Identification of novel genes regulated by LH in the primate corpus luteum: insight into their regulation during the late luteal phase

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The process of luteinization, during which granulosa cells are transformed into luteal cells, is accompanied by dramatic changes in the response of luteal cells to LH. Although luteal cells require LH-cAMP signalling cascade for survival, whether these cells respond to trophic factors through changes in gene expression remains poorly characterized. In an attempt to characterize gonadotrophin (LH)-regulated gene expression in the bonnet monkey corpus luteum (CL), changes in gene expression after GnRH antagonist treatment to inhibit LH secretion, different stages of CL and during hCG-simulated early pregnancy were examined using differential display RT–PCR, Northern blot and semiquantitative RT–PCR analyses. We have identified seven non-redundant cDNA’s whose expression were regulated by LH. The results show that inhibition of LH secretion not only leads to down-regulation in the expression of genes, e.g. low density lipoprotein (LDL) receptor and Aldose reductase, but expression of some of the genes was up-regulated, e.g. Humanin, RNA helicase, Lyric protein, Acidic ribosomal phosphoprotein and KIAA1750. mRNA levels of the genes identified as up-regulated after LH inhibition were higher during late compared to the late luteal phase

Key words: corpus luteum/differential display/GnRH antagonist/mRNA expression/bonnet monkey

Introduction

The corpus luteum (CL), an endocrine structure formed from the ruptured ovulatory follicle, is necessary, by way of production of progesterone, for establishment and maintenance of pregnancy in mammals (Zelesznik and Benyo, 1994; Niswender et al., 2000). It is well established that the development and function of the primate CL depends absolutely on the circulating levels of pituitary LH (Moudgal et al., 1972; Zelesznik and Benyo, 1994). Also, it is well established that choriionic gonadotrophin (CG) secreted from placental trophoblasts is responsible for transient prolongation of luteal function that occurs during early pregnancy in primates (Neill and Knobil, 1972; Ottobre and Stouffer, 1984). However, the cellular and molecular processes whereby LH and CG regulate luteal structure and function are poorly understood. Moreover, considering that both LH and CG activate the same receptors, it is not clear why the CL undergoes regression in non-fertile menstrual cycles despite the presence of circulating LH, whereas CG extends its functional lifespan in fertile cycles.

LH and CG bind and activate the same G protein-coupled receptor on target cells to primarily stimulate the adenylate cyclase–cAMP–protein kinase A (PKA) pathway (Vandervoort et al., 1988; Richards, 2001). At the nuclear level, the cAMP pathway regulates transcription mainly by PKA-dependent phosphorylation of the cAMP response element binding protein (CREB) family of transcription factors (CREB, ATF1, CREM etc.; Richards, 2001). In primates, intriguingly CREB has been reported to be extinct after ovulation and thus nuclear actions of LH appear to be compromised in the CL (Somers et al., 1995). Recent studies, however, seem to suggest that expression of some of the genes is altered by changes in the circulating gonadotrophin concentrations during luteolysis, e.g. Bax, Steroidogenic Acute Regulatory protein (StAR) and during simulated early pregnancy, e.g. StAR and low density lipoprotein receptor (LDLR) (Benyo et al., 1993; Sugino et al., 2000; Kohen et al., 2003). The results of these reports indicate that even though there is apparent disruption in the cAMP–PKA–CREB signalling pathway, the CL seems to respond to LH/CG by way of changes in transcription. Alternatively, LH and CG may mediate their actions by PKA-independent pathways such as through the Mitogen Activated Protein (MAP) kinase pathway (Srisuparp et al., 2003). Recently, however, we have observed expression of CREB in the monkey CL (Medhamurthy and Gupta, 2004). In light of the above observations, we hypothesize that at least some nuclear actions of LH may after all be intact in the primate luteal cells and that changes in the expression of genes occurs in response to varying levels of circulating LH/CG.

The objective of this study was to identify genes that are differentially expressed in CL collected from monkeys treated with GnRH antagonist to inhibit pituitary LH secretion. After identifying the differentially expressed genes, their expression under different gonadotrophin milieu was confirmed by Northern blot and semiquantitative
RT–PCR analyses. The identification and characterization of gene expression patterns occurring at different stages of CL development and function will contribute to a better understanding of the molecular determinants of CL rescue and regression.

Materials and methods

Reagents

GnRH antagonist (Cetrotelix®; CET) was a kind gift from Asta Medica (Germany). Human CG (hCG) was from Ares Serono (Switzerland). Avian Myeloblastoma Virus (AMV) RT, Taq DNA Polymerase, random hexamers, RNAsin, Wizard Plasmid Mini-preparation purification kit, qEMT easy cloning kit, dNTP, RNA markers and 100 bp DNA ladder were from Promega (USA). Oligonucleotide primers were synthesized by Sigma–Genosys (UK). Restriction enzymes were obtained from MBI Fermentas (Germany).

For random primer labelling, the Random Primer Extension Labelling System, [α-32P]dCTP and [α-32P]dATP were procured from Perkin Elmer Life Sciences Inc. (USA). Platinum Taq, Agarose and Trizol reagent were purchased from Invitrogen, Life Technologies (USA). All other reagents were purchased from Sigma Chemical Co. (USA), Invitrogen, Life Technologies (USA) or sourced locally.

Animal protocols and CL collections

Experimental protocols involving monkeys in this study were approved by the Institutional Animal Ethics Committee of the Indian Institute of Science. Care and management of monkeys were followed as per the guidelines outlined by the Indian National Science Academy, New Delhi for welfare of laboratory animals. Adult female bonnet monkeys (Macaca radiata) weighing 3–4.6 kg with a history of regular menstrual cyclicity (27–29 days) were utilized for the study. The general care and housing of monkeys at the Primate Research Laboratory, Indian Institute of Science, Bangalore have been described elsewhere (Srivinath, 1979). Monkeys were monitored daily for menses, and blood samples (~1.5 ml) from the lateral venuinpunctures were collected daily from day 8 following the onset of menses until day 12 for determining estradiol (E2) and LH surges. Further blood samples were collected either daily or at more frequent intervals until the day of CL retrieval. In this study, 1 day post LH surge was designated as day 1 of the luteal phase.

Corpus luteum on a designated day of the luteal phase (see below) was retrieved after accessing the ovary by performing laparotomy on ketamine hydrochloride (15 mg/kg body weight) anesthetized female monkeys under aseptic conditions. The excised CL was transferred to a sterile Petri dish containing filter paper, cut into four quarters, placed in individual sterile cryovials and snap-frozen in liquid nitrogen before storing under aseptic conditions. The excised CL was transferred to a sterile Petri dish containing filter paper, cut into four quarters, placed in individual sterile cryovials and snap-frozen in liquid nitrogen before storing under aseptic conditions.

Experiment I: Temporal changes in gene expression during GnRH antagonist-induced luteolysis

It is well established that administration of GnRH antagonist into monkeys during mid-luteal phase leads to suppression of LH secretion and abrupt termination of the luteal phase (Ravindranath et al., 1992; Duffy et al., 1999; Fraser et al., 1999). In the present study, Cetrotelix, a synthetic GnRH antagonist, was administered s.c., at a dose of 75 µg/kg body weight twice daily (09:00 and 21:00) on day 7 of the luteal phase of the menstrual cycle. This dose of Cetrotelix was chosen based on a pilot study in the bonnet monkey which indicated that Cetrorelix at a dose of 75 µg/kg body weight administered s.c., twice daily for 3 consecutive days, resulted in decreased circulating serum progesterone concentrations within 12 h after treatment and initiation of menstruation on day 3 or 4 after start of treatment. Twice daily administration of Cetrotelix was chosen since the half-life was reported to be 10 h (Duijkers et al., 1998). The GnRH antagonist was administered on day 7 of the luteal phase and CL (n = 3/time point) collected before, 12, 24 and 48 h after treatment. For differential display RT–PCR analysis, CL (n = 3/time point) collected from monkeys on day 9 of the luteal phase and from monkeys treated with Cetrotelix for 48 h served as control and treatment respectively.

**Table I. Primer sequences used in differential display RT–PCR**

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<thead>
<tr>
<th>Primer ID</th>
<th>Primer sequence 5’–3’</th>
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<tbody>
<tr>
<td>AP1</td>
<td>CGTGAATTCCGAGGCAAGCGA</td>
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<tr>
<td>AP2</td>
<td>CGTGAATTCCGAGCCGTGTG</td>
</tr>
<tr>
<td>AP3</td>
<td>CGTGAATTCCGAGGCTGGCT</td>
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<td>MG</td>
<td>CGGAATTCCGGTTTCTTTTTTVG</td>
</tr>
<tr>
<td>MC</td>
<td>CGGAATTCCGGTTTCTTTTTTVC</td>
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<tr>
<td>MT</td>
<td>CGGAATTCCGGTTTCTTTTTTVT</td>
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AP1, AP2, AP3, AP4, AP5 and AP6 are arbitrary primers and MA, MG, MC and MT are anchored primers.

Experiment II: Gene expression changes during various stages of CL development and function

During different stages of CL development, the pattern of expression of differentially expressed genes was examined by Northern blot and semiquantitative RT–PCR analyses. Corpora lutea (n = 3/group) were collected from monkeys experiencing spontaneous menstrual cycles at the early (day 5), mid- (days 8–9) and late (days 14–15) luteal phase of the menstrual cycle.

Experiment III: Gene expression changes during simulated early pregnancy

In order to determine the gene expression changes during rescue of the CL following conception and establishment of pregnancy, we have utilized a model system that involves simulation of early pregnancy in non-mated cycling bonnet monkeys. Studies carried out in rhesus (Ottobre and Stouffer, 1984) and bonnet (Benyo et al., 1992) monkeys have demonstrated that administration of hCG in increasing doses beginning day 9 of the luteal phase of the non-mated cycles mimics the circulating pattern of monkey CG, steroid profile and the expression profile of key steroidogenic enzymes in CL observed during early pregnancy. In the present study, in addition to administration of hCG (day 9–13 of the luteal phase, see below) in incremental doses to mimic early pregnancy, another group of monkeys was treated with hCG beginning in the early luteal phase similar to the protocol reported by Ottobre and Stouffer (1984), as that treatment regimen results in persistent rather than transient stimulation of luteal progesterone production, to examine whether the gene expression would be different from that observed during the simulated early pregnancy model system. Monkeys (n = 3) were administered increasing doses of hCG during days 5–13 (15, 30, 45, 90, 180, 360, 720, 1440 and 2880 IU) or days 9–13 (15, 30, 45, 90 and 180 IU) of the luteal phase i.m. twice daily (09:00 and 17:00), and CL collected on day 14 of the luteal phase from both the groups of monkeys. For comparison, CL collected on day 14 of the luteal phase, but without treatment (n = 3), was utilized as the untreated control.

RNA extraction

Total RNA was extracted from CL tissue using Trizol reagent according to the manufacturer’s recommendations. The quality and quantity of each RNA sample was assessed spectrophotometrically as well as on a 1% formaldehyde agarose gel.

Differential mRNA display

Differential display RT–PCR was performed by a variation of the method of Liang et al. (Liang and Pardee, 1992; Liang et al., 1993). Arbitrary (AP1–AP6) and anchored primers (MA, MG, MC and MT) designed for this study are listed in Table I. Total RNA of CL collected from individual monkeys from the control or treatment group were pooled within each group and analysed at least twice for differential expression. Pooled total RNA (200 ng) was reverse-transcribed using 2 µmol/l of anchored primer (HT11VM, where H: CCGAATTC; V is a variable nucleotide; and M is A, G, C or T), 250 µmol/l of dNTP, and 10 IU AMV RT in a 20 µl reaction volume with the following parameters: 37°C for 60 min, 75°C for 5 min. PCR with the same anchored primer and an arbitrary primer (e.g. AP2 primer: CGTGAATTCCGGACCGCTTGT) was performed using a variation of the method of Liang et al. (Liang and Pardee, 1992; Liang et al., 1993).
was carried out using 2 μl of the RT reaction in 1.5 mmol/l MgCl₂, 2 μmol/l dNTP, and 2 μl of [α-³²P]dATP and 1 IU Taq polymerase in a 20 μl reaction volume. The following PCR program was run in a Peltier Thermal Cycler PTC-200 MiniCycler™ (MJ Research, USA): 94°C for 30 s, 40°C for 2 min, 72°C for 30 s for 40 cycles following 72°C for 5 min. Aliquots of PCR mixture were run on a 6% sequencing gel and ampiclons were visualized by autoradiography. Different combinations of anchored and arbitrary primers were used initially in separate reactions to generate differential display from which fragments could be isolated. The primer combinations that resulted in consistent expression patterns on the differential display autoradiograms were subsequently repeated for reproducibility and those that were reproducible were chosen for further analysis. Details of the primer combinations used for isolation of differentially amplified products are provided in Table II. Differentially amplified products were recovered by aligning the gel with the film, excising the gel containing the ampiclons and soaking the excised gel with Whatman No. 3 paper in 100 μl of 3 mol/l sodium acetate pH 5.2, 1 μl of yeast tRNA and 450 μl of 100% ethanol at −70°C and DNA was dissolved in 10 μl of H₂O. Four microlitres of the resuspended cDNA was used to reamplify products with 1.5 mmol/l MgCl₂, 250 μmol/l dNTP, 2 μmol/l HT₁₁VM primer, 2 μmol/l arbitrary primer, and 5 IU Taq polymerase in a 40 μl reaction volume using the same PCR program as above.

cDNA cloning and sequencing

Re-amplified cDNA fragments eluted from the gel were cloned into pGEMT vector using TA cloning system according to the manufacturer’s instructions. The clones were confirmed by EcoR₁ digestion and release of the identical size product from the vector as observed before cloning. Using SP6/T7 primer, all clones were sequenced by Macrogen Inc. (Korea). The nucleotide sequences obtained were compared with GenBank databases using computer searches and sequence alignments at http://www.ncbi.nlm.nih.gov and http://searchlauncher.bcm.tmc.edu

Probe preparation and Northern blot analysis

One nanogram of each cloned PCR product was amplified using the specific primer combinations that resulted in consistent expression patterns on the differential display autoradiograms and quantified using UVI-Band Map software (1999).

Semiquantitative RT–PCR analysis

Semiquantitative RT–PCR was carried out essentially as described previously (Yadav et al., 2002).

<table>
<thead>
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<th>Table II. Summary of the cDNA clones differentially expressed in the monkey corpus luteum after GnRH antagonist treatment</th>
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Hormone assays

Estradiol (E₂), progesterone and LH concentrations in serum were determined by specific radioimmunoassays as reported previously (Selvaraj et al., 1996). The E₂ (GDN #244) and progesterone (GDN #337) antisera were kindly provided by Professor G.D.Niswender, University of Colorado, Fort Collins, Colorado, USA. Blood samples collected from hCG-treated monkeys were assayed for LH/hCG bioactivity using mouse Leydig cell bioassay as reported previously (Van Damme et al., 1974).

Statistical analysis

Wherever applicable, data were expressed as mean ± SEM. The data (hormone values and relative levels of mRNA) were analysed by one-way ANOVA, followed by the Newman–Keuls multiple comparison test (PRISM Graph Pad, version 2; Graph Pad Software, Inc., USA). P < 0.05 was considered statistically significant.

Results

Differential display of products amplified from CL collected from control and GnRH antagonist-treated monkeys

In order to examine regulation of gene expression by LH in the CL, we performed two independent rounds of differential display
RT–PCR on pooled total RNA isolated from CL of control and Cetrorelix-treated (48 h) monkeys (mean ± SEM progesterone concentrations in control and treatment monkeys were 4.77 ± 0.57 and 0.45 ± 0.24 ng/ml respectively). From the electrophoretic analysis of the amplified products on DNA sequencing gels, 13 differentially amplified products plus a single product not differentially regulated (to serve as an internal control for Northern blot and semiquantitative RT–PCR analyses) were identified (Figure 1A). All 14 bands were excised (Figure 1B), PCR-amplified and electrophoresed (Figure 1C). Sequence comparison of the 14 amplified products revealed nine non-redundant and five redundant cDNA. As differential display technique often results in false positives, total RNA extracted from CL of control and Cetrorelix-treated monkeys as well as CL collected from different experimental conditions were further subjected to Northern blot analysis in order to assess number of transcripts and transcript-specific changes in the mRNA expression of genes identified to be differentially expressed in RT–PCR. Additionally, semiquantitative RT–PCR analysis was performed to quantitatively determine changes in steady state mRNA levels of the differentially expressed genes in CL collected during different experimental conditions. Northern blot analysis confirmed the regulation in the mRNA expression of seven fragments (D1/2/4, D3, 5/9/11, 6, 8/12, 13 and 14) and revealed presence of multiple transcripts for some of the genes. One fragment (D10) was determined to be a false positive as mRNA expression levels between control and treatment were not different (data not shown). The differentially expressed genes identified were observed to be either up-regulated (D3, 6, 8/12, 13 and 14) or down-regulated (D1/2/4, 5/9/11) following inhibition of pituitary LH secretion. Nucleotide sequences of all seven differentially regulated non-redundant sequences and D7 (Ribosomal Protein Large P1; used as an internal control for Northern blot and semiquantitative RT–PCR analyses) have been deposited with GenBank and the details of each fragment are provided in Table II.

Changes in serum LH and progesterone during Cetrorelix treatment, different stages of CL development and simulated early pregnancy

Circulating serum LH (or bioactive LH equivalents) and progesterone concentrations determined during Cetrorelix treatment, different stages of CL (early, mid- and late luteal phase) and after hCG treatment are represented in Figure 2. Circulating serum LH and progesterone levels decreased significantly (P < 0.05) after Cetrorelix treatment within 12 h and remained low throughout the treatment period (Figure 2A). Although circulating serum progesterone levels were higher (P < 0.05) during the mid-luteal phase compared to other stages of the luteal phase, LH levels did not vary at different
Temporal changes in the expression of genes identified as LH responsive by differential display

Following identification of genes responsive to inhibition of pituitary LH secretion, their temporal pattern of expression at different times after GnRH antagonist treatment, during different stages of CL development and during simulated early pregnancy were examined by Northern blot and semiquantitative RT–PCR analyses. The expression of DDRT–PCR fragment D7 (RPLP1), which did not change under different experimental conditions (data not shown), was utilized as internal control for both the Northern blot and semiquantitative RT–PCR analyses. The expression pattern of five genes (Humanin, RNA helicase, Lyric protein, Acidic ribosomal phosphoprotein and KIAA1750) identified as up-regulated following inhibition of LH secretion (Figures 5–9). Humanin gene expression revealed the presence of three transcripts (1.6, 1.2 and 1.0 kb; Figure 5A) and semiquantitative RT–PCR data indicated that mRNA expression was significantly (P < 0.05) high at all time points after Cetrorelix treatment (Figure 5A–C). mRNA levels of Humanin increased progressively in mid- and late stage CL, but the expression was lower after hCG treatment (Figure 5A–C). RNA helicase gene expression revealed the presence of two transcripts (4.2 and 2.4 kb) and the expression of larger transcript increased after Cetrorelix treatment (Figure 6A) which was further confirmed by semiquantitative RT–PCR data (Figure 6B and C). The expression of RNA helicase progressively increased (P < 0.05) from mid- to late stage CL, but decreased (P < 0.05) significantly after hCG treatment (Figure 6A–C). Northern blot analysis of Lyric protein expression showed the presence of two transcripts (4.7 and 3.4 kb) and semiquantitative RT–PCR data indicated >2-fold increase in expression at 48 h after Cetrorelix treatment compared to the control (P < 0.05, Figure 7A–C). The expression of Lyric protein was significantly higher (P < 0.05) in mid- compared to early and late stage CL, whereas the expression was lower (P < 0.05) in CL retrieved from monkeys receiving 9–13 days of hCG treatment (Figure 7A–C). A single transcript (0.65 kb) was observed for Acidic ribosomal phosphoprotein gene (Figure 8A) and the expression was >3-fold higher (P < 0.05) 24–48 h after Cetrorelix treatment (Figure 8B and C). The expression progressively increased from mid- to late stage CL, but decreased (P < 0.05) after hCG treatment (Figure 8A–C). The expression of KIAA1750 was significantly higher (P < 0.05) in mid- and late stage CL (Figure 9A–C). The expression of KIAA1750 was higher 12–24 h after Cetrorelix treatment (Figure 9A–C). mRNA levels of aldose reductase were significantly higher (P < 0.05) during mid- and late stage CL compared to the early stage and the expression was also higher (P < 0.05) in CL retrieved from monkeys receiving 9–13 days of hCG treatment (Figure 4A–C). Next, we examined the expression pattern of five genes (Humanin, RNA helicase, Lyric protein, Acidic ribosomal phosphoprotein and KIAA1750) identified as up-regulated following inhibition of LH secretion (Figures 5–9). Humanin gene expression revealed the presence of three transcripts (1.6, 1.2 and 1.0 kb; Figure 5A) and semiquantitative RT–PCR data indicated that mRNA expression was significantly (P < 0.05) high at all time points after Cetrorelix treatment (Figure 5A–C). mRNA levels of Humanin increased progressively in mid- and late stage CL, but the expression was lower after hCG treatment (Figure 5A–C). RNA helicase gene expression revealed the presence of two transcripts (4.2 and 2.4 kb) and the expression of larger transcript increased after Cetrorelix treatment (Figure 6A) which was further confirmed by semiquantitative RT–PCR data (Figure 6B and C). The expression of RNA helicase progressively increased (P < 0.05) from mid- to late stage CL, but decreased (P < 0.05) significantly after hCG treatment (Figure 6A–C). Northern blot analysis of Lyric protein expression showed the presence of two transcripts (4.7 and 3.4 kb) and semiquantitative RT–PCR data indicated >2-fold increase in expression at 48 h after Cetrorelix treatment compared to the control (P < 0.05, Figure 7A–C). The expression of Lyric protein was significantly higher (P < 0.05) in mid- compared to early and late stage CL, whereas the expression was lower (P < 0.05) in CL retrieved from monkeys receiving 9–13 days of hCG treatment (Figure 7A–C). A single transcript (0.65 kb) was observed for Acidic ribosomal phosphoprotein gene (Figure 8A) and the expression was >3-fold higher (P < 0.05) 24–48 h after Cetrorelix treatment (Figure 8B and C). The expression progressively increased from mid- to late stage CL, but decreased (P < 0.05) after hCG treatment (Figure 8A–C). The expression of KIAA1750 was significantly higher (P < 0.05) in mid- and late stage CL, but became lower after hCG treatment (Figure 9A–C).
Figure 3. Representative autoradiograms of Northern blot analysis demonstrating transcript-specific changes in low density lipoprotein receptor (LDLr) mRNA expression (A) and representative ethidium bromide-stained agarose gels of semiquantitative RT–PCR analysis of LDLr and Ribosomal Protein Large P1 (RPLP1) expression (B) in Corpus Luteum before and after Cetrorelix treatment, different stages of development [early (E), mid- (M) and late (L) luteal phase] and following exogenous hCG treatment (from day 5 to 13/day 9 to 13 of the luteal phase) to simulate early pregnancy. (C) Relative levels (mean ± SEM) of LDLr mRNA in the CL with respect to time (h) of CET treatment, CL at different stages of the luteal phase, CL after CG administration to simulate early pregnancy after normalization against RPLP1 mRNA levels in each sample of semiquantitative RT–PCR gel. The estimated sizes of transcripts (kb) and PCR products (bp) are indicated. Bars with different letters are significantly different ($P < 0.05$).

Figure 4. Representative autoradiograms of Northern blot analysis demonstrating transcript-specific changes in Aldose reductase (AR) mRNA expression (A) and representative ethidium bromide-stained agarose gels of semiquantitative RT–PCR analysis of AR and RPLP1 expression (B) in corpus luteum (CL). (C) Relative levels (mean ± SEM) of AR mRNA in the CL after normalization against RPLP1 mRNA levels in each sample of semiquantitative RT–PCR gel. For details of experimental conditions and quantification of agarose gels, see Figure 3 legend. The estimated sizes of transcripts (kb) and PCR products (bp) are indicated. Bars with different letters are significantly different ($P < 0.05$).
Figure 5. Representative autoradiograms of Northern blot analysis demonstrating transcript-specific changes in Humanin mRNA expression (A) and representative ethidium bromide-stained agarose gels of semiquantitative RT–PCR analysis of Humanin and RPLP1 expression (B) in corpus luteum (CL). (C) Relative levels (mean ± SEM) of Humanin mRNA in the CL after normalization against RPLP1 mRNA levels in each sample of semiquantitative RT–PCR gel. For details of experimental conditions and quantification of agarose gels, see Figure 3 legend. The estimated sizes of transcripts (kb) and PCR products (bp) are indicated. Bars with different letters are significantly different ($P < 0.05$).

Figure 6. Representative autoradiograms of Northern blot analysis demonstrating transcript-specific changes in RNA helicase (RH) mRNA expression (A) and representative ethidium bromide-stained agarose gels of semiquantitative RT–PCR analysis of RH and RPLP1 expression (B) in corpus luteum (CL). (C) Relative levels (mean ± SEM) of RH mRNA in the CL after normalization against RPLP1 mRNA levels in each sample of semiquantitative RT–PCR gel. For details of experimental conditions and quantification of agarose gels, see Figure 3 legend. The estimated sizes of transcripts (kb) and PCR products (bp) are indicated. Bars with different letters are significantly different ($P < 0.05$).
Figure 7. Representative autoradiograms of Northern blot analysis demonstrating transcript-specific changes in Lyric protein (LP) mRNA expression (A) and representative ethidium bromide-stained agarose gels of semiquantitative RT–PCR analysis of LP and RPLP1 expression (B) in corpus luteum (CL). (C) Relative levels (mean ± SEM) of LP mRNA in the CL after normalization against RPLP1 mRNA levels in each sample of semiquantitative RT–PCR gel. For details of experimental conditions and quantification of agarose gels, see Figure 3 legend. The estimated sizes of transcripts (kb) and PCR products (bp) are indicated. Bars with different letters are significantly different ($P < 0.05$).

Figure 8. Representative autoradiograms of Northern blot analysis demonstrating transcript-specific changes in Acidic ribosomal phosphoprotein (ARPP) mRNA expression (A) and representative ethidium bromide-stained agarose gels of semiquantitative RT–PCR analysis of ARPP and RPLP1 expression (B) in corpus luteum (CL). (C) Relative levels (mean ± SEM) of ARPP mRNA in the CL after normalization against RPLP1 mRNA levels in each sample of semiquantitative RT–PCR gel. For details of experimental conditions and quantification of agarose gels, see Figure 3 legend. The estimated sizes of transcripts (kb) and PCR products (bp) are indicated. Bars with different letters are significantly different ($P < 0.05$).
Discussion

Although circulating LH is essential for function and survival of the primate CL during the menstrual cycle, its ability to sustain CL function appears to decline during the late luteal phase resulting in the regression of the CL (Ottobre and Stouffer, 1984; Zeleznik, 1998). In the present study, we sought to examine expression of genes regulated by LH in the monkey CL using differential mRNA display approach with emphasis on characterization of genes during the late luteal phase. Of the two genes identified to be down-regulated following inhibition of pituitary LH secretion, LDLr regulates the uptake of LDL from blood (Brown and Goldstein, 1986), the rich source of cholesterol, which is a key substrate required for steroidogenesis in the luteal tissue. To date, there are no reports of alterations in the expression levels of LDLr following LH inhibition. Two transcripts, 5.3 and 3.7 kb for LDLr in the primate CL and choriocarcinoma cells, have been reported by others (Benyo et al., 1993; Wittmaack et al., 1995). In the present study, an additional transcript of 2.0 kb was observed whose expression pattern paralleled other transcripts. The finding that the expression of LDLr was up-regulated following hCG treatment was in accordance with the similar findings reported by Benyo et al. (1993) for the rhesus monkey CL. Decreased expression observed following inhibition of LH secretion in the present study further confirms the importance of LH in the regulation of LDLr expression. Aldose reductase gene belongs to the highly conserved superfamily of Aldo-keto reductases whose products catalyse NADP(H)-dependent reduction of a wide variety of substrates such as carbohydrates, steroid hormones, prostaglandins and many aliphatic aldehydes and ketones (Jez et al., 1997; Yabe-Nishimura, 1998). Similar to the observations in the present study, others (Cao et al., 1998) also observed a single transcript for Aldose reductase by Northern blot analysis, but its role within the context of LH regulation of CL function is not clear. We speculate that it may be involved in clearance of toxic aldehydes generated by cellular metabolism (Vander Jagt et al., 1995).

Genes whose expressions were identified as up-regulated in response to inhibition of pituitary LH secretion in the monkey CL included translation machinery proteins (RNA helicase and Acidic ribosomal phosphoprotein), apoptosis regulating protein (Humanin), cell–cell interaction protein (Lyric protein) and a protein with predicted function for nucleotide binding (KIAA1750). Recently, Tang et al. (1999) reported characterization of a testis-specific RNA helicase also referred to as Gonadotrophin-regulated testis helicase that was observed to be up-regulated following desensitization of Leydig cells to a large bolus of hCG injection. RNA helicase has not been characterized in the ovary. Our results are the first to describe the expression of this gene in the ovary. In the monkey CL, although the expression was low during the mid-luteal phase, two transcripts that corresponded to the reported transcript sizes of testis-specific RNA helicase were observed (Endoh et al., 1999). In the present study, the observation that GnRH antagonist treatment resulted in a significant up-regulation of RNA helicase suggests that, as observed in the testis, a similar gonadotrophin-regulated RNA helicase expression appears to be present in the ovary.

It has recently been reported that Humanin functions as an anti-apoptotic factor in neuronal cells/tissue (Tajima et al., 2002) and it has been suggested that it could be used as a potential anti-apoptotic drug for treatment of Alzheimer’s disease (Maximov et al., 2002). In the present study, Humanin expression was increased during the late luteal phase and after GnRH antagonist treatment; in both conditions the process of luteolysis would be present, but the expression was decreased during simulated early pregnancy, a condition analogous to rescue of CL seen during early pregnancy. The results of Humanin expression in the present study appear to suggest a different function in the CL, a non-neuronal tissue, than reported in
the literature. However, in accordance with the findings of a previous report (Tajima et al., 2002), Humanin may play an important role in the survival/demise of the CL. Lyric protein is a novel protein and has been predicted to be associated with cell–cell adhesion molecules. An up-regulation of expression that occurs at the time of expected luteolysis leads us to speculate that it may be important for clearance of the luteal cells and replacement by the connective tissue during the process of luteolysis. Acidic ribosomal phosphoprotein identified in the present study may represent any of the three phosphoproteins P0, P1 and P2 that have previously been shown to be associated with the ribosomal machinery and all the three phosphoproteins have been reported to contain identical C-terminal tails. As differential display analysis represents the 3’ end of mRNA, it is not possible to know which form of protein was identified in the present study, and therefore the full length of the gene needs to be cloned to identify the fragment observed here. All three phosphoproteins, components of 60S ribosomal subunit, play important roles in the elongation step of protein synthesis (Rich and Steitz, 1987; Krowczynska et al., 1989), and observations in the present study point to their role in the regulation of translation machinery in the CL. The function of KIAA1750 is not clear and so far it has been electronically annotated as nucleotide binding protein (Human KIAA1750 protein accession number: NM_033512).

Based on the findings in the present study, we propose a general working model for gonadotropin-dependent gene regulation in the primate CL. During the early to mid-luteal phase, the LH-mediated regulation of gene expression involves both suppression and activation of LH-dependent genes that lead to maintenance of CL function. But as the luteal phase progresses, those genes whose expression is promoted by the circulating LH levels decrease and those genes whose expression is suppressed by LH increase in their expression, resulting in luteolysis. However, in the event of establishment of pregnancy the decreased gene responsiveness to LH expression, resulting in luteolysis. However, in the event of establishment of pregnancy the decreased gene responsiveness to LH observed during the late luteal phase is overcome by the higher concentrations of CG, such that the function of the CL is sustained until luteal–placental shift in progesterone secretion occurs. More recently, researchers at the Oregon National Primate Research Center using GnRH antagonist to inhibit LH secretion reported identification of several novel genes in the monkey CL employing DNA microarray analysis approach (Xu et al., 2003; Young et al., 2003) consistent with the findings in the present study that LH regulates gene expression in the primate CL.

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