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Expression of the IGF and insulin systems in the luteinizing macaque ovarian follicle

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Abstract

Objective—To determine intrafollicular hormone levels and characterize mRNA expression of the IGF receptors, IGF binding proteins (IGFBP), and pregnancy-associated plasma protein-A (PAPP-A) in granulosa cells before and after an ovulatory hCG stimulus.

Design—Experimental animal study.

Setting—Academic medical center.

Animal(s)—Adult rhesus macaques.

Intervention(s)—Animals received exogenous FSH to promote the development of multiple preovulatory follicles. Follicles were aspirated before (0 hr), 3, 6, 12, or 24 hr after an ovulatory hCG bolus.

Main Outcome Measure(s)—IGF1, IGF2, and insulin levels in follicular fluid were determined by radioimmunoassay. Messenger RNA (mRNA) levels in granulosa cells were determined by real-time RT-PCR. IGFBPs and PAPP-A in follicular fluid were determined by Western blot analysis and ELISA.

Result(s)—IGF1, IGF2 and insulin in follicular fluid did not change during luteinization. IGF1R, IGFBP1 and IGFBP2 mRNAs were unchanged by hCG. IGF2R, IGFBP3, 5, 6 and PAPP-A mRNAs increased following hCG, while insulin receptor and IGFBP4 mRNAs decreased following hCG treatment. IGFBP 3 and 6 and PAPP-A protein increased following hCG.

Conclusion(s)—Dynamic changes in the expression of the IGFBPs and PAPP-A suggest tight regulation of IGF action during ovulation and corpus luteum formation.

Keywords

luteinization; granulosa cell; IGF; IGFBP; primate

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INTRODUCTION

Luteinization of the primate follicle is associated with rapid and dramatic changes in steroidogenesis and differentiation of granulosa cells (1–5). The insulin and IGF families are closely associated with granulosa cell proliferation and differentiation, and may represent key signals in the development of the corpus luteum (6). However, the difficulty in obtaining non-luteinized (pre-LH/hCG) samples in humans and primates has precluded a systematic study of the expression of the insulin and IGF systems during the periovulatory interval in the non-human primate.

The IGF/IGFBP family is comprised of IGF1 and IGF2, IGF1R and IGF2R/mannose 6-phosphate receptor, and at least six IGF binding proteins (IGFBP1–6) with insulin and insulin receptor (IR) forming a closely related signaling system (7–10). IGF1R and insulin receptor are receptor tyrosine kinases signaling predominantly through AKT1-mediated pathways to stimulate cell proliferation, survival and steroidogenesis (11–13) while IGF2R is a candidate tumor suppressor that has been postulated to be important in IGF2 turnover, angiogenesis, and other intra-cellular events (14,15). IGF1 and IGF2 increase proliferation and steroidogenesis in IVF-derived human granulosa cells and bovine preovulatory granulosa cells (16–18).

IGF activity is regulated on several levels: gene expression, receptor expression and the presence of the IGFBPs (14). IGFBPs have a higher affinity for IGFs than does either IGF receptor and thus are capable of binding and inactivating circulating IGFs by preventing interaction with the IGF receptors (8). The IGFBPs also have actions independent of IGFs, including suppression of human granulosa cell steroid synthesis by IGFBP4 (19,20). IGFBPs are regulated at the transcriptional level and through proteolytic degradation, most notably by pregnancy-associated plasma protease-A (PAPP-A) (8,21).

All six IGFBPs are expressed in non-human primate or human follicles at varying stages of development (13,22–28). Despite this, there are no data demonstrating changes in either insulin or the IGF system during luteinization in non-human primates or women. This study seeks to describe follicular fluid concentrations of IGF1, IGF2, and insulin, the mRNA expression of IGF1 and IGF2, the IGF receptors, and granulosa cell mRNA and follicular fluid protein levels of PAPP-A and IGFBP1–6 before and up to 24 hours after an ovulatory hCG bolus given to rhesus monkeys undergoing controlled ovarian stimulation protocols.

MATERIALS AND METHODS

Animals

Adult female rhesus macaques (*Macaca mulatta*) were housed at the California National Primate Research Center (CNPRC) as described (29). Animal protocols and experiments were approved by the University of California, Davis Animal Care and Use Committee, and studies were conducted in accordance with Guide for the Care and Use of Laboratory Animals. Following the onset of menstruation, adult female rhesus monkeys were treated with recombinant human follicle-stimulating hormone for 7 days (r-hFSH, Organon, West Orange NJ, USA; 37.5 IU i.m. bid). The GnRH antagonist Antide (Ares-Serono, Randolph MA, USA, 5 mg/kg body weight, s.c. sid) was administered on days 6–7 to prevent endogenous gonadotropin secretion. Follicles were aspirated the morning after the last dose of r-hFSH by an ultrasound guided procedure (29). The characteristics of the follicular cohort in this model have been described elsewhere (30). The resulting cells are referred to as non-luteinized granulosa cells (NLGC). A subset of animals received an ovulatory bolus of r-hCG on the morning of day 8 and follicles were aspirated before (0h) 3, 6, 12 and 24 hr after hCG (n 3–5/time point). Aspirates represented the pooled contents of multiple follicles

from each animal. These were maintained at approximately 35 C within a temperature controlled isolette containing a fixed volume of media at all times. Cumulus oocyte complexes (COC) were removed by transferring the mixture of media, follicular fluid and cellular aspirate to a 24 mm diameter, 70 µm filter (Netwell inserts, #3479, Corning, Inc., Acton, MA, USA); granulosa cells were filtered through the mesh and COC retained. To collect granulosa cells for the corresponding time points, the tube was rinsed with fresh tyrode lactate (TL)-HEPES/polyvinylalcohol (PVA) medium (TL-HEPES/0.1 mg/ml PVA) that was also poured through the filter. The filter was further rinsed with fresh TL-HEPES-PVA medium until blood cells were removed. The rinse from the filter was saved for recovery of granulosa cells for various experiments (see below).

Preparation of macaque granulosa cells

Granulosa cells were recovered from the filter rinse by a modification of the method previously described (31). Briefly, the cell suspension was centrifuged for 5 min at 300g to pellet the red cells; speed was increased to 500g for an additional 5 min, resulting in a thin layer of granulosa cells over the red cell pellet. The supernatant was removed, and the layer of granulosa cells was transferred to a 40% Percoll gradient in medium 199 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged for 30 min at 500g. The supernatant was removed, and the granulosa cells were recovered from the surface of the Percoll with a Pasteur pipette and washed once with TL-HEPES/PVA before being counted on a hemocytometer. Cells were pelleted and snap frozen for shipment from CNPRC to the University of Maryland Baltimore and stored upon arrival at -80 C pending RNA isolation.

Preparation of macaque follicular fluid

The dilution of each sample was determined by assessing the final volume of the follicular fluid diluted into 1 ml of media. Follicular fluids were stored at -80 C until use.

Radioimmunoassay

IGF1 was measured using a coated-tube radioimmunoassay (Alpco Diagnostics, Salem, NH), and IGF2 by the double-antibody RIA (Alpco Diagnostics). Assay sensitivities were <0.1 ng/ml. Samples were acidified per manufacturer's instructions to liberate IGFs bound to IGFBPs. Insulin was measured using a coated-tube RIA (Siemens Medical Solutions Diagnostics, Deerfield, IL).

Real time RT-PCR

Total RNA was extracted from cells using RNAqueous Micro kit (Ambion Inc, Austin, TX) and reverse transcribed using MMLV reverse transcriptase (Invitrogen). All probes and primers, including the internal control ribosomal protein L19 (RPL19) were synthesized by Applied Biosystems (Foster city, CA). The target gene and the RPL19 were detected in the same reaction. Semi-quantitative real time experiments using Assays-on-Demand Gene Expression products were also performed according to manufacturer instruction (Applied Biosystems). The PCR protocol consisted of 40 cycles of denaturing at 95 C for 15 s and annealing/extending at 60 C for 1 min per cycle. Detection of the gene expression was performed during the 2nd step in a two step RT-PCR protocol. To relatively quantify mRNA levels, a standard curve was constructed using pooled macaque granulosa cell cDNA. Data were analyzed as the inverse log ([Ct-Y intercept]/slope of the standard curve) and expressed as a ratio of the target gene to endogenous control (32). Primer and probe sequences are shown in Table 1.

Western Blotting

Follicular fluid samples (1 or 2ul) were mixed with 2x loading buffer and resolved on a 12% separating SDS polyacrylamide gel for 45min–1hr depending on the size of the IGFBP. Kaleidoscope markers (Bio-Rad, Hercules CA) were also resolved to determine IGFBP band sizes. Proteins were transferred to PVDF membrane (Millipore, Bedford MA) for 60–90 min at 100V. Membranes were blocked in 5% non-fat dry milk (Carnation) in Tris-buffered saline with 0.05% Tween (TBST) for 1 hr at room temperature and then incubated overnight with the primary antibody (1:500 dilution for all IGFBPs) in TBST containing 5% milk. After 3 consecutive washes of 15 min each in TBST, the membrane was incubated with the secondary antibody (1:10,000) in TBST containing 5% milk for 60 min at room temperature. The membrane was washed and proteins were visualized using the Western Blue stabilized substrate (Promega, Madison WI) according to manufacturer's directions. Antibodies were purchased from the following sources: IGFBP2 (c-18, sc6001, Santa Cruz Biotechnology, Santa Cruz, CA), IGFBP3 (H-98, sc9028 Santa Cruz), IGFBP4 (c-20, sc-6005 Santa Cruz), IGFBP5 (MA-371-5, Austral Biological, San Ramone CA), IGFBP6 (PB-383-9, Austral Biological). Secondary antibodies were conjugated affinipure Donkey anti-Goat and Goat anti-Rabbit (Jackson ImmunoResearch, West Grove, PA) and anti-mouse alkaline phosphatase conjugate (Promega, Madison, WI).

ELISA

PAPP-A was measured using a solid phase sandwich ELISA (Alpco Diagnostics Salem, NH). Assay sensitivity was < 0.133 ug/ml.

Statistical Analysis

Data are presented as mean $\pm$ SEM. Bartlett's χ^2 was used to test for heterogeneity of variance, and data were logarithmically transformed ($\log+2$) if variances were significantly different. Data were analyzed by one-way ANOVA followed by Student Newman-Keuls means comparison test. Means were considered significantly different if $p < 0.05$.

RESULTS

Administration of hCG to rhesus monkeys undergoing controlled ovarian stimulation protocols increased intra-follicular concentrations of progesterone have been previously reported in these samples (32) and increased PR mRNA levels (data not presented), indicating a normal progression through the periovulatory interval.

Intra-follicular concentrations of IGF1 and IGF2 were determined by RIA following an acidification step to liberate IGFBP-bound IGFs. Both IGF1 and IGF2 levels in follicular fluid were approximately 250 ng/ml and did not change during luteinization (Fig. 1). Insulin was present at very low levels (<30 μ IU/ml) in follicular fluid, and did not change as a result of hCG. Interestingly, insulin was detectable in only 1 of 4 samples prior to hCG, 2/4 at 3 hr, 2/5 at 12 hr, and 6/7 at 24 hr. IGF1 mRNA was not detectable at appreciable levels in any sample, while IGF2 mRNA was present in all samples before and after hCG (data not presented).

The expression of IGF1 receptor mRNA in granulosa cells did not change with hCG treatment over 24 hr (Fig. 2). However, IGF2 receptor mRNA expression showed a dramatic increase over controls (0 hr) at both 3 and 24 hr post-hCG treatment. Insulin receptor (IR-A and IR-B; (33)) mRNA expression was reduced significantly ($p < 0.05$) 24 hrs after hCG treatment, although a trend of decreasing mRNA expression beginning at 3 hr was evident.

The mRNA expression of IGFBP1–6 was also examined as a part of this study (Fig. 3). Levels of IGFBP1 mRNA were highly variable during luteinization and did not statistically change following hCG (data not presented). IGFBP2 mRNA was present in all samples but did not change following hCG. In contrast, IGFBP3 mRNA increased (11-fold, $p<0.05$) 12 hr after hCG, while the expression of IGFBP4 declined (15-fold, $p<0.05$) 24 hr post-hCG. The expression of IGFBP5 mRNA increased at 6 and 12 hr post-hCG relative to controls (7- and 13-fold, respectively; $p<0.05$). IGFBP6 mRNA increased transiently 3 hr after hCG administration (17-fold, $p<0.05$).

Protein levels of IGFBP 2–6 were determined using Western blot (Fig 4). IGFBP2 was present in all samples and did not change following hCG treatment. In contrast, IGFBP3 increased significantly 3, 6 and 12 hrs post-hCG treatment. IGFBP4 levels decreased significantly at 3, 6 and 12 hrs compared to 0 and 24 hr post-hCG. IGFBP5 protein increased 3, 6 and 12 hr post-hCG. Levels of IGFBP6 protein were unchanged following hCG.

Pregnancy-associated plasma protein-A (PAPP-A) is a metalloproteinase that regulates IGF bioavailability through cleavage and inactivation of IGFBPs (22). The expression of PAPP-A mRNA tended to increase 3 hr after hCG ($p=0.07$) and was 4.4-fold higher ($p<0.05$) than controls at 6 hr post-hCG (Fig. 3). PAPP-A protein levels determined by ELISA increased significantly 24 hr after an ovulatory hCG bolus (Fig. 4).

DISCUSSION

The objective of this study was to characterize follicular fluid levels of IGF1, IGF2, and insulin, and determine the mRNA and protein expression of the IGF system in granulosa cells and follicular fluid from rhesus monkeys undergoing controlled ovarian stimulation. Intra-follicular concentrations of IGF1 and IGF2 do not change during luteinization, while insulin is present at very low levels. The mRNA and follicular fluid levels of IGF binding proteins (IGFBP) 3, 5, and 6, as well as pregnancy-associated plasma protease-A (PAPP-A), increase following hCG administration. These data suggest tight control of the IGF signal in the luteinizing monkey follicle is exerted through IGFBPs, as well as a possible IGF-independent role for IGFBPs (8).

IGF1 and IGF2

IGFs are important intra-ovarian factors, acting as mitogens, survival factors, and co-gonadotropins supporting steroid synthesis (22). While IGF2 mRNA is expressed by primate granulosa cell, IGF1 mRNA is undetectable, supporting the hypothesis that follicular fluid IGF1 is of seric origin (22,34). However, whether luteinizing theca cells in primates/human contribute to follicular levels remains to be elucidated. The functions of IGF1 and IGF2 are likely diverse, acting as co-gonadotropins to regulate a diversity of processes, including the expression of vascular endothelial growth factor, steroidogenesis, and oocyte maturation (17,18,35,36). Whether IGF1 and IGF2 have roles in periovulatory events distinct from one another, or partially or completely overlapping remains to be determined, but a patient with genetic loss of IGF1 was still capable of spontaneous ovulation (37), suggesting that at least some overlap exists. The levels of IGF1 and 2 determined in the present study, however, represent total rather than bioactive concentrations. The actual IGF signal is predicted to be much lower on the basis of IGFBP expression.

IGF and insulin receptors

The expression of IGF1 receptor mRNA suggests important local roles for the IGFs. Several studies have shown that human and primate granulosa cells express IGF1R and IGF2R

mRNA (23,25,38,39), although there are surprisingly few reports of expression in human granulosa cells aspirated after an ovulatory hCG bolus to women undergoing IVF cycles (13). IGF1R can mediate the actions of both IGF1 and IGF2 leading to enhanced steroidogenesis and granulosa cell survival (18), and there is ample evidence to indicate a critical role for IGF1R-mediated signaling through PKB/AKT in granulosa cell function (40). In contrast, most IGF2R protein is intracellular and transports mannose 6-phosphate to lysosomes; the limited cell surface protein internalizes IGF2, although there is growing list of potential ligands for this receptor (41). IGF2R is a candidate tumor suppressor (42) and thus the role(s) of this protein leading to ovulation and CL formation are likely very complex. The dynamic nature of IGF2R mRNA expression during luteinization, with distinctive and dramatic peaks at 3 and 24 hr post-hCG, supports the hypothesis that this receptor has important, but undetermined, actions in periovulatory events. However, these changes in IGF2R mRNA are largely driven by very low levels of expression at 0, 6, and 12 hr, and so the consequences of this induction are currently obscure.

The expression of the insulin receptor is reduced 24 hr post-hCG. A role for insulin in normal physiologic processes leading to ovulation and luteal formation is unclear, although insulin has been reported to promote progesterone synthesis in human IVF granulosa cells (14). The low levels of insulin and the declining expression of the insulin receptor may suggest a more limited role in periovulatory events.

IGFBPs

The IGFBPs form a group of proteins that bind IGFs with high affinity and block their interaction with IGF receptors (8). IGFBP1–6 have all been variously reported to be expressed in human IVF-derived granulosa cells and the primate ovary (15,22,23), although the current data are the first to systematically evaluate expression over the course of the ovulatory cascade and luteal formation. While the mRNA expression of IGFBP1–6 is diverse and consistent with complex regulation, there is a generalized increase in mRNA levels of IGFBP3, 5, and 6 during the first 12 hr following hCG, with a concomitant increase in protein for IGFBP3 and 5 in the follicular fluid. This suggests a corresponding reduction in IGF bioavailability facilitating the earliest steps in granulosa cell luteinization. Interestingly, a reduction in mRNA levels of IGFBP4, 5, and 6 occurs at 24 hr as well as a decrease in IGFBP 3 and 5 follicular fluid protein,, consistent with the hypothesis that IGFs are important in luteal steroidogenesis and survival, as well supporting oocyte maturation and embryo development (35,43). The fact that protein levels increase in follicular fluid before observable increases granulosa cell mRNA levels suggests that vesicle-stored IGFBPs in granulosa cells are rapidly secreted after hCG. As the contents of the vesicles are depleted, follicular fluid concentrations decline until new granulosa cell synthesis occurs (new ref: J. Endocrinol. 2001 Feb;168(2):307–315). Alternatively, the rapid increase in follicular fluid levels of IGFBP3 and 5 could originate from serum through increasing vascular permeability associated with luteal formation, although it is unlikely that sufficient changes in the local vascular network occur within the first three hours following hCG to support the observed increases (44,45). The expression of PAPP-A mRNA also increases rapidly following hCG, while PAPP-A protein is highest at 24 hr of hCG treatment. This may represent an additional means to control IGFBP activity.

In summary, high concentrations of total IGF1 and IGF2 are present before and after an ovulatory stimulus in rhesus macaques undergoing controlled ovarian stimulation cycles. IGF1R mRNA is expressed by granulosa cells before and after an ovulatory hCG bolus, while IGF2R is strongly induced by hCG and insulin receptor declines. There is dynamic regulation of the IGFBP mRNAs and follicular fluid protein levels, with a generalized increase in expression apparent during the first 12 hr following hCG. Overall, these data suggest a complex regulation of IGF-I and IGF-II activity during the periovulatory interval

and point to a potentially important role for these signals leading to ovulation and corpus luteum formation in primates.

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Figure 1. Intra-follicular concentrations of IGF1, IGF2, and insulin during luteinization
IGF1, IGF2, and insulin concentrations were measured using specific radioimmunoassays before (0 hr), 3, 6, 12, and 24 hr after an ovulatory hCG stimulus (n=5, 5, 5, 4, 5). IGF1 and IGF2 samples were acidified prior to assay in order to free IGFs bound to IGFBPs.

Figure 2. Expression of IGF1R, IGF2R, and Insulin Receptor (IR) mRNAs during luteinization Granulosa cells aspirated from rhesus monkeys undergoing controlled ovarian stimulation before (0 hr), 3, 6, 12 and 24 hr following an ovulatory hCG bolus (n=5,5,4,3,3). The mRNA expression of IGF1R (left panel), IGF2R (middle panel) and total insulin receptor (right panel) was determined by real-time RT-PCR. Data were normalized to RPL19 mRNA and expressed as relative units. Asterisk indicates significant differences ($P < 0.05$) from control (0 hr) samples.

Figure 3. Granulosa cell expression of IGFBPs during luteinization

Granulosa cells aspirated from rhesus monkeys undergoing controlled ovarian stimulation before (0 hr), 3, 6, 12 and 24 hr following an ovulatory hCG bolus ($n=5,5,4,3,3$). The mRNA expression of IGFBP2 (top left panel), IGFBP3 (top right), IGFBP4 (middle left), IGFBP5 (middle right), IGFBP6 (lower left), and PAPP-A (lower right) was determined by real-time RT-PCR. Data were normalized to RPL19 mRNA and expressed as relative units. Asterisk indicates significant differences ($P < 0.05$) from control (0 hr) samples.

Figure 4. Follicular fluid levels of IGFBPs and PAPP-A during luteinization

Follicular fluid was collected from rhesus monkeys undergoing controlled ovarian stimulation before (0 hr), 3, 6, 12 and 24 hr following an ovulatory hCG bolus (N=3,3,2,3,5). (A) Representative example of a western blot showing the IGFBP2 bands during the time course of hCG treatment, (top panel) normalized to the large proteins remaining in the gel post transfer (bottom panel). (B). The follicular fluid protein profiles of IGFBP2–6 shown in graphical form. The western blots were completed, membranes and gels scanned into a PDF file and IGFBP band density was normalized to coomassie blue stained protein using the program U-SCAN IT (Silk-Scientific Corporation). Asterisk indicates significant differences ($P<0.05$) from control (0 hr) samples. #, significant differences between 3 and 6 hrs of hCG treatment (IGFBP4). C. follicular fluid levels of PAPP-A determined by ELISA.

Table 1

Gene detection primer sequences

Gene Name	Sequence
RPL19	Forward: CCCCAATGAGACCAATGAAATC Reverse: CAGCCCATCTTTGATGAGCTT Probe: ATGCCAACTCCCGTCAGCAGATC
IGF1	Assay-on-Demand (Applied biosystems)
IGF2	Assay-on-Demand (Applied biosystems)
IGF1R	Forward: TGAAAGTGACGTCCTGCATTTTC Reverse: TACCGGGTGCCAGGTTATGAT Probe: CACCACCACGTCGAAGAATCGCA
IGF2R	Forward: TTTGAGTGGCGAACGCAGTAT Reverse: CCAGCCCCATCTTTGAATGA Probe: CTGCCCCACCTTTCGATCTGACTGAAT
Insulin Rec.	Forward: GGGTCCCTGTCCCAAGGT Reverse: GACGTCACCGAGTCGATGGT Probe: CACCTCCTAGAAGGC * targets IR-A and IR-B
IGFBP2	Forward: GCCCTCTGGAGCACCTCTACT Reverse: TCTTGCACTGTTTGAGGTTGTACA Probe: CATCCCCAACTGTGACAAGCATGGC
IGFBP3	Forward: ACGCACCGGGTGCTCTGA Reverse: TGCCCTTTCTTGATGATGATTATC Probe: CCCAAGTTCCACCCCTCCATTCA
IGFBP4	Forward: ACAACAGCTTCAGCCCCTGTA Reverse: CGAATTTTGGCGAAGTGCTT Probe: CGCCCATGACCGCAGGTGC
IGFBP5	Forward: AGGAGACCTACTCCCCAAGAT Reverse: TTCTTCACTGCTTCAGCCTTCA Probe: CGGCCCAAACACACCCGCA
IGFBP6	Forward: TGCAGAGGAGAATCCTAAGGAGAGT Reverse: TGGTAGAGGTGCCTGGATTCC Probe: CCAAGCAGGCACTGCCCCG
PAPP-A	Assay-on-Demand (Applied biosystems)