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Expression of Vascular Endothelial Growth Factor A During Ligand-Induced Down-Regulation of Luteinizing Hormone Receptor in the Ovary☆

M. Harada, **H. Peegel**, and **K. M. J. Menon***
Departments of Obstetrics/Gynecology and Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0278

Abstract

Vascular endothelial growth factor A (VEGF-A) is one of the most important regulators of ovarian angiogenesis. In this study, we examined the temporal relationship between VEGF-A and luteinizing hormone receptor (LHR) mRNA expression during ligand-induced down-regulation of LHR. Immature female rats were treated with pregnant mare's serum gonadotropin followed by 25 IU hCG 56h later (day 0). On day 5, treatment with hCG (50 IU) to down-regulate LHR showed a temporal decrease in VEGF-A mRNA and protein levels in parallel with decreasing LHR mRNA. This effect was specific since the expression of CYP11A1 mRNA showed no decline. Examination of VEGF-A mRNA expression, using *in situ* hybridization histochemistry with ^{35}S-labeled antisense VEGF-A mRNA probe, showed intense signal in the corpora lutea on day 5. Treatment with 50 IU hCG to down-regulate LHR mRNA showed a decline in the intensity of VEGF-A mRNA in the corpora lutea. VEGF-A mRNA expression returned to control level 53 hours later when the expression of LHR mRNA also recovered. These results show that the transient down-regulation of VEGF-A mRNA and protein closely parallels the ligand-induced down-regulation of LHR mRNA. The present study establishes a close association between VEGF-A and LHR mRNA expression, suggesting the possibility that VEGF-A-induced vascularization of the ovary is dictated by the expression of LHR and this might play a regulatory role in ovarian physiology.

Keywords

luteinizing hormone receptor; vascular endothelial growth factor; ovary; ovarian hyperstimulation syndrome

1. Introduction

The mammalian ovary undergoes programmed angiogenic processes during the ovarian cycle (Stouffer et al., 2001). Among many candidates, vascular endothelial growth factor A

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*Corresponding author: Dr. K. M. J. Menon, Departments of Obstetrics/Gynecology and Biological Chemistry, University of Michigan Medical School, 6428 Medical Science 1, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109-0617. Tel.: 1 734 764 8142; Fax: 1 734 764 936 8617. kmjmenon@umich.edu.

(VEGF-A), a member of the VEGF family, has emerged as one of the most important regulators of angiogenesis in the ovary. VEGF-A has been known to promote proliferation and migration of vascular endothelial cells, and to enhance vascular permeability (Leung et al., 1989; Pepper et al., 1992; Senger et al., 1983). VEGF-A expression in the ovary undergoes dynamic changes during follicle maturation, ovulation and luteinization, suggesting that its expression is regulated by gonadotropin and might play an important role in ovarian physiology (Stouffer et al., 2001). Multiple transcript variants of VEGF-A have been identified in different species. In the human, five transcript variants of VEGF-A have been identified (Neufeld et al., 1999). While three major transcript variants have been reported for murine VEGF-A ($VEGF_{120, 164, 188}$), two of these, ($VEGF_{120}$ and $VGEF_{164}$), are expressed in the ovary (Gomez et al., 2002; Ishikawa et al., 2003). Previous *in vitro* studies have shown that human chorionic gonadotropin (hCG) stimulates VEGF-A expression in granulosa-lutein cells (Lee et al., 1997; Neulen et al., 1995). Evidence suggests that LH/hCG and products of its action in target cells are the major regulators of angiogenesis and VEGF-A expression in the ovary (Martinez-Chequer et al., 2003; Schams et al., 2001; Stouffer et al., 2001; van den Driesche et al., 2008). Non-endocrine factors such as hypoxia are also known to induce VEGF-A expression in most tissues (Ladoux and Frelin, 1993; Neeman et al., 1997; Shweiki et al., 1992).

While VEGF-A plays a crucial role in angiogenesis in the ovary, under pathological conditions, the excess production of VEGF-A has been implicated in inducing ovarian hyperstimulation syndrome (OHSS), the most serious complication of controlled ovarian hyperstimulation. Using well-established OHSS model rats, Gomez and colleagues (Gomez et al., 2006; Gomez et al., 2002) showed that hCG administration to rats primed with pregnant mare serum gonadotropin (PMSG) for 4 consecutive days resulted in an increase in systemic vascular permeability and VEGF-A mRNA expression in the ovary 24 hours later, and this effect was not seen in the mesenteric tissue (Gomez et al., 2002). The vital role of VEGF-A in OHSS has also been strengthened by the observation that agents interfering with VEGF-A signaling inhibited the increase in vascular permeability induced by hCG (Gomez et al., 2006; Gomez et al., 2002). In inducing OHSS, hCG plays a critical role since the syndrome disappears or fails to develop if hCG injection is not instituted at the end of controlled hyperstimulation of ovarian follicles (Schenker, 1993). The vital role of hCG/LH has been further supported by the observation, using a rodent model (Gomez et al., 2002), that although PMSG treatment alone produced a slight increase in vascular permeability and VEGF-A expression, further treatment with hCG augmented these responses.

During the ovarian cycle, LH/hCG receptor (LHR) expression itself shows remarkable changes, as manifested by the acquisition of LHR by the growing follicles in response to the combined actions of FSH and estradiol (Camp et al., 1991) followed by a transient loss of LHR in response to the preovulatory LH surge, and its subsequent recovery to support the corpus luteum function (Hoffman et al., 1991; LaPolt et al., 1990; Peegel et al., 1994). Studies from our laboratory have elucidated that the transient ligand-induced down-regulation of LHR during this transition period is a result of post-transcriptional regulation of LHR mRNA via accelerated degradation, involving a specific mRNA binding protein (Kash and Menon, 1998; Nair et al., 2002; Nair and Menon, 2004). In the present study, we examined the temporal association between LHR and VEGF-A expression during ligand-induced down-regulation of LHR mRNA to test whether VEGF-A expression is acutely dependent on LHR expression.

2. Materials and Methods

2.1. Reagents

PMSG was purchased from Calbiochem (La Jolla, CA). HCG, β-nicotinamide adenine dinucleotide, nitro blue tetrazolium, dehydroepiandrosterone, and pregnenolone were purchased from Sigma Chemical Co. (St. Louis, MO). DMEM/Ham's F-12 medium and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). Tissue collagenase CLS4 was purchased from Worthington Biochemical (Lakewood, NJ). RNase free DNase-I, RNase inhibitor, and pGEM T-Easy vector system were purchased from Promega (Madison, WI). O.C.T. compound was purchased from Sakura Finetek (Torrance, CA). [^{35}S] UTP was purchased from PerkinElmer Life and Analytical Sciences (Shelton, CT). Ambion MAXIscript Kit was purchased from Applied Biosystems (Foster City, CA). KODAK NTB emulsion was purchased from Carestream Health, Inc. (Rochester, NY).

2.2. Animals

Sprague-Dawley female rats (23 days old) were purchased from Charles River Laboratories (Wilmington, MA). To establish pseudopregnancy, animals were injected sc with 50IU PMSG, followed by 25 IU hCG 56h later to induce superovulation and subsequent luteinization (Peegel et al.,1994). The pseudopregnant animals were treated with 50 IU hCG on day 5 of pseudopregnancy; control rats received an equal volume of saline. At specific time intervals, the animals were euthanized by CO_2 asphyxiation and ovaries were collected. They were frozen in O.C.T. compound in dry ice-isopentane for *in situ* hybridization histochemistry. For the studies using purified luteal cells, ovaries were processed immediately after collection as indicated below. All the experimental protocols used in this study were approved by the University Committee on the Use and Care of Animals (U.C.U.C.A).

2.3. Isolation of steroidogenic cells

Ovaries were removed under sterile conditions, trimmed free of fat, minced, and incubated for 1.5 h at 37 C in DMEM/Ham's F-12 medium containing collagenase CLS4 (500 U/ml, 20 ml/g of tissue), 2 U/ml RNase free DNase-I, and 40 U/ml RNase inhibitor. After rinsing, dispersed cells were resuspended in DMEM/Ham's F-12 medium and purified by a previously published procedure (Nelson et al., 1992) with minor modification. The cell suspensions were filtered through Isopore Membrane Filters, 10 μm TCTP (Millipore Corp., Billerica, MA) separating the large luteal cells from other contaminating cell types. The purity of these isolated cells was confirmed by 3β-Hydroxysteroid dehydrogenase staining as indicated below.

2.4. 3β-Hydroxysteroid dehydrogenase (3β HSD) staining

The isolated cells were stained for 3βHSD activity according to the procedure of Arioua et al., 1997 with minor modification. An aliquot (5×10^5 cells/100 μl) of the cells was incubated for 1h at 37 C with 0.3ml staining solution (0.07 M phosphate buffer, pH 7.2, containing 1 mg/ml nicotinamide, 6 mg/ml β-nicotinamide adenine dinucleotide, 1.5 mg/ml nitro blue tetrazolium, 100 μg/ml dehydroepiandrosterone, and 100 μg/ml pregnenolone). The cells were mounted and coverslipped on slides and the images were captured using a brightfield microscope (Leica DMR, Wetzlar, Germany).

2.5. RNA extraction, RT, and real-time quantitative PCR

Total RNA was isolated using TRIzol reagent, following the manufacturer's protocol. Total RNA (100ng) was reverse transcribed in a volume of 20 μl using 2.5 μM random hexamer, 500 μM dNTPs, 5.5 mM $MgCl_2$, 8 U ribonuclease inhibitor, and 25 U multiscribe reverse

transcriptase. The reactions were carried out in a PTC-100 (MJ Research, Watertown, MA) thermal controller (25 C for 10 min, 48 C for 30 min, and 95 C for 5 min). The resulting cDNAs were diluted with water. The real-time PCR quantification was performed using 5 μl of the diluted cDNAs in triplicate with predesigned primers and probes for rat VEGF-A (*Vegfa*), designed to detect all three major transcript variants ($VEGF_{120, 164, 188}$), LHR (*Lhcgr*), CYP11A1 (*Cyp11a1*) and 18S rRNA (TaqMan Assay on Demand Gene Expression Products, Applied Biosystems, Foster City, CA). Reactions were carried out in a final volume of 25 μl using Applied Biosystems 7300 Real-Time PCR system (95 C for 15 sec, 60 C for 1 min) after initial incubation for 10 min at 95 C. The fold changes in VEGF-A, LHR, and cyt $P450_{scc}$ mRNA expression were calculated by using the (2-delta, delta C_T) method as described in the Applied Biosystems User Bulletin, with 18S rRNA as the internal control.

2.6. In situ hybridization

In situ hybridization was performed as reported previously (Li et al., 1998; Peegel et al., 1994). Briefly, ovaries frozen in O.C.T. compound were cut at −20 C using a Reichert 2800 Frigocut-N cryostat (Leica, Deerfield, IL), and 10-μm sections were mounted on silane-coated slides. Sections were fixed in paraformaldehyde, washed in PBS, incubated with proteinase-K, and rinsed with deionized water. Subsequently, slides were placed in triethanoleamine and acetic anhydride was added. Sections were then washed with 2 x SCC (0.15 M NaCl, 0.015 M sodium citrate), dehydrated in graded alcohols (50–100%), and dried. The RNA probe (3×10^7 cpm/ml in hybridization buffer [75% formamide, 3 x SCC, 1 x Denhardt's reagent (0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 0.05 M sodium phosphate (pH 7.4), 10% dextran sulfate, 1 mM dithiothreitol, and 200 μg/ml yeast tRNA]) was applied to the tissue sections, coverslips were overlaid and sealed with rubber cement, and slides were incubated at 58 C overnight in a moist chamber. After hybridization, sections were washed in 2 x SCC, treated with RNase-A and RNase-T1 and washed in increasingly lower concentrations of SCC (2-0.2 x). After a final incubation in 0.1 x SCC-0.1% sodium dodecyl sulfate for 1h at 58 C, sections were dehydrated through graded alcohols and dried. The slides were processed for liquid emulsion autoradiography using KODAK NTB emulsion and developed after 10 days. For visualizing morphology, tissue sections adjacent to those designated for *in situ* hybridization were applied to slides and stained with hematoxylin-eosin (H–E). The slides were examined at 40x magnification using an Olympus BX-51A under both bright and darkfield.

2.7. Synthesis of probes for in situ hybridization

Antisense and sense [^{35}S] UTP-labeled VEGF-A RNA probes were synthesized from 591-bp rat VEGF-A cDNA template subcloned in pGEM T-Easy vector, using Ambion MAXIscript Kit. This cDNA sequence corresponded to nucleotides 648-1238 (NCBI accession: BC168708), common to the three major transcript variants, and was verified by sequencing using ABI 3730 DNA Analyzer (Applied Biosystems).

2.8 Western blot analysis

The isolated large luteal cells were solubilized with RIPA buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS). Cell lysates were then briefly sonicated and centrifuged for 10 min at 13,000 × g. The samples were concentrated using Amicon® Ultra centrifugal filter units (Millipore Corp., Billerica, MA) with 3,000 MWCO membranes. Proteins were measured using BCA Protein Assay (Thermo Scientific, Rockford, IL). Proteins (60ug/lane) were separated by SDS-PAGE using 4–20% TRIS-HCL gradient gels (BioRad Laboratories, Hercules, CA and transferred to nitrocellulose membranes. Rat VEGF-A was detected using mouse monoclonal anti-recombinant VEGF-A IgG (Santa Cruz Biotechnology, CA), followed by HRP-linked anti-mouse IgG. (Cell

Signaling, Beverly, MA). The presence of immune complexes was detected by chemiluminescence using Super Signal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL). Protein loading was monitored by stripping the blots (Restore™ Western Blot Stripping Buffer, Thermo Scientific, Rockford, IL), and hybridizing with mouse monoclonal anti-β-tubulin IgG (Sigma, St. Louis, MO), followed by the HRP-linked anti-mouse IgG and then the Super Signal Substrate. Films of the blots were scanned with an AGFA ARCUS II scanner and bands were quantitated using NIH Image 1.63 program.

2.9. Statistical analysis

Statistical analysis was carried out using unpaired *t* test with GraphPad Prism software (version 3.0; GraphPad Inc., San Diego, CA). Values were considered statistically significant at $P < 0.05$. Each experiment was repeated at least three times with similar results. Graphs represent the mean ± SE of three replicates.

3. Results

3.1. Purification and characterization of luteal cells

VEGF-A is reported to be expressed both in steroidogenic cells and vascular cells (i.e. vascular endothelial cells and pericytes) in the ovary (Papa et al., 2007; Redmer et al., 2001). Considering that approximately half of the cells constituting the corpus luteum are known to be vascular cells (Reynolds et al., 2000), the possibility exists that changes in VEGF mRNA in the whole ovary might also include a component of vascular cells, whose proliferation might be stimulated by LH/hCG itself or other factors induced by LH/hCG (Berndt et al., 2006; Redmer et al., 2001). Thus, in order to determine the expression of VEGF-A in steroidogenic cells devoid of contaminating vascular cells, luteal cells were purified from collagenase-dispersed ovarian cells. The purity of the isolated cells was examined by 3βHSD staining. The results presented in Fig. 1A show that greater than 85 per cent of the cells stained positive for 3βHSD activity, suggesting that the majority of non-steroidogenic cells was excluded by the purification procedure employed. The control cells without substrates for 3βHSD did not show appreciable staining (Fig. 1B).

3.2. VEGF-A and LHR mRNA expression in purified luteal cells

We have previously shown that treatment of day 5 pseudopregnant rats with 50 IU of hCG causes down-regulation of LHR mRNA expression (Peegel et al., 1994). In order to examine whether the LHR down-regulation leads to similar changes in the expression of VEGF-A mRNA, day 5 pseudopregnant rats were treated with 50 IU hCG and the levels of VEGF mRNA were determined in purified luteal cells isolated at 0, 12, 24, and 53h post-injection using real-time PCR. Results presented in Fig. 2A show that there was a significant reduction in the expression of VEGF mRNA 12h following administration of this dose of hCG compared to the control. Concominantly, this treatment also produced a significant reduction in the expression of LHR mRNA (Fig. 2B). A comparison of the pattern of expression of VEGF mRNA and LHR mRNA at different intervals shows that there was a time-dependent recovery of both. The effect of hCG on the down-regulation of VEGF-A mRNA and LHR mRNA was specific, and not the result of a general suppression of ovarian function, since the expression of a crucial steroidogenic enzyme, CYP11A1, failed to show a similar decline throughout the same time course (Fig. 2C).

3.3. In situ hybridization of VEGF-A mRNA during hCG-induced down-regulation

In situ hybridization analysis was then performed to determine the cell-specific expression of VEGF-A mRNA, using the ^{35}S-labeled antisense RNA strand of VEGF-A as the probe.

On day 5 of pseudopregnancy, one group of rats received a dose of 50IU hCG that has been known to cause down-regulation of LHR mRNA expression (Peegel et al., 1994). Controls received an equal volume of saline. Figure 3, panelA, shows intense hybridization of the ^{35}S-labeled VEGF-A antisense probe to the luteal cells in the numerous corpora lutea that are present on day 5 of pseudopregnancy, which is also indicated as T=0. Twelve hours after hCG injection, the intensity of the VEGF-A mRNA signal in the tissue sections prepared from the hCG-treated rats dramatically declined compared to that in the control ovaries (panel E versus panel C, respectively). These results are in agreement with the changes in mRNA expression levels assessed by real-time PCR measurements in Fig. 2A. At 24h, a substantial reappearance of VEGF-A mRNA in situ hybridization signal was seen in the hCG-treated ovarian sections (panel I) when compared to the 12h hCG-treated ovarian sections (panel E), but the intensity of the 24h hCG signal was lower than that in the 24h control sections (panel G) as evidenced by the decreased number of individual grains in the hCG down-regulated panel (I) compared to the corresponding control panel (G). At 53h, the signal intensity in the hCG-treated group became stronger (panel M), perhaps surpassing the 53h control (panel K). Panels (B, D, H and L) and (F, J and M) represent hematoxylin/eosin (H/E) stained sections of corresponding control and hCG-treated tissues, respectively. No hybridization signal was detected when the tissue sections were pretreated with RNase or hybridized with ^{35}S-labeled sense probe (data not shown) indicating that the hybridization reaction was specific. The hCG treatment produced no adverse effects on ovarian morphology as evidenced by the normal appearing corpora lutea in H-E stained sections under brightfield microscopy as seen in Fig. 3 and in Fig. 4a, Panel B. Examination of the magnified darkfield image in Panel A of Fig. 4a showed that VEGF-A mRNA was localized exclusively in the corpus luteum (CL) with no appreciable signals detected in the non-luteal cells, seen as the dark areas surrounding the corpus luteum. Figure 4a, Panel B shows the H/E stained luteal cells (L) and the surrounding non-luteal cells, which can be identified as fibroblasts (F) and collagen bundles (C), and blood vessels, which are indicated by circles.

We have previously shown that during hCG-induced down-regulation, the *in situ* hybridization profile of LHR mRNA expression shows a marked decline followed by recovery to control levels (Peegel et al., 1994). The results in this study indicate that treatment with a high dose of hCG causes down-regulation of VEGF-A mRNA expression with a subsequent recovery, a comparable to that seen with LHR mRNA.

3.4 Western blot analysis of VEGF-A protein expression in purified luteal cells

Since hCG treatment produced a decline in the expression of VEGF-A mRNA, further studies were carried out to determine whether changes in VEGF-A mRNA expression resulted in similar changes in VEGF-A protein expression. Western blot analysis of large luteal cells was performed at 12, 24 and 53h after hCG treatment. The results presented in Fig. 4b show that there was a significant reduction of the VEGF-A protein level at 12h, followed by increases at 24 and 53 hours. These results show that the VEGF-A protein expression profile during down regulation exhibits a trend similar to that seen for VEGF-A mRNA. The differences in the extent of changes of the two can be attributed to different sensitivities of the two assays.

4. Discussion

In the present study we show that the transient down-regulation of VEGF-A mRNA expression in rat ovaries in response to a high dose of hCG occurs in parallel to a decline in LHR mRNA expression. To demonstrate that this phenomenon occurs specifically in steroidogenic cells, we purified luteal cells free of other contaminating cell types, and a marked correlation between the expression of VEGF-A and LHR mRNA expression was seen in the purified luteal cells. These observations are supported by the *in situ* hybridization

data that clearly show a temporal down-regulation of VEGF-A mRNA in the corpora lutea of ovaries from down-regulated rats and subsequent recovery. No decline in the CYP11A1 mRNA expression was seen during the same time course, showing that the hCG-induced transient down-regulation of VEGF-A and LHR mRNA expression is specific. The present study is the first demonstration of the relationship between VEGF-A and LHR mRNA expression during ligand-induced down-regulation of LHR, a phenomenon which also occurs in the ovary following the preovulatory surge.

It is conceivable that down-regulation of VEGF-A mRNA is due to a diminution of the hCG-induced signal as a result of the loss of LHR expression, since there is substantial evidence that a LH/hCG-induced signal stimulates VEGF-A expression in the ovary (Gomez et al., 2002; Lee et al., 1997; Neulen et al., 1995). The earlier recovery of VEGF-A mRNA after LHR down regulation can be explained on the basis of the possible participation of other regulators of VEGF-A expression such as cytokines and HIF (Martinez-Chequer et al., 2003; Schams et al., 2001; van den Driesche et al., 2008). These factors might act by overwhelming the inhibitory effect produced by the loss of LHR. The changes in the expression of VEGF-A mRNA were also reflected in similar changes in VEGF-A protein expression. The close association between ligand-induced down-regulation of LHR mRNA and the decrease in VEGF-A mRNA expression makes it plausible that the decrease in LHR expression in response to the preovulatory LH surge might be responsible for preventing the onset of ovarian hyperstimulation during the normal ovarian cycle. Furthermore, in pathological conditions, it is also likely that a defect in the fine tuning of VEGF-A expression in steroidogenic cells as a result of abberant LHR down-regulation might cause or trigger the excess production of VEGF that may play a role in the pathophysiology of OHSS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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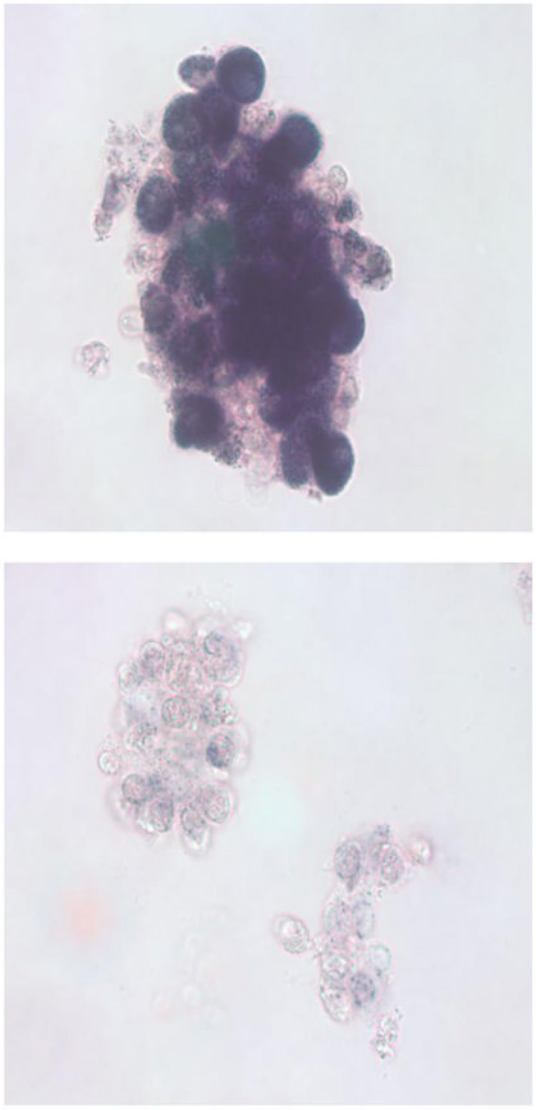


Fig. 1.

3β-HSD staining of purified luteal cells. Isolated ovaries were minced and incubated with collagenase, RNase free DNase-I, and RNase inhibitor. Dispersed cells were purified based on size as described in Materials and Methods. An aliquot of the cells was incubated for 1h at 37 C with staining solution containing 100 μg/ml dehydroepiandrosterone and 100 μg/ml pregnenolone as substrates (A). Cells incubated with staining solution without substrates are shown in (B).

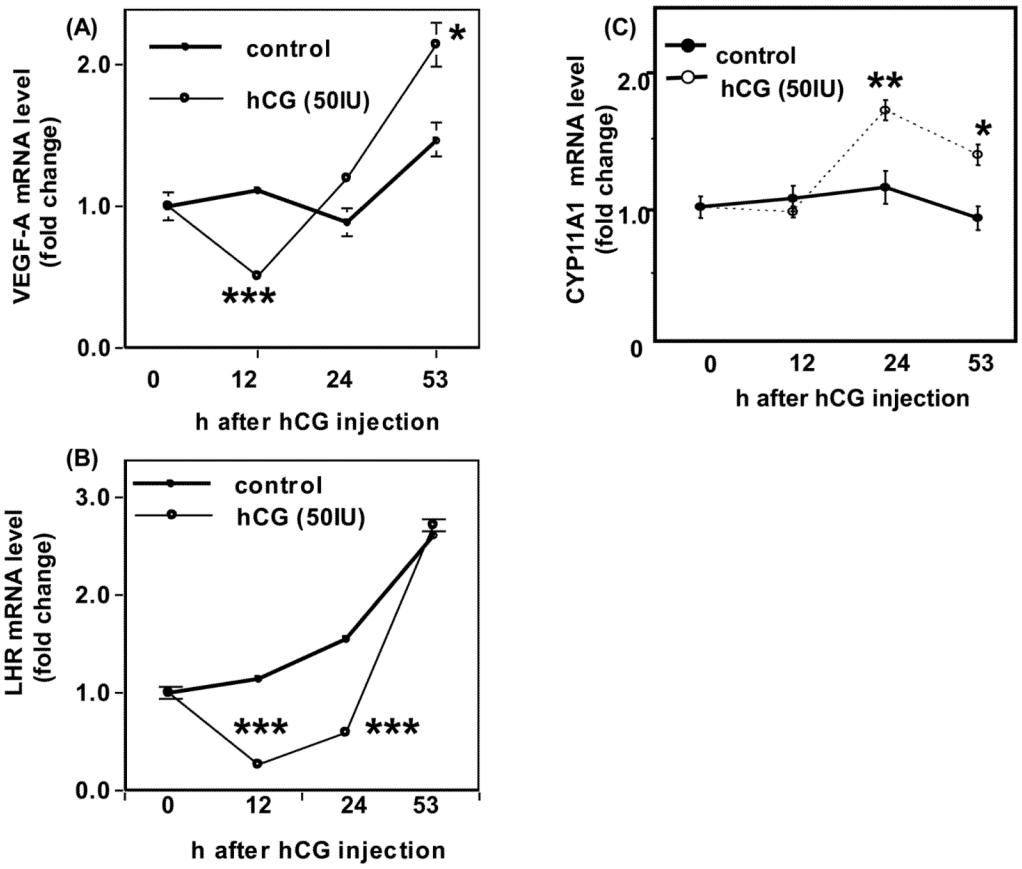


Fig. 2.

VEGF-A, LHR, and CYP11A1 mRNA expression in purified luteal cells during hCG-induced down-regulation of LHR in pseudopregnant rats. 23-day-old female rats were injected sc with 50IU PMSG, followed by 25 IU hCG 56h later (day 0). On day 5 (T=0), one group of animals was treated with an additional dose of 50 IU hCG; control rats received an equal volume of saline. Purified luteal cells were isolated from ovaries collected at the indicated time intervals. Total RNA from the purified luteal cells was reverse transcribed, and resulting cDNAs were subjected to real-time PCR using predesigned primers and probes for rat VEGF-A, LHR and CYP11A1 as described in Materials and Methods. The graphs represent the changes in (A) VEGF-A, (B) LHR and (C) CYP11A1 mRNA expression normalized for 18S rRNA. Error bars represent the mean ± SE of three experiments. (A) *, $P<0.01$; ***, $P<0.0001$ compared to control at indicated time points. (B) ***, $P<0.0001$ compared to control at indicated time points. (C) *, $P<0.05$; **, $P<0.01$ compared to control at indicated time points.

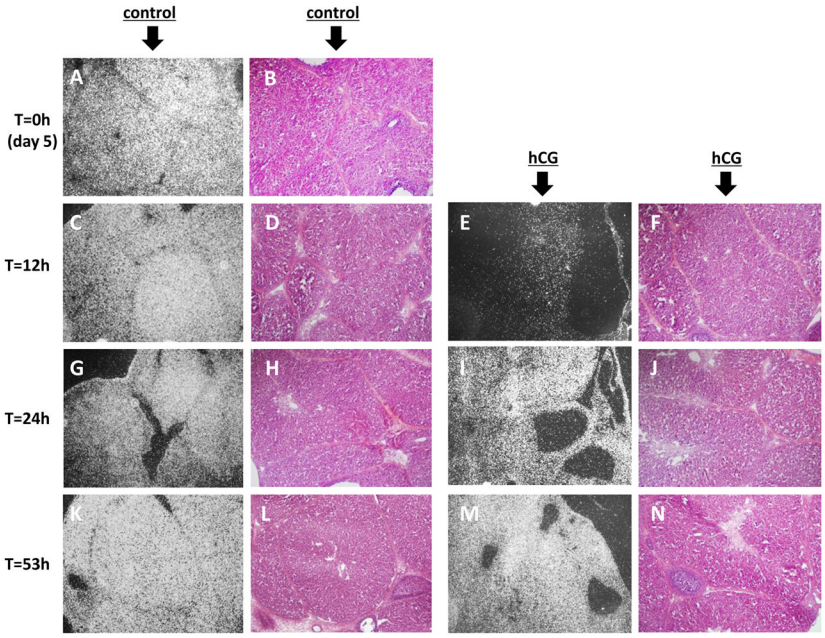
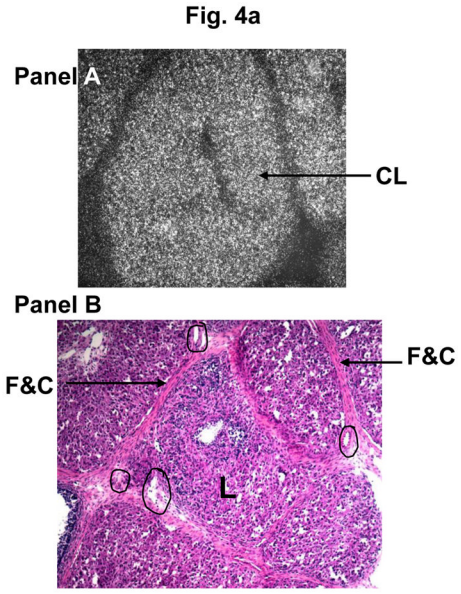


Fig. 3.

Expression of VEGF-A mRNA in the pseudopregnant control and down-regulated rat ovaries. Tissue sections of ovaries from day 5 of pseudopregnancy (T=0) were hybridized with ^{35}S-labeled antisense VEGF-A probe (panel A) or stained with H-E (panel B). On that day 5, one group of animals was treated with 50 IU hCG; controls received an equal volume of saline. Tissue sections were collected at T=12h (panels C through F), at T=24h (panels G through J) and at T=53h (panels K through N). Tissue sections from control ovaries are shown in panels C and D, panels G and H, and panels K and L). Tissue sections from hCG-treated ovaries are shown in panels E and F, panels I and J, and panels M and N. One half of the sections were hybridized with ^{35}S-labeled antisense VEGF-A probe (panels C and E, G and I, and K and M) and the other half stained with H-E (panels D, F, H, J, L, and N). The images were captured as described in Materials and Methods, using brightfield optics to show the corpora lutea (panels B, D, F, H, J, L, and N) or darkfield optics to show ^{35}S RNA hybridization signals (panels A, C, E, G, I, K, and M).



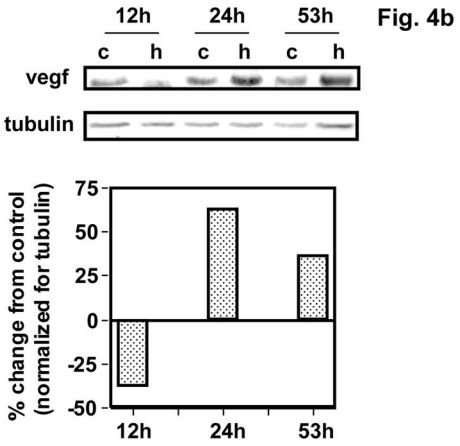


Figure 4.

Fig. 4a. Expression of VEGF-A mRNA in the day 5 pseudopregnant control ovary. Tissue sections were hybridized with ^{35}S-labeled antisense VEGF-A probe (Panel A) or stained with H-E (Panel B). The images were captured as described in Materials and Methods, using darkfield optics at 40x magnification to show ^{35}S RNA hybridization signals in the luteal cells (Panel A) or brightfield optics at 40x magnification to show cells in and around the corpora lutea (Panel B). The abbreviations used are: L, luteal cells; F, fibroblasts; C, collagen bundles; CL, corpus luteum. Circles indicate blood vessels. Fig. 4b. Western blot analysis of VEGF-A protein expression in purified luteal cells

On day 5 of pseudopregnancy, one group of rats was treated with 50 IU hCG; control animals received an equal volume of saline. Purified luteal cells were isolated from ovaries collected at the indicated time intervals. Western blot analysis was performed as described in Materials and Methods. The upper two panels are blots of VEGF-A protein and tubulin, respectively. The bar graph indicates VEGF-A protein in hCG treated cells compared to control cells at the same time point, expressed as per cent change from control and normalized for tubulin, and derived from the densitometric scans. The abbreviations used are: c, control; h, hCG treated.