogenic capacity of the CL and luteal expression of StAR and 3β-hydroxysteroid dehydrogenase mRNA and protein (8).

Our recent findings indicate that StAR mRNA and protein are highly expressed in early and midluteal phase CL, and those plasma P4 concentrations are highly correlated with luteal expression of both premature and mature StAR proteins (9). These observations corroborate a key role for StAR gene expression in the regulation of human luteal steroidogenesis. We hypothesized that StAR mRNA and protein expression plays a key role in controlling the CL steroidogenic response to exogenous hCG throughout the luteal phase. In the present study we determined whether the hCG-stimulated steroidogenic response of CL throughout the luteal phase is dependent on the age of the CL and is correlated with changes in StAR mRNA abundance and StAR protein levels. Additionally, we examined the cellular localization of StAR protein by immunohistochemistry to determine whether there are differences in the activities of large and small luteal cells, respectively.

Materials and Methods

Subjects and experimental design

The experimental protocol was approved by the San Borja-Arriaran Medical ethics committee, and signed informed consent was obtained from all women participating in these studies.

Corpora lutea were enucleated at the time of minilaparotomy for tubal sterilization, as described previously (10). The surgery was scheduled at varying times throughout the luteal phase at the Hospital Clínico San Borja-Arriarán, National Health Service, Universidad de Chile (Santiago, Chile). All women were healthy (n = 7 control and n = 20 treated), aged 31–40 yr, with a body mass index ranging from 22–30, with regular menstrual cycles, and they had not received any form of hormonal contraception for at least 6 months before participating in the study. Fourteen women undergoing surgery were administered 10,000 IU hCG (Profasi, Serono, Milan, Italy) im during the early (n = 3), mid (n = 7), and late (n = 4) luteal phase, respectively, 20–24 h before surgery. Seven women undergoing tubal ligation were scheduled for surgery at different stages of the luteal phase without receiving hCG and represent the control group. Serial plasma samples were obtained before hCG administration (time zero) and at 1, 2, 4, and 8 h after hCG treatment. Specific RIAs were used to determine P4, testosterone (T), and estradiol (E2) plasma levels as previously reported (10) or using commercial kits for 17α-hydroxyprogesterone (17αOHP) and hCGβ according to the manufacturer's instructions (Diagnostic Products, Los Angeles, CA). Three women undergoing tubal ligation were given 2 mg GnRH antagonist (Cetrorelix, Serono), sc, during the early luteal phase for 24 or 48 h (n = 3) before surgery, respectively. Another three women received Cetrorelix for 48 h, followed by hCG treatment (10,000 IU), im, 24 h before surgery. Plasma steroids were determined before treatment (time zero) and on a daily basis after treatment.

Dating of the CL and tissue preparation

The CL were dated on the basis of the presumptive day of ovulation, which was determined by vaginal ultrasound scan and the urinary LH peak as previously reported (10). Furthermore, plasma P4 and endometrial and CL histological features were used to confirm tissue dating. Briefly, the morphological criteria used in determining the age of the CL included sprouting of capillaries into granulosa cells and luteinization, the appearance of fibroblasts in the central cavity, and shrinkage and vacuolization of granulosa cells. The histological dating of the endometrial tissue followed the Noyes criteria (11). The CL were classified as early (2–4 d post-LH peak; n = 3), mid (5–9 d post-LH peak; n = 7), and late (10–12 post-LH peak; n = 4).

The entire CL was enucleated from the ovary, transported to the laboratory, and processed as previously reported (10). Tissue was weighed, and pieces were collected, fixed in 4% buffered paraformaldehyde, and embedded in paraffin wax for histology, immunohistochemistry, and *in situ* hybridization. Other pieces of tissue were snap-frozen in liquid nitrogen and stored at –70 C for subsequent RNA and protein extraction.

Northern blot analysis

Total RNA was isolated by the method of Chomczynski and Sacchi (12), and its concentration was quantified by absorbance at 260 nm. Northern analysis was accomplished using a human StAR cDNA as a probe with minor modifications as reported previously (9). Briefly, 10 μg total RNA were resolved on 1% agarose-formaldehyde gels, blotted onto GeneScreen Plus nylon membranes (NEN Life Science Products, Boston, MA), and cross-linked by UV irradiation. The membranes were prehybridized for 30 min at 42 C in Ultrahyb hybridization solution (Ambion, Inc., Austin, TX). Hybridization was carried out overnight at 42 C in the same solution after addition of the ^{32}P-labeled probe (10^6 cpm/ml solution). Membranes were washed twice for 5 min each time at 42 C in 0.5× standard saline citrate (SSC)/0.1% sodium dodecyl sulfate, followed by two washes in 0.1× SSC/0.1% sodium dodecyl sulfate for 15 min each time at 42 C. Blots were exposed to x-ray film (Kodak, Rochester, NY) for 24 h. StAR signals were normalized for loading differences with the densitometric values obtained for 18S rRNA detection by ethidium bromide staining. The intensities of autoradiographic signal/ethidium staining were estimated by densitometric scanning using a BioImage Scanner UMAX Vista Scan T630 (software NIH 1.6, BioImage, Ann Arbor, MI).

Western blot analysis

Western blot analysis of StAR protein was carried out with minor modifications as reported previously (9). Briefly, luteal tissues were homogenized in a buffer containing 20 mM HEPES, 100 mM KCl, 1 mM dithiothreitol, 2 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, and 50 mM benzamidine and centrifuged at 10,000 × *g* for 15 min. Protein extract (5 μg) was loaded onto a 10% acrylamide gel for SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Hybridon, Millipore Corp., Bedford, MA). The membranes were incubated with a rabbit polyclonal antihuman StAR antibody as previously described (9). The antirabbit Vistra ECF Western blotting kit (Amersham Pharmacia Biotech, Arlington Heights, IL) was used to detect the primary antibody and generate a chemifluorescence signal that was quantified on a Storm PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) using the blue fluorescence/chemifluorescence mode. ImageQuant 1.1 software (Molecular Dynamics, Inc.) was used to analyze the signals for both premature and mature StAR protein.

In situ hybridization

In situ hybridization for StAR mRNA was performed using digoxygenin-labeled riboprobes on sections of midluteal phase CL (13). Riboprobes were synthesized by *in vitro* transcription from the plasmid preparation used for Northern hybridization, using DIG RNA labeling mix (Roche, Indianapolis, IN) with T7/T3 polymerase, purified, quantified, and stored at −80 C. Briefly, paraffin-embedded sections (4 μm thick) were mounted on slides and deparaffinized with a xylol substitute solvent (Poly/clear, DAKO Corp., Carpinteria, CA). Sections were rehydrated through a series of decreasing concentrations of ethanol. Tissue sections were thereafter digested with 20 μg/ml proteinase K (Sigma-Aldrich Corp., St. Louis, MO) for 10 min, acetylated in 0.25% acetic anhydride in the presence of 0.1 M triethanolamine for 10 min, washed thoroughly in 0.1 M PBS, dehydrated through a series of ascending concentrations of ethanol solutions, and air-dried.

Sense and antisense digoxigenin-labeled RNA probes for StAR (200 ng/ml) were applied in hybridization buffer containing 10% dextran sulfate/50% formamide and incubated for 17 h at 54 C. After hybridization, the tissue sections were washed four times in SSC solutions to a maximum stringency of 0.1× SSC at 58 C. An enzyme-catalyzed color

reaction using nitro blue tetrazolium chloride (Roche) visualized specifically bound riboprobe as a chromogen. Slides were counterstained with 2 μg/ml bisbenzimide (Hoechst 33258) before mounting with Paramount aqueous mounting medium (DAKO Corp.).

Immunohistochemical localization of StAR in hCG-stimulated human CL

Immunohistochemistry was conducted as previously reported with minor modifications (9). Briefly, tissue sections (5 μm) of hCG-treated CL obtained during the early (n = 3), mid (n = 5), and late CL (n = 4) luteal phases and from placenta were mounted on ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA) and deparaffinized with a xylol substitute solvent (Poly/clear solvent, DAKO Corp.). The sections were stained by the peroxidase conjugate technique (Calbiochem-Novabiochem Co., La Jolla, CA) using capillary technology (MicroProbe Staining System, Fisher Scientific) and incubated in 3% H_2O_2 to block endogenous peroxidase activity. Nonspecific binding was suppressed with normal goat serum diluted 1:10 in PBS containing 4% BSA. The sections were incubated with a rabbit polyclonal anti-StAR antibody (1:500). After washing, the sections were incubated with a goat antirabbit peroxidase-conjugated immunoglobulin G and processed as reported previously (9). Color was developed with 3-amino-9-ethylcarbazole (DAKO Corp.). The sections were counterstained with Mayer's hematoxylin solution (DAKO Corp.).

Histological sections of CL incubated with normal rabbit serum in place of anti-StAR antibody served as a negative control. Staining of placental tissue, which does not express StAR, was also used as a negative control. We have previously shown that the StAR antibody specifically detects StAR in sections of human tissue and that the specific signal is ablated by neutralization of the antiserum with recombinant human StAR antigen. Slides were evaluated by two observers using a subjective semiquantitative scale of: −, no staining; +, minimal staining; 2+, moderate staining; and 2++, intense staining.

Statistical analysis

The increment in steroid production after hCG treatment was defined by the ratio of stimulated steroid concentration/basal steroid concentration. The increment is expressed as the fold increase for each steroid. Experimental data are presented as the mean ± SEM. To compare the maximal plasma levels of the hormones to the basal levels, the area under the curve (AUC) for steroids was analyzed by the paired *t* test for normally distributed variables. Northern and Western blotting experiments performed with CL tissue included at least three subjects for each stage of the luteal phase. ANOVA and Tukey's multiple comparison tests were used to analyze these data employing PRISM version 3.00 for Windows (GraphPad Software, Inc., San Diego, CA). Statistical significance was considered at the $P < 0.05$ level.

Results

Differential responses of plasma steroid hormones to hCG stimulation at different stages of the luteal phase

Table 1 summarizes the clinical features of the study participants, including mean age, body mass index, range of endometrial dates at the time of study, mean CL weights, and basal and hCG treatment plasma concentrations for P4, 17αOHP, T, and E2. There were no significant differences in mean age and body mass index among the subjects studied at the three different stages of the luteal phase. The mean weights of the control CL exhibited a significant decline in the late luteal phase. In contrast, the mean weights of the hCG-treated CL did not differ significantly throughout the luteal phase. However, the mean weights of CL exposed to hCG were greater in the mid and late luteal phases compared with those of control CL. Thus, hCG administration prevented the significant decline in CL weight that typically occurs during the late luteal phase.

Administration of 10,000 IU hCG caused the greatest increment in plasma P4 (4-fold) and 17αOHP (3-fold) during the late luteal phase. In the midluteal phase there was a significant response to the hCG challenge in plasma P4, 17αOHP, and E2. However, hCG did not increase T levels. Although P4 and E2 levels in plasma increased after the administration of hCG in the early luteal phase, the increments in the level of these steroids were not statistically significant. In contrast, 17αOHP increased significantly after hCG administration in the early luteal phase. Thus, there is a differential ovarian response to hCG in terms of progestin production in the early luteal phase.

The histological dating of endometrial biopsies taken at the time of surgery was correlated with the preovulatory LH peak. Dating of only one specimen showed a more than 2-d advance in stromal maturation compared with the endometrial glands. Thus, administration of hCG did not overtly modify the histological characteristics of the endometrium used in dating within the 24-h period between hCG injection and endometrial biopsy.

Figure 1 presents the plasma concentrations of hormones at 0, 1, 2, 4, and 8 h after hCG treatment during the early, mid, or late luteal phase. Figure 1A shows the mean plasma concentrations of hCGβ over the 8-h sampling period in women

TABLE 1. Clinical and endocrine characteristics of study subjects and results of hCG challenge at different stages of the luteal phase

Variable	Early (n = 3)		Mid (n = 7)		Late (n = 4)	
	Basal	hCG	Basal	hCG	Basal	hCG
Age (yr)	39 ± 1.0		34.9 ± 3.4		37.7 ± 1.3	
Body mass index (kg/m^2)	25.7 ± 3.5		25.4 ± 0.89		30.9 ± 3.5	
Weight of CL (g)	1.69 ± 0.56	1.87 ± 0.36	1.51 ± 0.1	1.9 ± 0.16[a]	0.81 ± 0.14[b]	1.62 ± 0.25[a]
Progesterone (nmol/liter)	10.8 ± 5.7	15.9 ± 4.1	31.5 ± 5.1	50.2 ± 7.3[c]	10.8 ± 2.2	43.8 ± 4.4[c]
17αOHP (nmol/liter)	5.5 ± 1.8	11.4 ± 0.9[c]	8.9 ± 2.8	20.1 ± 5.0[c]	3.2 ± 1.3	10.3 ± 2.2[c]
Testosterone (nmol/liter)	2.0 ± 0.3	1.9 ± 0.3	1.6 ± 0.2	1.6 ± 0.4	1.2 ± 0.2	1.6 ± 0.2
Estradiol (pmol/liter)	374 ± 73	470 ± 40	440 ± 77	653 ± 99[c]	470 ± 91	587 ± 51
Histological dating of endometrium (days of cycle)	16–20		21–24		25–27	

Values are the means ± SEM.
[a] Significant difference ($P < 0.05$) in hCG effect on CL weight *vs.* basal condition.
[b] Significant difference ($P < 0.05$) in CL weight throughout the luteal phase.
[c] Significant difference ($P < 0.05$) in hCG *vs.* basal steroid concentrations.

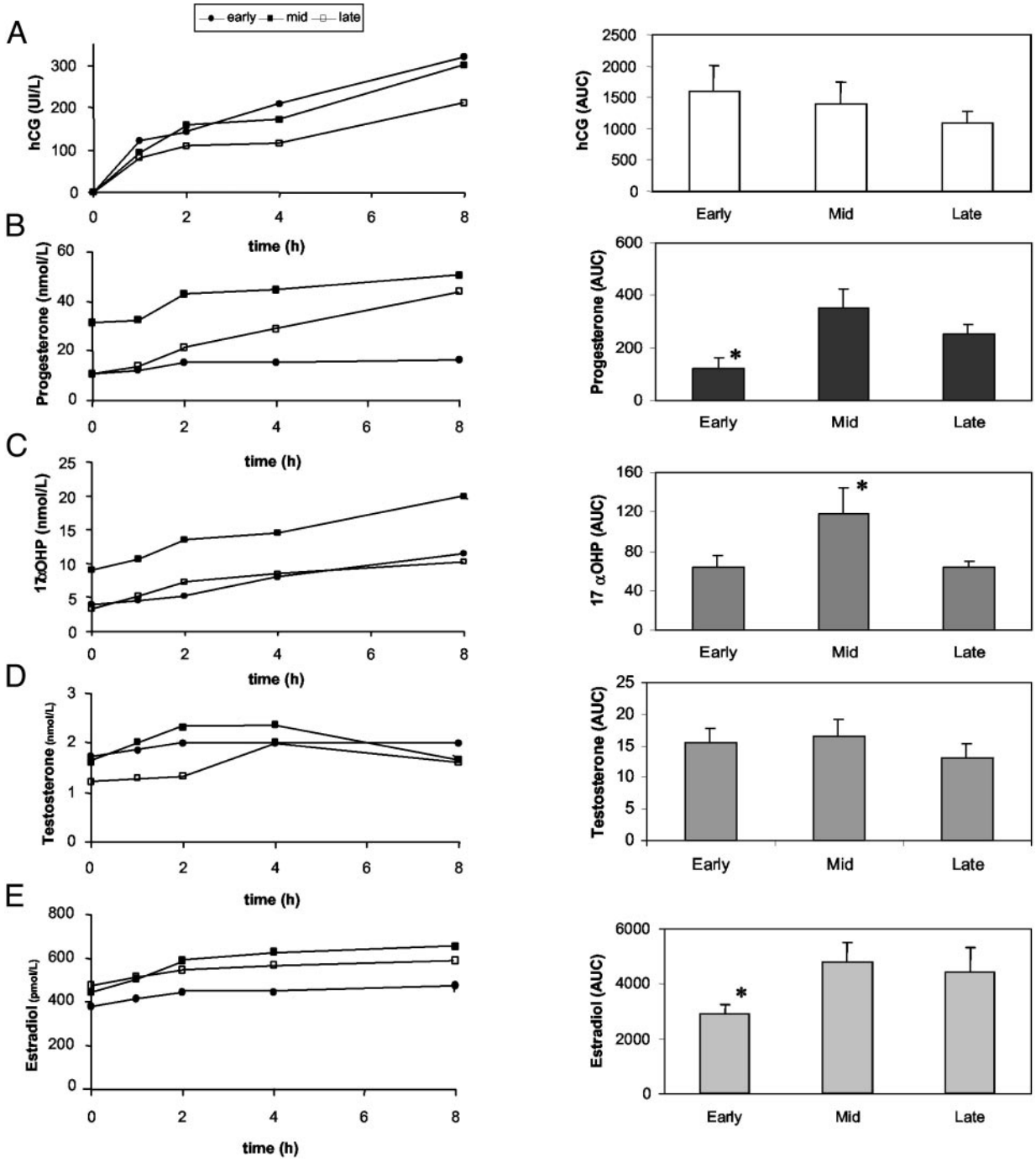

FIG. 1. Plasma concentrations of hCGβ, P4, 17αOHP, T, and E2 after hCG treatment of women during early, mid, or late luteal phase over an 8-h sampling period. The AUC was determined for each steroid at every stage of the luteal phase. *, The mean ± SEM are significantly ($P < 0.05$) different at every stage of the luteal phase

given a single dose of 10,000 IU hCG, im, at the three different stages of the luteal phase. Plasma hCGβ concentrations increased progressively in the majority of subjects during the study period, and they were not affected by the stage of the luteal phase.

Figure 1, B and C, illustrate the mean plasma concentrations of P4 and 17αOHP in women treated with hCG during the early, mid, and late luteal phases. Plasma P4 concentrations in the mid and late luteal phases displayed a progressive rise throughout the study period, being steepest during the first hour after hCG administration in the midluteal phase. Concentrations of P4 remained largely unchanged during the hCG challenge in the early luteal phase. In contrast, the plasma level of 17αOHP, which is produced predominantly by the CL, exhibited a progressive rise at all stages of the luteal phase, reaching highest levels 4–8 h after hCG treatment during the midluteal phase. The AUC for plasma P4 was significantly less during the early luteal phase

compared with the mid and late luteal phases. In contrast, the AUC for plasma 17αOHP was greatest during the midluteal phase ($P < 0.05$). Thus, the ovarian response to hCG in terms of progestin production displays temporal differences as well as CL age dependency.

Figure 1D shows the effect of hCG on plasma T levels throughout the luteal phase. hCG did not significantly stimulate T production in the early, mid, or late luteal phase. However, a trend for increasing T production was observed in the first 2 h after hCG administration.

Figure 1E illustrates the mean plasma E2 concentrations during early, mid, and late luteal phases, respectively. There was an increase in mid and late luteal phase E2 levels 8 h after hCG administration compared with basal values ($P < 0.05$). However, E2 levels did not increase significantly in the early luteal phase after the hCG challenge.

StAR mRNA expression in CL challenged with hCG at different times in the luteal phase

A representative Northern blot for StAR mRNA expression by CL from different stages of the luteal phase and after the administration of hCG is shown in Fig. 2 Treatment with hCG caused a pronounced increase in the abundance of both 1.6- and 4.4-kb StAR transcripts in late luteal phase CL and to a lesser extent in midluteal phase CL. However, hCG did not stimulate the expression of StAR transcripts in early luteal phase CL. The densitometric analysis of StAR mRNA (1.6-kb transcript) levels in control CL compared with CL after hCG administration reveals that the relative abundance of StAR mRNA in hCG-stimulated CL was increased 1.6-fold in midluteal phase CL and 3.5-fold in late luteal phase CL ($P < 0.05$).

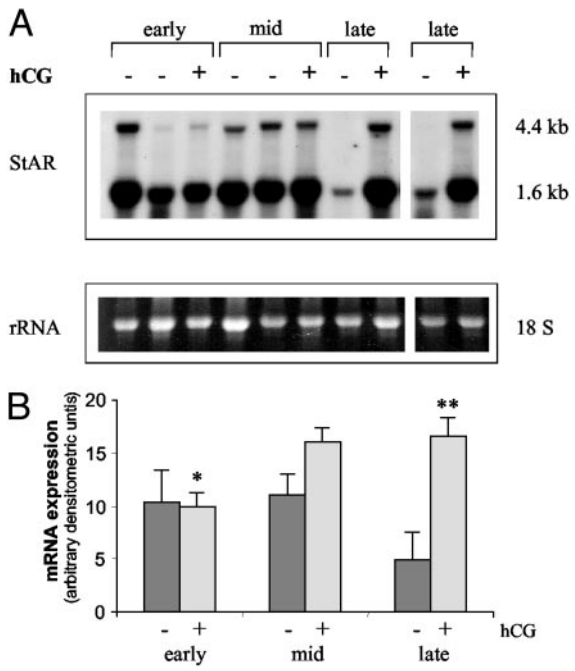

FIG. 2. StAR mRNA levels in luteal tissue. A, representative Northern blot of StAR mRNA in luteal tissue collected during early (n = 3), mid (n = 7), and late (n = 4) luteal phase and after hCG administration. The blot illustrates the 1.6- and 4.4-kb StAR transcripts, and the 18S rRNA loading control is also shown. B, Histogram of Northern blot data for the 1.6-kb StAR transcript in CL of different ages. StAR mRNA levels were normalized to 18S rRNA. *, The mean ± SEM are significantly ($P < 0.05$) different from the mid and late hCG-treated CL. **, The mean ± SEM are significantly ($P < 0.05$) different from the control CL.

Expression of StAR protein in CL treated with hCG at different stages of the luteal phase

Figure 3A is a representative Western blot for StAR protein in luteal tissue from different stages of the luteal phase in control tissue and 24 h after hCG administration. The Western blot shows immunoreactive bands of 37 and 30 kDa that represent the premature and mature StAR proteins, respectively. hCG treatment increased the abundance of both the 37- and 30-kDa StAR proteins in mid and late luteal phase CL. However, the treatment did not affect the expression of StAR protein in early luteal phase CL when 37-kDa StAR protein was detectable even in unstimulated CL. The relative abundance of the 30-kDa StAR protein was 1.4-fold greater in midluteal phase and 1.8-fold greater in late luteal phase hCG-stimulated CL ($P < 0.05$), as shown in Fig. 3B.

Localization of StAR mRNA and protein in midluteal phase CL and effects of hCG treatment

The expression and localization of StAR mRNA were further analyzed by *in situ* hybridization in midluteal phase CL and after receiving hCG on d 6–8 post-LH peak. The CL were collected 24 h later (Fig. 4). The StAR mRNA signal was limited to theca (small) and granulosa (large) luteal cells of the midluteal phase CL (Fig. 4B). In contrast, no signal was observed when luteal tissue sections were hybridized with the sense strand probe (Fig. 4A). Figure 4, C and D, shows intense StAR signal in hCG-treated midluteal phase CL, with the predominance of the signal localized in theca-lutein cells.

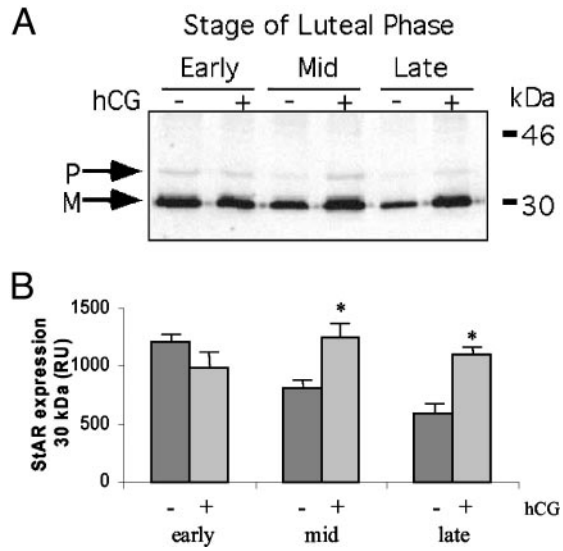

FIG. 3. StAR protein levels in luteal tissue. A, Representative Western blot of StAR preprotein (37 kDa) and mature protein (30 kDa) in luteal tissue collected during early, mid, and late luteal phase and after hCG administration. B, Histogram of Western blot data for mature StAR protein throughout the luteal phase. *, The mean ± SEM are significantly ($P < 0.05$) different from the control.

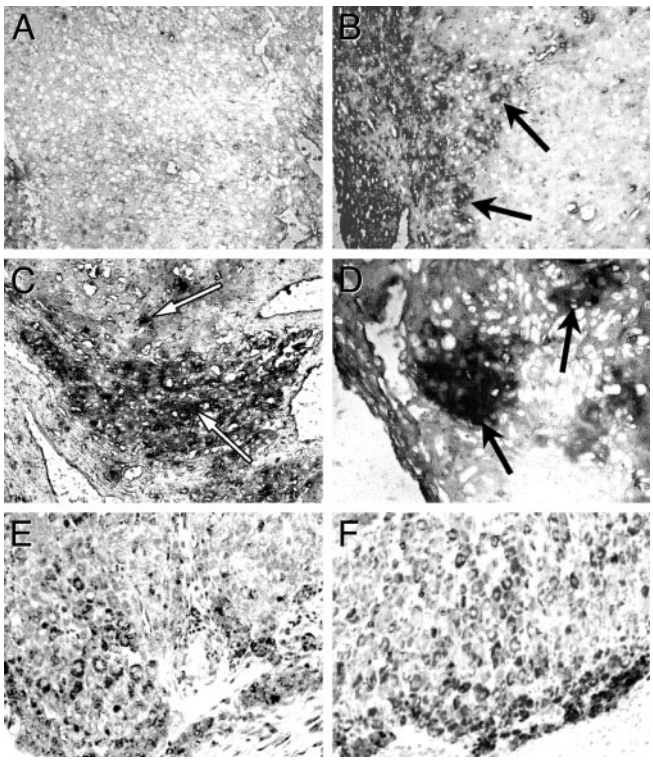

FIG. 4. Localization of StAR mRNA and protein in normal and hCG-treated midluteal phase CL. A, No signal was detected in a CL section hybridized with the sense strand probe. B, The StAR antisense probe signal was localized in the theca- and granulosa-lutein cells, being prominent in theca-lutein cells (magnification, ×200). C and D, StAR mRNA localization in hCG-treated CL. The StAR mRNA signal is marked in theca and luteinized granulosa cells (magnification, ×200 and ×400). E, StAR protein in midluteal tissue. The intense signal is localized in the cytoplasm of theca-lutein cells (magnification, ×200). F, hCG-treated CL, denoting an intense StAR signal in the theca-lutein cells compared with the granulosa-lutein cells. The capillary networks of both the thecal and granulosa cell layer are expanded (magnification, ×200). The *black* and *open arrows* indicate StAR mRNA localization.

Figure 4, E and F, shows the immunohistochemical identification of StAR protein within control midluteal phase CL and after hCG treatment, respectively. StAR was detected in the cytoplasm of both theca-lutein and granulosa-lutein cells. As reported previously, staining for StAR was heterogeneous, particularly in granulosa-lutein cells of midluteal phase CL. As shown in Fig. 4F, hCG administration caused a moderate increase in the staining of granulosa-lutein cells of midluteal phase CL. However, a more pronounced effect was observed in the periphery of the midluteal phase CL, with increased staining intensity in patches of theca-lutein cells. These data suggest that hCG administration causes a moderate increase in the StAR mRNA and protein signal in granulosa-lutein cells of midluteal phase CL and a more intense effect in theca-lutein cells. Thus, there is evidence for a difference in the magnitude of response to hCG in specific luteal cell types.

Immunohistochemical localization of StAR in late luteal phase CL and effect of hCG treatment

Figure 5 shows the immunohistochemical localization of StAR protein within the CL collected during the late luteal phase and from women who received hCG on LH d 11–13. The CL were collected 24 h after hCG administration. Figure 5A depicts the classical histological features of late luteal phase CL, including contraction of the lutein cell layers and fibroblasts in the central cavity. Figure 5B shows minimal to moderate staining for StAR protein in both steroidogenic cell types for the late luteal phase control CL. StAR protein staining intensity in both theca and granulosa lutein cells increased dramatically after hCG treatment. The granulosa-lutein cells exhibited cytoplasmatic expansion with granular staining of greater intensity (Fig. 5, C and D). These observations indicate that StAR protein levels in both theca- and granulosa-lutein cells are increased in late luteal phase CL within 24 h of hCG treatment.

Plasma steroid hormone responses to GnRH antagonist treatment in the presence and absence of hCG administration during the early luteal phase

Figure 6 shows the effect of the GnRH antagonist on plasma steroid levels during the early luteal phase. Cetrorelix administered on d 2 or 3 post-LH peak caused a marked decline in plasma LH, P4, 17αOHP, and E2 levels within 2–4 h after administration (data not shown) that declined further by 24 and 48 h ($P < 0.05$). Administration of hCG to patients treated with Cetrorelix for 48 h increased P4 (2.0-fold) and 17αOHP (2.2-fold) over basal plasma concentrations. In comparison to Cetrorelix treatment alone, plasma P4 and 17αOHP concentrations levels were 7- and 5.2-fold greater in the hCG- plus Cetrorelix-treated patients, respectively.

Plasma T concentrations decreased slightly (20%) after Cetrorelix administration, and hCG treatment increased T plasma levels compared with those in patients treated with Cetrorelix for 48 h in the early luteal phase ($P < 0.05$).

Cetrorelix administration reduced plasma E2 concentrations by 40% in the early luteal phase, and hCG administration restored values to those observed in early luteal phase women.

The weights of CL from Cetrorelix-treated women during the early luteal phase (1.12 $\pm$ 0.25 g) were slightly reduced compared with those of control CL (1.69 $\pm$ 0.56 g; $P > 0.05$). Administration of hCG to Cetrorelix-treated patients increased CL weight (2.2 $\pm$ 0.2 g) compared with that of Cetrorelix-treated alone CL ($P < 0.05$).

Figure 7 presents a Northern blot of early luteal phase CL from GnRH antagonist-treated women. The blot demonstrates a loss of the 4.4-kb StAR mRNA by 48 h after GnRH antagonist administration. The 1.6-kb StAR mRNA declined by roughly 50% and 80%, respectively, after 24 and 48 h of Cetrorelix administration. hCG administration reestablished the abundance of both StAR transcripts to levels similar to those found in midluteal phase CL.

Figure 8 shows that the immunohistochemical staining for StAR is diminished in both theca and granulosa lutein cells after Cetrorelix treatment (C) compared with control early luteal phase tissue (B). The intensity of the immunohistochemical signal for StAR was weak, and a limited number of groups of cells retained the signal. Administration of hCG for 24 h partially reestablished the immunohistochemical StAR signal in both theca- and granulosa-lutein cells (Fig. 8D). The

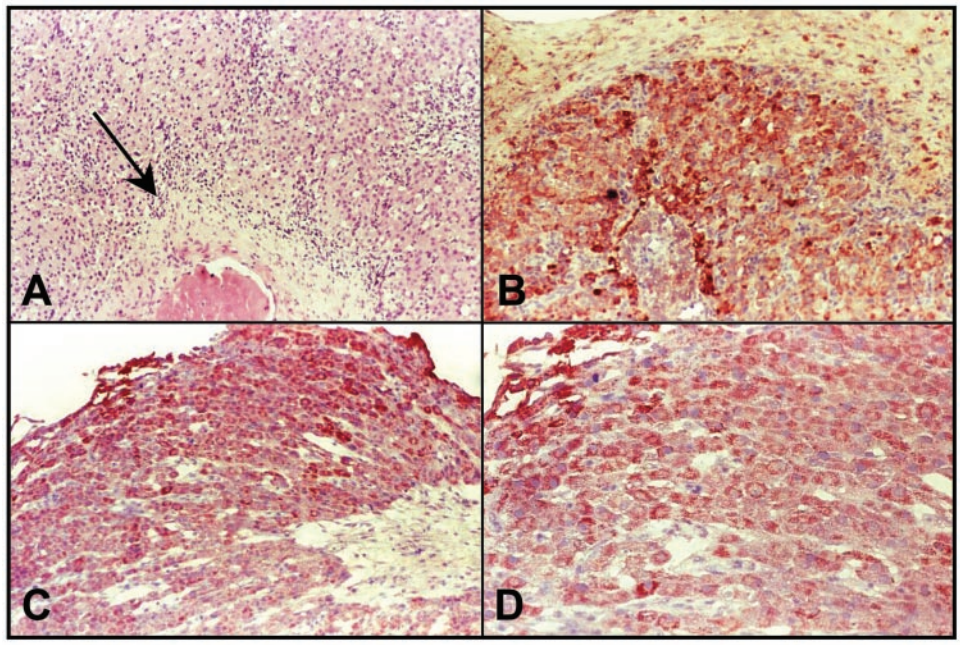

FIG. 5. Immunolocalization of StAR protein in the late luteal phase CL. A, Histological section from late luteal phase CL stained with hematoxylin-eosin (magnification, ×100). The *black arrow* indicates contraction of granulosa cells and organization of central cavity. B, Immunohistochemistry for StAR protein showing a moderate granular staining in the cytoplasm of theca- and granulosa-lutein cells of a late luteal phase CL (magnification, ×100). C, Immunohistochemical localization of StAR protein in a CL obtained from a woman on d 12 who was treated with hCG for 24 h (magnification, ×100). D, Expansion of the vascular network of the theca and granulosa cell layers with intense StAR signal detected in the cytoplasm of steroidogenic cells (magnification, ×200).

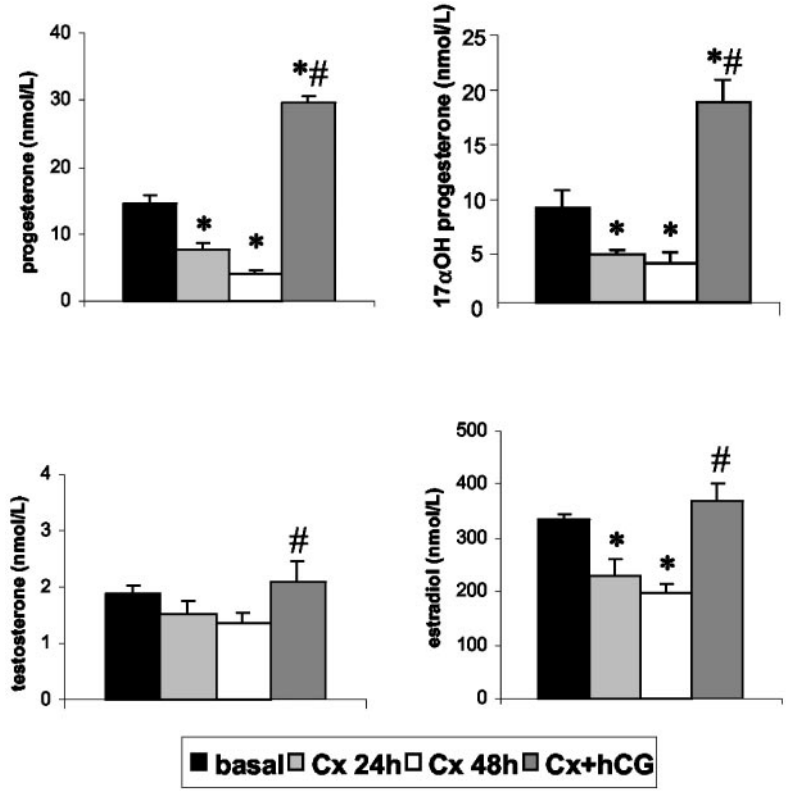

FIG. 6. Endocrine response after the hCG challenge in women treated with GnRH antagonist (2 mg) sc during the early luteal phase. Plasma P4, 17αOHP, T, and E2 concentrations in women receiving Cetrorelix (Cx) for 24 or 48 h and Cetrorelix plus hCG. *, The mean ± SEM are significantly ($P < 0.05$) different from the basal steroid level observed in the early luteal phase. #, The mean ± SEM are significantly ($P < 0.05$) different from 48 h Cx-treated *vs.* Cx- plus hCG-treated steroid levels.

staining among luteal cells was heterogeneous; groups of cells with cytoplasmatic granular staining were visualized among unstained luteal cells with apparently normal morphology.

Discussion

P4 secreted from the CL is required for the development of endometrial receptivity and maintenance of early pregnancy. It is thought that tropic hormones, particularly LH/hCG acting through the cAMP signaling pathway, regulate the transcription of several genes and the activity of their products that participate in the steroidogenic pathway leading to P4 synthesis. This action encompasses increased levels and/or activity of cholesterol esterase lipoprotein receptor-mediated cholesterol uptake and StAR and steroidogenic enzyme expression (14). Cholesterol transport to the inner

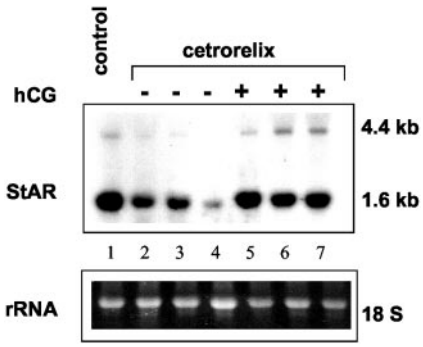

FIG. 7. StAR expression in CL from women treated with the GnRH antagonist. A Northern blot of StAR transcripts from early luteal phase CL collected 24 h (lanes 2 and 3) and 48 h (lane 4) after Cetrorelix administration are depicted. Lanes 5–7 represent luteal tissue StAR transcripts after hCG treatment for 24 h in women treated with Cetrorelix. The 1.6- and 4.4-kb StAR transcripts are indicated. The *lower panel* shows the 18S rRNA loading control.

mitochondrial membrane is a StAR-dependent process, which is considered to be the rate-limiting step in steroidogenesis (2). Previous studies from our laboratories demonstrated that the steroidogenic capacity of the human CL is correlated with the abundance of StAR transcripts and protein (9). Thus, the StAR gene is presumably a key factor governing luteal P4 production at the time of implantation and luteal regression and during CL rescue. The present study represents the first *in vivo* simultaneous appraisal of the hCG-stimulated steroidogenic response and analysis of StAR expression within human CL. Moreover, the steroidogenic response and StAR expression profile were assessed in CL from Cetrorelix-hCG-treated women in the early luteal phase to examine the role of endogenous LH in controlling luteal function. Therefore, the present investigation extends our knowledge concerning the regulatory mechanisms of StAR expression in hCG-stimulated CL throughout the luteal phase.

Numerous studies of cultured monkey or human luteal cells have demonstrated an age-dependent differential steroidogenic response to hCG (15, 16). Luteal cells isolated from midluteal phase CL synthesize the greatest quantities of P4 and E2 (17). Moreover, age-dependent hCG steroidogenic responses of the CL have been demonstrated *in vivo* in monkeys and women (5, 6). Our observations confirm the age-dependent response of the CL to hCG, but also reveal differential effects on progestin production and different sensitivities with respect to StAR gene and protein expression.

Under physiological circumstances the early CL is not exposed to hCG. Exogenous hCG administration in the early luteal phase failed to significantly stimulate P4 and E2 biosynthesis as assessed by monitoring plasma hormone concentrations. The apparent failure of early luteal phase CL to respond to hCG is consistent with our earlier *in vitro* observations that suggest an inadequate coupling between LH/hCG receptor and adenylate cyclase in early CL (16). Other investigators have proposed different explanations for the relative insensitivity of the early CL to hCG, suggesting that the poorly developed capillary network supplying the cells of the early CL is the cause (5, 18).

Interestingly, 17αOHP levels significantly increased 8 h after hCG administration in the early luteal phase. This differential steroidogenic response of the early CL to hCG administration probably reflects the differential sensitivity of the granulosa- and theca-lutein cells (17, 19). Theca-lutein cells (small luteal cells) are the site of expression of 17α-hydroxylase that converts progestins into 17αOHP as well as androgens in the primate CL (20). Small luteal cells isolated from the human CL produce more androgens in culture than granulosa-lutein cells (large luteal cells) (19). Moreover, human and bovine theca-lutein cells exhibit greater binding capacity for [^{125}I]hCG compared with granulosa-lutein cells (17).

The poor steroidogenic response of the early luteal phase CL to hCG administration, in terms of P4, was correlated with the absence of a significant change in StAR transcript abundance and mRNA size, and protein levels after hCG administration, compared with control tissue (unstimulated early luteal phase CL). This observation suggests that the failure of hCG to elicit a rise in plasma P4 levels is not the consequence of insufficient lipoprotein-carried cholesterol substrate, but, rather, a failure of the steroidogenic machinery in the majority of luteal cells to respond to hCG signal. The increment in 17αOHP noted previously may be associated with increased StAR levels in a specific population of CL cells (theca-lutein cells) that is obscured when total CL mRNA and protein are analyzed. Indeed, our *in situ* hybridization and immunohistochemical studies suggest that this is indeed the case.

To determine whether luteal steroidogenesis is LH dependent during the early luteal phase a selective reduction in LH levels was achieved by the administration of a GnRH antagonist. This resulted in a marked decline in LH, followed by a pronounced fall in plasma P4. The molecular assessment of early luteal phase CL tissue 48 h after the administration of GnRH antagonist showed a pattern of StAR expression characterized by the absence of the 4.4-kb transcript and the reduction of the 1.6-kb transcript by 80% (9). The light microscopic appraisal of CL from women treated with GnRH antagonist disclosed morphological changes in both theca and granulosa cell layers, characterized by shrinkage of both cellular compartments. Similar findings have been reported in CL from GnRH antagonist-treated monkeys in the early luteal phase (21). Additionally, we observed a markedly reduced immunohistochemical signal for StAR in both theca- and granulosa-lutein cells. Interestingly, the treatment of these women with hCG for 24 h restored P4 and 17αOHP and StAR transcript levels to those seen in the midluteal phase. Notably, after Cetrorelix treatment, hCG administration caused a more pronounced StAR signal in theca-lutein than in granulosa-lutein cells, again suggesting differential sensitivity of the two luteal cells types. This observation coupled with the observations of differential responses of P4 and 17αOHP in the early luteal phase to exogenous hCG suggest that granulosa-lutein cells are dependent on pituitary LH for the maintenance of StAR expression in the early luteal phase and that endogenous LH sustains maximal StAR expression. In contrast, the theca-lutein cells may not be maximally stimulated by endogenous LH, retaining the ability to respond to exogenous hCG.

The ability to recover endocrine function and StAR ex-

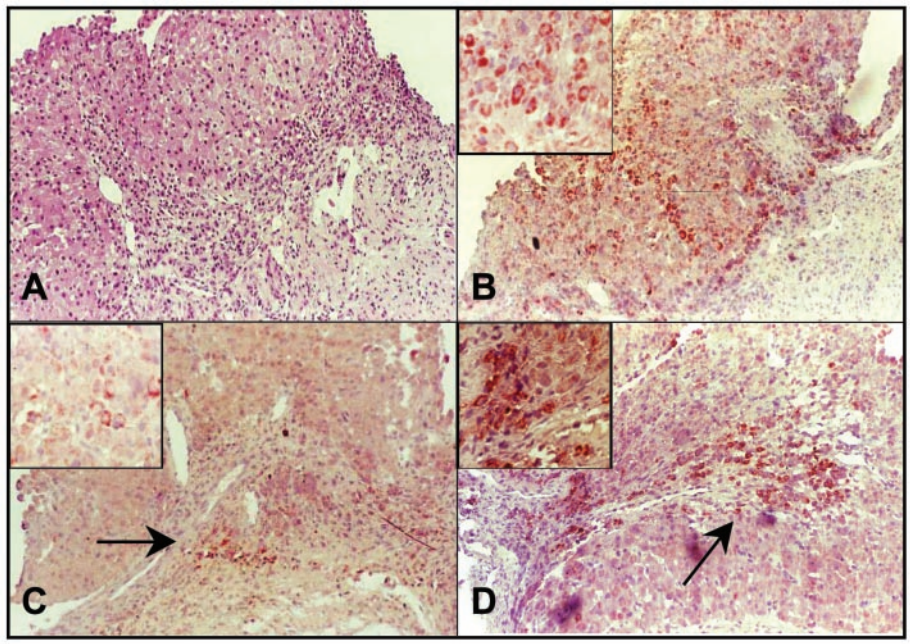

FIG. 8. Immunohistochemical localization of StAR in early CL from Cetrorelix-treated women. A, Histological section of an early luteal phase CL stained with hematoxylin-eosin (magnification, ×100). B, Immunohistochemistry for StAR protein detected in the same early luteal phase CL. Moderate staining for the StAR protein was localized in the cytoplasm of theca- and granulosa-lutein cells. C, StAR protein in luteal tissue from a women treated with Cetrorelix for 48 h. Only small patches of StAR-immunopositive cells (*black arrow*) are seen, and these appear to be predominantly in theca-lutein cells. D, StAR protein (*black arrow*) in luteal tissue from a Cetrorelix- plus hCG-treated women. Granular cytoplasmatic StAR staining was partially reestablished in both theca- and granulosa-lutein cells. The *inset panels* (magnification, ×200) illustrate the granular nature of the StAR cytoplasmatic staining consistent with localization in the mitochondria.

pression is consonant with the findings of Hutchinson and Zeleznik (22), who found that CL viability can be preserved for about 3 d without LH support.

However, in contrast to our observations, Dubourdieu *et al.* (23) found that in the human, hCG administration only partially rescued CL function in women treated with GnRH antagonist (Nal-Glu) for 48 h. The difference in magnitude of the steroidogenic response in our study and that of Dubourdieu *et al.,* (23) could be explained by the different types and doses of GnRH antagonist used and the timing of hCG administration.

The highest concentrations for P4, 17αOHP, and E2 after hCG administration were achieved in the midluteal phase. This is not surprising given that the human midluteal phase CL has a well developed capillary network (24) and exhibits the highest binding capacity for LH/hCG (1) and the greatest StAR mRNA and protein abundance (9).

The moderate, although significant, steroidogenic response of the midluteal phase CL to hCG suggests that the steroidogenic machinery of this tissue is at near-maximal capacity in the normal midluteal phase. This is consistent with our observation of the moderate increase in StAR mRNA abundance and protein levels in response to hCG challenge in the midluteal phase. *In situ* hybridization studies for StAR mRNA suggested that hCG treatment causes a more pronounced expression of StAR in theca-lutein cells than in granulosa lutein cells.

The CL of the late luteal phase is of particular interest. P4 and 17αOHP increased significantly in the plasma after hCG administration in the late luteal phase, showing the greatest increment above baseline values. We recently reported that StAR mRNA abundance and protein StAR expression, encompassing both the preprotein (37 kDa) and the mature protein (30 kDa), decline markedly in late luteal phase human CL (9). The steroidogenic responsiveness of late luteal phase CL treated with hCG is consistent with our Northern and Western blot analyses, which displayed the greatest increase in both StAR mRNA transcripts and protein. The rise in both P4 and 17αOHP and the increase in the immunohistochemical signal for StAR in both large and small luteal cells indicate that both granulosa- and theca-lutein cells of the late luteal phase CL respond to hCG, unlike the situation in the early luteal phase. Our data suggest that the increase in P4 concentrations by 24 h after hCG administration and probably at earlier time points reflects changes in StAR mRNA, presumably determined by rates of StAR gene transcription. Increased StAR activity resulting from phosphorylation may also contribute to increased P4 synthesis, particularly at early time points after hCG administration.

In conclusion, we have documented age-dependent responses of the human CL to hCG, provided evidence for differential CL responses with respect to production of P4 and 17αOHP that may reflect different sensitivities of granulosa- and theca-lutein cells, and demonstrated the dependence of the early luteal phase CL on endogenous LH. Finally, our studies indicate that the increase in hCG-stimulated StAR mRNA and protein within late luteal phase CL is associated with increased P4 and 17αOHP production. The response of the late luteal phase CL to hCG may mimic in molecular and endocrine terms the rescue of the CL in a cycle of conception.

Acknowledgments

We thank Veronica Alam, M.D., Serono Medical Director for Latin America, for providing Profasi and Cetrorelix.

Received December 5, 2002. Accepted March 18, 2003.

Address all correspondence and requests for reprints to: Dr. Luigi Devoto, Instituto de Investigaciones Materno Infantil y Departamento de Obstetricia y Ginecología, Facultad de Medicina, Universidad de Chile. P.O. Box 226-3, PC 6519100 Santiago, Chile. E-mail: ldevoto@machi.med.uchile.cl.

This work was supported in part by CONICYT/FONDECYT-Programa Fondap Grant 15010006 and NIH Grants TW-001485 and HD-06274.

References

1. **Nakano R** 1997 Control of the luteal function in humans. Semin Reprod Endocrinol 15:335–344
2. **Stocco DM, Clark BL** 1996 Regulation of the acute production of steroids in steroidogenic cells. Endocr Rev 17:221–244
3. **Strauss III JF, Kallen CB, Christenson LK, Watari H, Devoto L, Arakane F, Kiriakidou M, Sugawara T** 1999 The steroidogenic acute regulatory protein (StAR): a window into the complexities of intracellular cholesterol trafficking. Recent Prog Horm Res 54:369–395
4. **Momoeda M, Tsutsumi O, Morita Y, Igarachi T, Suenaga A, Osuga Y, Shiotsu H, Yano T, Taketani Y** 1998. Differential effect of exogenous chorionic gonadotrophin on progesterone production fron normal or malfunctioning corpus luteum. Hum Reprod 13:1907–1911
5. **Wilks JW, Noble AS** 1983 Steroidogenic responsiveness of the monkey corpus luteum to exogenous chorionic gonadotropin. Endocrinology 113:1256–1266
6. **Tay PY, Lenton EA** 2000 Corpus luteum response to exogenous HCG during the mid-luteal phase of the menstrual cycle. Clin Endocrinol (Oxf) 53:345–350
7. **Penzias AS** 1995 Luteal phase support. Semin Reprod Endocrinol 13:32–38
8. **Duncan WC, Cowen GM, Illingworth PJ** 1999 Steroidogenic enzyme expression in human corpora lutea in the presence and absence of exogenous human chorionic gonadothophin (HCG). Mol Hum Reprod 5:291–298
9. **Devoto L, Kohen P, González RR, Castro O, Retamales HI, Vega M, Carvallo P, Christenson LK, Strauss III JF** 2001 Expression of steroidogenic acute regulatory protein in the human corpus luteum throughout the luteal phase. J Clin Endocrinol Metab 86:5633–5639
10. **Devoto L, Kohen P, Castro O, Vega M, Troncoso JL, Charreau E** 1995 Multihormonal regulation of progesterone synthesis in cultured human midluteal cells. J Clin Endocrinol Metab 80:1566–1570
11. **Noyes RW, Hertig AT, Rock J** 1950 Dating the endometrial biopsy. Fertil Steril 1:3–12
12. **Chomczynski P, Sacchi N** 1987 Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
13. **Yang H, Wanner IB, Roper SD, Chaudhari N** 1999 An optimized method for in situ hybridization with signal amplification that allows the detection of rare mRNAs. J Histochem Cytochem 47:431–445
14. **Strauss III JF, Penning TM 1999** Synthesis of the sex steroid hormones: molecular and structural biology with application to clinical practice. In: Fauser BCJM, Rutherford AJ, Strauss III JF, Steirteghem AV, eds. Molecular biology in reproductive medicine. New York: Parthenon; 201–232
15. **Brannian JD, Stouffer RL** 1991 Progesterone production by monkey luteal cell subpopulations at different stages of the menstrual cycle: changes in agonist responsiveness. Biol Reprod 44:141–149
16. **Vega M, Devoto L, Navarro V, Castro O, Kohen P** 1987 In vitro net progesterone production by human corpora lutea: effects of human chorionic gonadotropin, dibutyryl adenosine 3′,5′-monophosphate, cholera toxin and forskolin. J Clin Endocrinol Metab 65:747–752
17. **Retamales I, Carrasco I, Troncoso JL, Las Heras J, Devoto L, Vega M** 1994 Morpho-functional study of human luteal cell subpopulations. Hum Reprod 9:591–596
18. **Corner GW** 1956 The histological dating of the human corpus luteum of menstruation. Am J Anat 98:377
19. **Ohara A, Mori T, Taii S, Ban C, Narimoto K** 1987 Functional differentiation in steroidogenesis of two types of luteal cells isolated from mature human corpora lutea of menstrual cycles. J Clin Endocrinol Metab 65:1192–1200
20. **Sander SL, Stouffer RL** 1997 Localization of steroidogenic enzyme in macaque luteal tissue during the menstrual cycle and simulated early pregnancy: immunohistochemical evidence supporting the two-cell model for estrogen production in the primate corpus luteum. Biol Reprod 56:1077–1087
21. **Dickson SE, Fraser HM** 2000 Inhibition of early luteal angiogenesis by gonadotropin. releasing hormone antagonist treatment in the primate. J Clin Endocrinol Metab 85:2339–2344
22. **Hutchison JS, Zeleznik AJ** 1984 The rhesus monkey corpus luteum is dependent on pituitary gonadotropin secretion throughout the luteal phase of the menstrual cycle. Endocrinology 115:1780
23. **Dubourdieu S, Charbonnel B, Massai MR, Marraoui J, Spitz I, Bouchard P** 1991 Suppression of corpus luteum function by the gonadotropin-releasing hormone antagonist Nal-Glu: effect of the dose and timing of human chorionic gonadotropin administration. Fertil Steril 56:440–445
24. **Fraser HM, Lunn SF** 2001 Regulation and manipulation of angiogenesis in the primate corpus luteum. Reproduction 121:355–362